TP53 as a Biomarker in Head and Neck Squamous Cell Carcinoma

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A

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MASTER OF SCIENCE

By

Thomas J. Ow, MD

Houston, Texas

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Dedication

This work is dedicated to patients who are suffering from head and neck squamous cell cancer and to those who have succumbed to this disease.
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Abstract

*TP53 as a Biomarker in Head and Neck Squamous Cell Carcinoma*

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Currently, there are no molecular biomarkers that guide treatment decisions for patients with head and neck squamous cell carcinoma (HNSCC). Several retrospective studies have evaluated TP53 in HNSCC, and results have suggested that specific mutations are associated with poor outcome. However, there exists heterogeneity among these studies in the site and stage of disease of the patients reviewed, the treatments rendered, and methods of evaluating TP53 mutation. Thus, it remains unclear as to which patients and in which clinical settings TP53 mutation is most useful in predicting treatment failure.

In the current study, we reviewed the records of a cohort of patients with advanced, resectable HNSCC who received surgery and post-operative radiation (PORT) and had DNA isolated from fresh tumor tissue obtained at the time of surgery. TP53 mutations were identified using Sanger sequencing of exons 2-11 and the associated splice regions of the TP53 gene. We have found that the group of patients with either non-disruptive or disruptive TP53 mutations had decreased overall survival, disease-free survival, and an increased rate of distant metastasis. When examined as an independent factor, disruptive mutation was strongly associated with the development of distant metastasis.
As a second aim of this project, we performed a pilot study examining the utility of the AmpliChip® p53 test as a practical method for TP53 sequencing in the clinical setting. AmpliChip® testing and Sanger sequencing was performed on a separate cohort of patients with HNSCC. Our study demonstrated the ability of the AmpliChip® to call TP53 mutation from a single formalin-fixed paraffin-embedded slide. The results from AmpliChip® testing were identical with the Sanger method in 11 of 19 cases, with a higher rate of mutation calls using the AmpliChip® test.

TP53 mutation is a potential prognostic biomarker among patients with advanced, resectable HNSCC treated with surgery and PORT. Whether this subgroup of patients could benefit from the addition of concurrent or induction chemotherapy remains to be evaluated in prospective clinical trials. Our pilot study of the p53 AmpliChip® suggests this could be a practical and reliable method of TP53 analysis in the clinical setting.
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Abbreviations

AJCC -- American Joint Committee on Cancer
CCR -- Concurrent Chemoradiation
           (chemotherapy given during the same period as radiation treatment)
DFS -- Disease Free Survival
DM -- Distant Metastasis
DNA -- Deoxyribonucleic acid
DNE -- Dominant Negative Effects
ECE -- Extracapsular Extension of lymph node metastatic disease
EGFR -- Epidermal Growth Factor Receptor
FFPE -- Formalin-Fixed, Paraffin-Embedded
H&E -- Hematoxylin and Eosin
HNSCC -- Head and Neck Squamous Cell Carcinoma
HPV -- Human Papilloma Virus
Indels -- nucleotide base-pair insertion or deletion mutations
IRB -- Institutional Review Board
LR -- Local Recurrence
LRR -- Local-Regional Recurrence
LVI -- Lymphovascular Invasion
NED -- No Evidence of Disease
OCT -- Optimal Cutting Compound (Sakura®, Torrance, CA)
OS -- Overall Survival
PBS -- Phosphate Buffered Saline
PNI -- Perineural Invasion (PNI)
POCRT-- Post-operative Concurrent Chemoradiation
PORT-- Post-operative radiation treatment
TNM – Tumor, Node, Metastasis
UTMDACC – University of Texas, M.D. Anderson Cancer Center
Introduction

The Clinical Challenges of Head and Neck Squamous Cell Carcinoma

Squamous cell carcinoma of the head and neck (HNSCC) ranks among the top ten cancers worldwide for both incidence and mortality, with over 600,000 new cases diagnosed and over 300,000 estimated deaths each year(1). Approximately 45,000 new cases of HNSCC were diagnosed, with approximately 8,000 deaths occurring in the United States during 2010(2). Head and neck squamous cell carcinoma (HNSCC) can arise anywhere in the upper aerodigestive tract, particularly in the oral cavity, larynx, and pharynx.

The treatment options for HNSCC primarily include surgical resection, external beam radiation, and the use of chemotherapy(3, 4), most commonly platinum-based cytotoxic regimens(5, 6) or the EGFR inhibitor, cetuximab(7, 8). The strategies are largely chosen based on site of disease and staging according to the AJCC TNM criteria(9). In general, surgical resection is used as the primary modality to treat squamous carcinomas of the oral cavity due to the increased morbidity of definitive radiation to this region, particularly the risk of osteoradionecrosis of the mandible (10). Organ-sparing protocols, with radiation as the primary modality, are typically recommended for pharyngeal and laryngeal squamous cancers(6). Definitive treatment of advanced-stage disease (AJCC Stage III and IV) requires multimodality approaches, generally surgical resection with post-operative radiation (PORT), or concurrent chemoradiation (CCR) for organ-sparing approaches (3, 4, 6). Several studies have evaluated
induction/neoadjuvant chemotherapeutic strategies for the treatment of stage III and IV HNSCC (5, 6, 11, 12), further adding to the complexity of treatment options under consideration for the patient with advanced HNSCC, though the indications and measurable benefit from these strategies remain to be further delineated.

In 2004, two multi-institutional, phase-III randomized controlled trials evaluated the efficacy of adding concomitant cisplatin to PORT for advanced, resectable HNSCC, and each independently supported the use of post-operative chemoradiation (POCRT) in this setting (13, 14). When considering these two studies together, it became clear that patients with extracapsular spread of lymph node disease (ECE) and those with positive surgical margins after surgical resection represented a high-risk group for recurrence and mortality, and these factors are now strong indications for POCRT (15).

Figure-1 summarizes the current approach to treating patients with HNSCC based on the guidelines detailed above.

Figure-1 – Algorithm summarizing the current approach to the treatment of HNSCC.
*in specific cases (eg. laryngeal microsurgery for T1 glottic cancer)
**in specific cases (eg. advanced T4 laryngeal cancer)

Please note that the management of an individual patient with HNSCC is much more complex, with multiple considerations dependent on disease site, stage, and patient factors. The algorithm is presented here to highlight key factors that play a role in determining the treatment regimen of a typical patient, and is not meant to provide treatment guidelines to be used in the management of patients in the clinical setting.
This is a simplified view of the decision tree for the clinical team for the typical patient with HNSCC, but the algorithm highlights the important factors leading to the multidisciplinary treatment strategy of this disease. Two principles are evident regarding the management of HNSCC. First, multimodality strategies are crucial to increase the likelihood of control of advanced disease, and these options become more complicated as disease stage increases. Second, contemporary management decisions remain largely founded upon disease site, stage, and additional clinical and pathologic factors.

Despite the multitude of treatment options available to treat HNSCC, many patients continue to suffer from the morbidity of treatment, treatment failure, disease recurrence, and the development of metastasis. The recurrence and survival rates vary greatly depending on disease site in the head and neck. For example, outcomes with early stage tumors of the glottic larynx can be quite favorable with 5-year disease-free rates as high as 80 – 90%(16), whereas treatment of early-stage oral tongue cancer can have local failure rates as high as 30-50%(17). Patients with advanced, resectable head and neck squamous cell carcinoma, even after POCRT (the most rigorous strategy available), are faced with progression-free survival rates in the range of 50-60%(13, 14). As described above, the treatment decisions for patients with HNSCC are made based upon disease site and stage, and it is evident that these clinicopathologic considerations are limited in their ability to accurately predict the outcome of patients with specific treatments. Furthermore, the unacceptable but consistent failure rate, even for early-stage lesions in some cases, suggest that additional factors are necessary to aid our ability to predict
which HNSCC tumors will be best managed with either simple or more comprehensive treatments selecting from surgery, radiation, or our growing number of chemotherapeutic options. To this end, there is currently a paucity of molecular biomarkers that are employed today to guide the management of HNSCC. A single, yet notable exception that has been elucidated in recent years is the presence of HPV infection and/or p16 expression in oropharyngeal cancer.

Recent data has identified an increasing incidence of patients diagnosed with oropharyngeal squamous cell carcinoma (18-20), and this disease appears to be increasing most among middle-aged, white male patients who are non-smokers(19, 21, 22). Epidemiology studies have established that the human papilloma virus (HPV), particularly the high-risk serotypes 16 and 18 (22), are present at a high rate in tumor specimens of these patients. There is substantial prospective and retrospective evidence that the subgroup of patients with HPV-positive oropharyngeal cancer has a more favorable prognosis as compared to patients with oropharynx cancer that is negative for HPV(21, 23). Active HPV infection results in several alterations in key cell signaling pathways that promote tumorigenesis. In particular, expression of the E6 and E7 viral proteins lead to the inactivation of two key tumor suppressors, p53 and Rb, along with several other key factors in DNA damage signaling and cell cycle regulation(24). The ensuing dysregulation of normal cell cycle regulation and inhibition of feedback loop mechanisms lead to overexpression of p16, and recent studies have shown that p16 overexpression is also associated with improved outcome in HNSCC (21, 22). P16 overexpression is certainly a surrogate for active HPV infection in tumor cells, and there are reports
supporting p16 overexpression as a prognostic marker independent of HPV, but this remains to be further clarified(21, 22). Evaluation of HPV infection and/or p16 overexpression have become prognostic biomarkers in the workup of oropharyngeal squamous carcinoma(21, 23). HPV appears to play a much less significant role in other sites of HNSCC, and the overall utility of HPV as a biomarker in these subsites is still being explored (25-27). Thus, molecular biomarkers that can be used for prognosis and for guiding management decisions are lacking for HPV-negative oropharyngeal carcinomas as well as for other sites of HNSCC.

HPV and p16 have become prognostic biomarkers for patients with oropharyngeal squamous cell carcinoma, and the current outstanding question is if patients with HPV-positive oropharynx cancer can receive a de-escalated treatment regimen (eg. lower total dose of radiation, or withholding concurrent chemotherapy) without decreasing disease control and survival, thus potentially making this the first biologic marker used to alter management decisions that would otherwise proceed based on site and stage alone.

Despite the advances in our understanding of HPV in oropharyngeal cancer, there are still no biomarkers currently used to assist with clinical decision-making in the treatment of patients with HNSCC, leaving the clinician to rely strictly upon clinicopathologic factors to determine treatment for HPV-negative oropharynx cancer, and HNSCC from other anatomic sites. There is a need for biomarkers that identify those patients with HNSCC that could benefit from additional treatment, and the current study aims to explore the potential utility of TP53 mutation in this role.
**Description of the TP53 gene and p53 tumor suppressor**

TP53 is the most frequently mutated gene in human cancer (28-30), and has been well-studied since its first description over 30 years ago (31). P53 was determined to be a tumor suppressor roughly 10 years after its discovery (32, 33), and since that time alteration of p53 has been shown to play a key role in the pathogenesis and progression of numerous, if not all human cancers. P53 is involved in coordinating the response to cell stressors such as DNA damage, hypoxia, and oncogenic stress (34). P53 functions largely as a sequence-specific transcription factor with hundreds of targets in the human genome (35). Described targets include genes that induce cell cycle arrest (eg. p21, GADD45), activate DNA repair mechanisms (eg. p48, XPC), and drive apoptosis (eg. Bax, Apaf1, Noxa) (35, 36). P53 also has cytoplasmic and mitochondrial targets that regulate apoptosis, autophagy, and metabolism (36). Because radiation therapy and cytotoxic chemotherapeutic agents act through many of these same pathways, p53 also plays an important role in the response to these cancer therapies (37, 38). In summary, p53 responds to many stressors that promote carcinogenesis, and it protects against the development of malignancy by guiding the cell either toward a state of repair or self-destruction. A summary of p53 function and results of mutation are summarized in Figure-2.

The myriad functions of p53 can be attributed to its complicated structure and regulation. P53 is a protein made up of 393 amino acids encoded by 11 exons found in the TP53 gene on chromosome 17 (39, 40). The protein product is composed of several domains, including a DNA-binding domain, an oligomerization
region, and a tetramerization site (39) (see Figure-3, results section). At the latter region, p53 forms a dimer with another p53 molecule, which in turn combines with a second p53 dimer to form the final functional complex (39). Under basal conditions, p53 is maintained at a very low level secondary to regulation by MDM2, which directly binds and ubiquitinates p53, resulting in proteosomal degradation of the p53 protein (34). Cell stressors generally cause p53 stabilization via inhibition of MDM2 degradation. For example, ATM, a protein activated by DNA damage, phosphorylates p53, stabilizing the molecule and inhibiting MDM2-mediated degradation (34). This archetypal model of p53 regulation is just one example of p53 regulation. There are several amino acids of the p53 protein that are targets of enzymatic modification, which include phosphorylation, ubiquitination, neddylation, sumoylation, acetylation, and methylation. These sites are found in every domain of the p53 protein, including the transactivation domain, the DNA-binding region, and the tetramerization site (41). In addition to enzymatic modification, there are

**Figure 2.** Summary of p53 function and consequences of TP53 mutation. (*Model of mutant p53 shows codon sites where mutations were found in the current study*)
additional levels of regulation of the p53 molecule and its function (eg. alternative splicing, regulation by microRNA, etc.), however a full discussion of these processes is beyond the scope of this report. Each alteration in the molecular structure of p53 may change its stability, its capacity to translocate to the nucleus, or its influence on transcriptional and cytoplasmic targets.

In summary, the complexity of the TP53 gene and the p53 protein leads to its diverse role in tumor biology, and the effects of mutation of TP53 are equally complex.

Overview of TP53 mutation in Human Cancer

The patterns of mutations found in TP53 in human cancer have several interesting and unique features. Whereas most tumor suppressor genes are altered by truncating mutations or deletions, the mutation pattern of p53 is unique in that there appears to be a strong selection bias for missense mutations, particularly involving amino acids in the DNA-binding domain (29, 30, 42, 43). For this reason, several studies of TP53 in HNSCC have relied on sequencing exons from this region alone to assess for mutations in the TP53 gene (44, 45). Missense mutation often leads to p53 stabilization and accumulation of p53 in tumor cells that can be noted on immunohistochemical examination (46-48).

Mutation in p53 can be categorized as either ‘loss of function’ or ‘gain of function’. If a mutation renders p53 expression absent, then all p53 function is lost. However, mutant forms of p53 (especially those carrying missense mutations) often retain their expression, and therefore may remain partially functional. Loss of p53
function may also occur secondary to a dominant negative effect (DNE) by mutant p53. DNE occurs when mutant p53 directly inhibits the remaining wild-type p53 produced by the normal allele. Mutant p53 can also exhibit new, oncogenic properties that are not attributable to the loss of p53 alone. These ‘gain-of-function’ mutations were first identified in 1991(49) and later definitively confirmed in 1993(50) through in vivo mouse studies in which B-cell tumors that were p53-null became significantly more aggressive after mutant forms of p53 were introduced. Since that time, many examples of p53 gain-of-function mutations have been identified(51). The varying sequelae of mutation in TP53 are summarized above in Figure-2.

It should be noted that mutation is not the only mechanism for p53 inactivation. P53 can be inactivated by tumor-associated viral proteins, as in the abrogation of p53 by the HPV E6 protein, as described above. MDM2 amplification is another well described mechanism of disruption of the p53 axis described in human cancer (52), however MDM2 expression is more often diminished or lost in HNSCC, and TP53 mutation predominates (53).

The 30-year history of p53 research has contributed significantly to our understanding of this important protein, but the complexity of TP53 mutation has limited the utility of this information in the clinical setting.

**The role of TP53 mutation in HNSCC**

TP53 mutation is very common in HNSCC, and cancer of the head and neck ranks among breast, lung, and colorectal cancer as those in which TP53 mutations
are most commonly found (IARC database version R15, (42)). There are, of course, several genetic alterations that consistently contribute to the development of HNSCC. Cyclin D1 or p16 overexpression, signaling through EGFR or MET, SMAD4 mutation, PTEN loss, and PIK3CA activating mutations have all been noted to contribute to the development of head and neck squamous cell carcinoma at a relatively high frequency (54). Two recent studies using whole exome sequencing of HNSCC specimens have provided the most detailed examination of mutation in this disease to date (55, 56). Several mutations and gene alterations were discovered and described, and interestingly both studies found frequent alterations of NOTCH-1. Additionally, as expected, TP53 mutations predominated in both studies. Mutation in TP53 is present in 40 - 60% of reported cases of HNSCC (42, 44, 57, 58). Wild-type p53 is disrupted in an additional subset of HNSCC infected by HPV, commonly found in tumors of the oropharynx, as described above. Similar to other models of cancer progression (59), loss of heterozygosity of TP53 was identified as an early event in the development of many head and neck cancers (58, 60), and mutations in TP53 have been associated with poor outcome in several studies (44, 45, 57, 61).

In 1998, Koch, et al. (44) examined exons 5-9 of TP53 in tumors harvested from 110 patients with HNSCC who received either primary radiation or adjuvant radiation for treatment of their disease, and 48 (44%) were found to have a mutation. Though not associated with decreased overall survival, TP53 mutation was found to be associated with decreased progression-free survival, which remained an independent factor after multivariate regression analysis. Erber et. al,
in 1998(45), evaluated exons 5-8 of TP53 in tumors from 86 previously untreated patients with HNSCC, and did not find a significant difference in overall survival between the group with TP53 mutation versus the group with no mutation identified. The authors did, however, classify TP53 mutation into structural and contact mutations based on the location of the mutation in the L3, H2, S10, or Zinc binding amino acids (contact mutations) proposed by Cho, et al.(62) to make up the core DNA-binding domain. They found that patients with TP53 contact mutations had decreased overall and recurrence-free survival. More recently, a large study conducted by Poeta, et al. in 2007(57) evaluated TP53 in 420 patients with HNSCC and classified mutations into disruptive (ie. nonsense mutations or missense mutations in the L2-L3 region leading to an amino acid change with a different polarity or charge) or nondisruptive (ie. all other non-conservative mutations). In this study, representing the largest to date, TP53 mutation was associated with decreased overall survival, and disruptive mutation was associated with the group of patients with the poorest survival. After multivariate analysis, disruptive TP53 mutation remained strongly associated with decreased overall survival. Most recently, Lindenbergh, et al. in June, 2011(61) published a study evaluating 141 patients with HNSCC who either received surgery or surgery and radiation, and found that truncating TP53 mutations were more strongly associated with poor outcome than disruptive mutations.

There exists ample data to suggest that TP53 can be used to predict outcome in HNSCC, however TP53 mutation in HNSCC tumors has not become part of the routine evaluation in the clinical setting.
Current limitations to TP53 as a biomarker in HNSCC

Despite several studies that have supported the potential of TP53 mutation as a predictor of poor outcome in HNSCC, TP53 mutational analysis has not become a routine diagnostic in the clinical setting. There are currently several limitations to the implementation of TP53 mutation analysis. One major limitation is that previous studies have largely reviewed HNSCC patients with varying tumor sites, disease stage, and treatments received (44, 45, 57, 61), and thus it is not clear which group of patients would benefit most from the evaluation of TP53 mutation analysis. Second, methods used to detect TP53 mutation have varied between studies, as well, adding to the difficulty of arriving at generalizable conclusions from a collective review of the literature (63). Finally, technical and economic limitations have further hindered the implementation of TP53 mutation analysis in the clinical setting. A more detailed review of these limitations is discussed in the following sections.

Patients evaluated in studies examining TP53 in HNSCC have been heterogeneous with regard to site, stage, and treatment. Several factors play a role in the eventual outcome of patients with HNSCC, including disease site, stage, HPV status, and the treatments that are provided. The difficulty of arriving at generalizable conclusions from the published literature studying p53 in HNSCC due to these factors are well-summarized in the systematic review by Tandon (63). Among the studies that have evaluated TP53 mutation and prognosis among
patients with HNSCC, only a few have evaluated HPV status(61, 64), which is now known to dramatically alter patient prognosis with current treatment strategies. Furthermore, in the largest study to evaluate TP53, the patients examined received a diverse array of treatments(57), and several other studies include at least two, if not several treatment strategies among the patient cohorts(44, 45, 61). Therefore, though TP53 mutation may be relevant to patients with HNSCC as a whole, it is currently unclear for which patients TP53 mutation may be most useful in predicting outcome or for guiding treatment decisions.

**The method of detecting TP53 mutation has varied from study to study.** There are a multitude of methods for evaluating p53 in tumor specimens, most common of which are immunohistochemical (IHC) evaluation for p53 protein expression (with a vast array of potential antibodies) and direct sequencing of genomic DNA covering either a subset or all of the exonic regions of TP53. Several of these methods have been used in the published literature with varying results, making it difficult to arrive at definitive conclusions from the available data(63). Because missense mutation in TP53 often results in p53 overexpression, high p53 expression on IHC has been used as a marker for TP53 mutation, and the majority of studies evaluating p53 in HNSCC have used IHC to assess p53 status(63). The poor concordance between TP53 mutation and IHC evaluation has been demonstrated(47). Furthermore, several studies have limited TP53 gene sequencing to a subset of TP53 exons focusing on the DNA-binding domain(44, 45), whereas more recent studies have evaluated the entire coding region of TP53(57, 61), perhaps leading to varying
results or selection bias based on the mutations that were considered versus those that were not.

The feasibility of sequencing DNA from HNSCC tumor samples has been limited outside of the research setting. Both technical and economical considerations have limited the use of gene sequencing in the clinical setting. First, standard processing of tissue samples involves formalin-fixation and paraffin-embedding followed by staining for pathological review. Many patients are initially identified at small, community-based hospitals and are subsequently referred to multidisciplinary cancer centers for treatment. Furthermore, head and neck cancer is commonly diagnosed from small biopsy samples, and a large fraction of patients are never treated surgically. Techniques for sequencing large genomic regions from formalin-fixed, paraffin-embedded tissue is limited, and PCR amplification is often of poor quality or impossible due to DNA cross-linking and fragmentation secondary to the embedding process. Therefore, current standard clinical practices would require repeat acquisition of tumor for genetic analysis after initial diagnosis, which would add increased and unnecessary risk to patients and additional, and perhaps unnecessary medical costs. Furthermore, gene sequencing, until recently, has been prohibitively expensive, and though costs have significantly decreased, they remain substantial when considered for routine clinical practice. Finally, the inherent and variable presence of normal tissue in biopsy samples can lead to false-negative results when sequencing for mutations in a background of contaminating
wildtype copies, which is yet another limitation to routine TP53 mutation detection from biopsy samples.

**Study Rationale, Hypothesis, and Aims**

The evidence to date suggests that the TP53 gene is a potential prognostic biomarker for patients with HNSCC, but the limitations highlighted above are hurdles that must be overcome before TP53 mutation is deemed useful in the clinical setting. There is a growing need to evaluate TP53 as a potential biomarker in specific subsets of HNSCC patients, in particular patient groups in which current standards of treatment are failing in a significant fraction, or those in which a significant proportion of patients are at high risk of treatment-related morbidity yet excellent outcomes are expected. Furthermore, techniques to evaluate TP53 in the clinical setting must be rapid, reliable, and best performed on specimens that are most readily available in the clinical setting—ie. formalin-fixed, paraffin-embedded (FFPE) biopsy specimens. To further advance our knowledge of the utility of TP53 mutation as a clinical biomarker in HNSCC, we proposed the following hypothesis and aims:

**We hypothesize that TP53 mutation is associated with poor outcome in patients with advanced, resectable HNSCC, and that TP53 mutation can be reliably identified with the TP53 AmpliChip® test (Roche Molecular Systems®, Pleasanton, CA) from tissue that is readily available from standard biopsy specimens.**
Specific Aim #1: To determine whether TP53 mutation could be used as a predictor of poor outcome in advanced, resectable HNSCC treated with surgical resection and post-operative radiation.

Before TP53 mutation can become clinically useful, it must be determined for which subset of patients with HNSCC this potential biomarker would be most informative. Patients with advanced, resectable disease are currently faced with the most complex treatment options, many of which involve cytotoxic therapies (eg. radiation and cisplatin) for which functional p53 would perhaps be the most relevant. We also felt it was important to examine a cohort of patients who were all treated similarly in order to determine the influence of TP53 status on outcome without the added consideration of variability in treatments provided. In this study, we therefore examined tumor samples from patients with advanced HNSCC who were uniformly treated with surgery and PORT. If TP53 mutation could be used to predict poor outcome among this cohort of patients, it would suggest that the presence of a TP53 mutation may be an indication for the addition of chemotherapy to surgical resection and PORT. We therefore sequenced the entire coding region of TP53 from the DNA from tumors among this cohort of patients using standard Sanger sequencing, and patient records were reviewed in order to determine if the outcomes of local-regional recurrence, metastasis, and death were associated with mutation inTP53. Our intent was to examine TP53 mutation as a prognostic factor, as well as to classify TP53 mutation into the nondisruptive and disruptive
categories, as proposed by Poeta, et al.(57), in order to determine if these factors were associated with poor outcome in our patient cohort.

**Specific Aim#2:** To assess the ability of the AmpliChip® TP53 test to identify TP53 mutation from formalin-fixed, paraffin-embedded specimens.

Previous studies have relied on sequencing from fresh frozen tissue enriched for high tumor content with screening and/or microdissection. The availability of fresh tissue for sequencing in the clinical setting, and the potential false-negative results secondary to wild-type p53 contamination from surrounding or infiltrating normal tissue often found in standard biopsy samples, remain two hurdles limiting the routine use of TP53 mutation analysis. Recent studies have evaluated a new technology, the TP53 AmpliChip® (Roche Molecular Systems®, Pleasanton, CA) to identify TP53 mutation in breast cancer and chronic lymphocytic leukemia(65, 66). The AmpliChip® is a microarray-based technique that interrogates each nucleotide in exons 2-11 of the TP53 coding regions and splice sites. The AmpliChip® is therefore designed to detect single base-pair alterations only, and cannot detect alterations ≥ 2 bases in size. DNA processing involves isolation, fragmentation, PCR amplification, and labeling. The resulting processed DNA is then hybridized to the microarray and scanned. These procedures overcome the limitations of poor quality DNA in FFPE tissue, and the array is designed to detect the presence of single nucleotide changes in the midst of a high wild-type sequence background. We therefore designed a pilot study to assess the utility of the TP53 AmpliChip® to
detect mutations in HNSCC samples. Formalin-fixed paraffin-embedded (FFPE) specimens from 19 HNSCC samples were analyzed using this new technology and results were compared to sequencing results obtained from the standard Sanger technique performed on DNA isolated from corresponding fresh frozen tumor tissue. The pilot study is designed to assess the ability of the AmpliChip® to identify TP53 mutations from a single slide of FFPE tissue, equivalent to a typical tissue sample that would be available with current standard biopsy protocols for HNSCC.

The overall goal is to determine whether TP53 mutation can be evaluated with a rapid, reliable method from paraffin-embedded biopsy samples, and also to determine if TP53 mutation can be used to predict poor outcome among patients with advanced, resectable HNSCC treated with surgery and PORT. In achieving these aims we intend to further advance TP53 mutation as a potential biomarker in HNSCC.
Methods

Specific Aim #1: To demonstrate that TP53 mutation is associated with poor outcome in advanced, resectable HNSCC treated with surgical resection and post-operative radiation.

Patient Selection and Tissue Procurement

Patients evaluated and treated at The University of Texas MD Anderson Cancer Center (UTMDACC) from 1992 – 2002 were recruited to participate in a head and neck cancer tissue banking program under a protocol approved by the institutional review board (IRB). Clinical records were reviewed retrospectively for these patients and TP53 gene status was determined according to a second protocol approved by the IRB at UTMDACC authorizing TP53 genomic analysis on previously banked samples. All patients were previously untreated and received primary treatment of squamous cell carcinoma of the oral cavity, pharynx, or larynx, undergoing surgical resection followed by PORT. Specimens were harvested from the tumor primary site and snap frozen at the time of surgery.

Patients who received neoadjuvant or adjuvant chemotherapy were excluded. Frozen sections from all specimens were reviewed by a member of the UTMDACC Pathology Department and were confirmed to be HNSCC with at least 70% of the specimen determined to be tumor. Clinical and pathologic factors were recorded, including T stage, N stage, surgical margin status, extracapsular lymph node extension (ECE), lymphovascular invasion (LVI), and perineural invasion.
For the purposes of this study, pathology reports that did not explicitly identify LVI or PNI were considered to be negative for these features. Additional review of the pathology slides was performed if the original pathology reports did not explicitly state the presence or absence of ECE or positive surgical margins.

Generally, all patients were evaluated every 2 - 3 months for the first year following treatment, every 3 - 4 months the second year, and then every six months, thereafter. The outcomes and dates of local recurrence (LR), local-regional recurrence (LRR), development of distant metastasis (DM), disease-status at last contact, and death were recorded.

**DNA Extraction and Isolation**

Approximately 10mg of tumor sample was minced and resuspended in pure PBS. The sample then underwent overnight digestion with proteinase K at 55°C. DNA was isolated using the QIAmp® DNA mini Kit (Qiagen®, Maryland, USA), following the manufacturer’s protocol. Eluted DNA was quantified using the Quant-It™ PicoGreen® system (Invitrogen®, Carlsbad, CA, USA).

**TP53 Sequencing and Classification**

The coding regions and surrounding splice sites from exons 2-11 of the TP53 gene were evaluated via direct sequencing from genomic DNA, which was sent to Beckman Coulter Genomics (Beckman Coulter®, Brea, CA, USA). Standard Sanger sequencing using BigDye® Terminator chemistry (Applied Biosystems® Life Technologies®, Carlsbad, CA, USA) was performed through the service provided
by the company. TP53 point mutations were categorized as “disruptive” and “non-disruptive”. Disruptive mutations were defined as stop codons, or missense mutations occurring within the L2 or L3 loop of the key DNA-binding domain, leading to substitution with an amino acid of a different polarity or charge group, as described by Poeta, et al(57). All other point mutations, except for splice site and insertion or deletion mutations (indels), were defined as non-disruptive mutations. Splice site mutations and indels were classified together as a separate group, and the disruptive and non-disruptive mutations were also categorized collectively as “point mutations” for portions of the analysis. In cases where more than one mutation was identified and each mutation was classified into two different groups, TP53 status was classified according to the mutation suspected to have the more deleterious effect (eg. if a disruptive and a non-disruptive mutation were both identified, then the tumor would be classified in the TP53 disruptive mutation category).

**Statistical Analysis**

The proportion of TP53 mutations and disruptive TP53 mutations between different groups of patients were compared using \( \chi^2 \) statistics, with or without Yates’ correction, or Fisher exact tests where appropriate. Each patient’s follow-up period was defined as the date of surgery to the date of last contact or date of death. Median follow-up time was calculated via the method of Schemper and Smith(67). Overall survival was defined as the period from the date of completion of definitive treatment to the date of death. Disease-free survival (DFS) was defined as the
period from the date of completion of definitive treatment to the date of first recurrence or date of death. Time to distant metastasis (DM) was defined as the date of the completion of definitive treatment to the date of development of distant metastasis. Patients who died prior to developing distant metastasis were censored for analysis of this outcome. Curves for overall survival (OS), DFS, and the development of DM between TP53 mutant and wild-type groups, as well as between disruptive, non-disruptive, and splice site/indels were estimated using the Kaplan-Meier method, and Log Rank statistics were used to calculate the significance of differences observed. Univariate and multivariate proportional hazards models were generated to identify prognostic factors that were significantly associated with reduced OS, DFS, and DM. For all values, a threshold of p < 0.05 was accepted as statistically significant.

**Specific Aim#2:** To assess the ability of the TP53 AmpliChip® to identify TP53 mutation from formalin-fixed, paraffin-embedded specimens.

**Tumor Specimens and Sample Preparation**

Additional HNSCC tumor specimens were evaluated using both Sanger sequencing of TP53 and using the AmpliChip® TP53 assay (Roche Molecular Systems®, Pleasanton, CA). These samples were obtained from patients who had consented to provide tumor specimens at the time of surgical resection, to be stored as part of a Head and Neck Cancer tumor bank at UTMDACC, opened and maintained under an active IRB approved protocol. An additional IRB-approved
protocol was obtained authorizing TP53 genomic analysis. Fresh tissue was snap frozen in liquid nitrogen at the time of acquisition and stored at -80 °C. Tissue samples were deemed to be HNSCC after review by a pathologist at the time of biopsy.

Each specimen was divided in half, with one half designated for Sanger sequencing and the other half designated for TP53 AmpliChip® testing. For Sanger sequencing, samples were embedded in Optimal Cutting Compound (OCT) (Sakura®, Torrance, CA), frozen in dry ice conditions, and stored at -80 °C until DNA isolation. For TP53 AmpliChip® analysis, specimens were fixed in formalin and embedded in paraffin. A representative slide was cut from this block and stained with hematoxylin and eosin (H&E) stains. Each slide was reviewed by two independent members of the laboratory (one postdoctoral fellow, and one research instructor with formal pathology training) who graded each slide for percent tumor in the entire specimen, and percent tumor composing the most dense region of each sample- these two percentages were recorded and averaged to arrive at the final call. The time from tissue acquisition to paraffin-embedding, acquisition to TP53 analysis, and from paraffin-embedding to TP53 analysis was recorded.

Patient Data

A retrospective chart review was performed under an IRB-approved protocol, and the following data were recorded: date of diagnosis, date of surgical resection, disease stage, primary or recurrent disease, treatments rendered, date of last treatment of initial disease, date of last contact, presence or absence of LRR, the
date LRR presented, presence or absence of the development of DM, date that DM presented, date of last contact, and vital status.

**DNA Isolation and TP53 Sequencing**

For Sanger sequencing analysis, a cryotome was used to cut fifteen 10 uM sections from the specimens previously embedded in OCT. From these sections, the RecoverAll™ Total Nucleic Acid Isolation Kit (Ambion®, Applied Biosystems®, Austin, Tx) was used per the manufacturer protocol to extract genomic DNA. The amount of DNA isolated and the concentration was then analyzed using the NanoDrop™ 2000 (Thermo Fisher Scientific®, Wilmington, DE). DNA samples were then sent to the Baylor College of Medicine Human Genome Sequencing Center for sequencing of the coding regions and splice regions of TP53 for exons 2-11.

**Sample Preparation for the AmpliChip® p53 Assay and Analysis**

The DNA isolation, preparation, and analysis for the AmpliChip® p53 assay are under development by Roche Molecular Systems® and not yet commercially available. Paraffin-embedded slides were processed by Roche® under a research agreement with our group. After formalin-fixation and paraffin embedding, one slide was cut from each block for H&E staining, and a second 10um-thick section was cut and mounted on a second slide. In cases of small samples, two or three sections were mounted per slide. H&E slides were evaluated by two independent members of the laboratory (one of whom is a pathologist by training) who provided an
estimate of percent tumor of the entire sample and for the most dense regions of tumor for each section. The estimates from the two evaluators were averaged to arrive at the reported result. Sections of most dense tumor were circled for macrodissection. These slides were then sent to the Roche® laboratory, where they were macrodissected and prepared for AmpliChip® testing as previously reported (66, 68).

Briefly, slides were deparaffinized, DNA was extracted and isolated, fragmented, PCR-amplified, biotin-labeled, and hybridized to the AmpliChip® p53 arrays. The arrays were then scanned using an Affymetrix® Dx2 Scanner and data was analyzed to arrive at the TP53 sequence of each sample.

**Statistical Analysis**

Kaplan-Meier survival analysis for the patients was stratified by TP53 status, and generated for each of the two TP53 analysis methods for comparative purposes. This analysis was used to arrive at median disease-free survival for each group.
Results

Specific Aim #1: To demonstrate that TP53 mutation is associated with poor outcome in advanced, resectable HNSCC treated with surgical resection and post-operative radiation.

Patient characteristics and follow-up

We initially identified 88 patients that had received surgical resection and PORT as treatment for HNSCC who had DNA available for this study. Five patients were excluded because their tumors arose in sites that the authors felt were not appropriate for evaluation in this cohort (2 maxillary sinus, 1 lip, and 2 from perioral skin), and five additional patients were excluded because they had received neoadjuvant or adjuvant chemotherapy in addition to PORT as part of their treatment regimen. A significant portion of the TP53 gene in 4 additional samples could not be amplified and sequenced. Therefore, a total of 74 patients were evaluated in this study (Table-1).

The median age of the patients evaluated was 57 years (range 34 – 82). There were 50 male and 24 female patients who had primary tumors from the oral cavity (n=32), hypopharynx (n = 18), larynx (n = 16) or oropharynx (n=8). Sixty-five patients were determined to have stage IV disease, 3 patients stage III, and only 6 patients stage I or II. Sixty-two patients (84%) presented with T3 or T4 tumors at the primary site, and 46 (62%) had N2b or greater nodal burden at diagnosis. Twenty-seven (36%) patients were noted on pathology to have PNI, and 17 (23%) had signs of LVI. Forty-eight patients (65%) had ECE, and 13 (18%) had positive
Table-1. Patients included and excluded, and TP53 mutations identified.

<table>
<thead>
<tr>
<th>Patient #</th>
<th>Tumor Site</th>
<th>Mutation type</th>
<th>Site</th>
<th>Disruptive/Non-Disruptive</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Oral Cavity</td>
<td>missense</td>
<td>F270C, T211I</td>
<td>Non-disruptive</td>
</tr>
<tr>
<td>2</td>
<td>Oral Cavity</td>
<td>missense</td>
<td>C135F</td>
<td>Non-disruptive</td>
</tr>
<tr>
<td>3</td>
<td>Oral Cavity</td>
<td>missense</td>
<td>G245S</td>
<td>Non-disruptive</td>
</tr>
<tr>
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<td>Oral Cavity</td>
<td>missense</td>
<td>R273C</td>
<td>Non-disruptive</td>
</tr>
<tr>
<td>5</td>
<td>Oral Cavity</td>
<td>missense</td>
<td>Y220N</td>
<td>Non-disruptive</td>
</tr>
<tr>
<td>6</td>
<td>Oral Cavity</td>
<td>missense</td>
<td>R273H</td>
<td>Non-disruptive</td>
</tr>
<tr>
<td>7</td>
<td>Hypopharynx</td>
<td>missense</td>
<td>R273C, R213G</td>
<td>Non-disruptive</td>
</tr>
<tr>
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<td>missense</td>
<td>R175H</td>
<td>Non-disruptive</td>
</tr>
<tr>
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<td>Hypopharynx</td>
<td>missense</td>
<td>E258K</td>
<td>Non-disruptive</td>
</tr>
<tr>
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<td>Hypopharynx</td>
<td>missense</td>
<td>M133K</td>
<td>Non-disruptive</td>
</tr>
<tr>
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<td>Larynx</td>
<td>missense</td>
<td>V167F</td>
<td>Non-disruptive</td>
</tr>
<tr>
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<td>Larynx</td>
<td>missense</td>
<td>G245C</td>
<td>Non-disruptive</td>
</tr>
<tr>
<td>13</td>
<td>Larynx</td>
<td>missense</td>
<td>Y236C</td>
<td>Non-disruptive</td>
</tr>
<tr>
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<td>missense</td>
<td>R175H</td>
<td>Non-disruptive</td>
</tr>
<tr>
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<td>missense</td>
<td>F134C</td>
<td>Non-disruptive</td>
</tr>
<tr>
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<td>missense</td>
<td>L145Q</td>
<td>Non-disruptive</td>
</tr>
<tr>
<td>17</td>
<td>Oral Cavity</td>
<td>missense</td>
<td>H179Y</td>
<td>Disruptive</td>
</tr>
<tr>
<td>18</td>
<td>Oral Cavity</td>
<td>missense</td>
<td>G245D</td>
<td>Disruptive</td>
</tr>
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<td>Oral Cavity</td>
<td>missense</td>
<td>C238F</td>
<td>Disruptive</td>
</tr>
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<td>missense</td>
<td>C176F</td>
<td>Disruptive</td>
</tr>
<tr>
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<td>missense</td>
<td>H179Y</td>
<td>Disruptive</td>
</tr>
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<td>Oral Cavity</td>
<td>nonsense</td>
<td>R342stop</td>
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</tr>
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<td>nonsense</td>
<td>K291stop</td>
<td>Disruptive</td>
</tr>
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<td>Disruptive</td>
</tr>
<tr>
<td>25</td>
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<td>nonsense</td>
<td>R213stop</td>
<td>Disruptive</td>
</tr>
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<td>Hypopharynx</td>
<td>nonsense</td>
<td>R196stop</td>
<td>Disruptive</td>
</tr>
<tr>
<td>27</td>
<td>Hypopharynx</td>
<td>nonsense</td>
<td>G266stop, Q52stop</td>
<td>Disruptive</td>
</tr>
<tr>
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<td>nonsense</td>
<td>R306stop</td>
<td>Disruptive</td>
</tr>
<tr>
<td>29</td>
<td>Hypopharynx</td>
<td>nonsense</td>
<td>E339stop</td>
<td>Disruptive</td>
</tr>
<tr>
<td>30</td>
<td>Hypopharynx</td>
<td>nonsense</td>
<td>R196stop</td>
<td>Disruptive</td>
</tr>
<tr>
<td>31</td>
<td>Larynx</td>
<td>nonsense</td>
<td>C104Xstop</td>
<td>Disruptive</td>
</tr>
<tr>
<td>32</td>
<td>Larynx</td>
<td>nonsense</td>
<td>R342stop</td>
<td>Disruptive</td>
</tr>
<tr>
<td>33</td>
<td>Oral Cavity</td>
<td>insertion-deletion</td>
<td></td>
<td>Disruptive</td>
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<td>34</td>
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<td>insertion-deletion</td>
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<td>Disruptive</td>
</tr>
<tr>
<td>35</td>
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<td>insertion-deletion</td>
<td></td>
<td>Disruptive</td>
</tr>
<tr>
<td>36</td>
<td>Hypopharynx</td>
<td>insertion-deletion</td>
<td></td>
<td>Disruptive</td>
</tr>
<tr>
<td>37</td>
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<td>insertion-deletion</td>
<td></td>
<td>Disruptive</td>
</tr>
<tr>
<td>38</td>
<td>Hypopharynx</td>
<td>insertion-deletion</td>
<td></td>
<td>Disruptive</td>
</tr>
<tr>
<td>39</td>
<td>Oropharynx</td>
<td>insertion-deletion</td>
<td></td>
<td>Disruptive</td>
</tr>
<tr>
<td>40</td>
<td>Oral Cavity</td>
<td>splice-site</td>
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<td>Disruptive</td>
</tr>
<tr>
<td>41</td>
<td>Hypopharynx</td>
<td>splice-site</td>
<td></td>
<td>Disruptive</td>
</tr>
<tr>
<td>42</td>
<td>Larynx</td>
<td>splice-site</td>
<td></td>
<td>Disruptive</td>
</tr>
<tr>
<td>43</td>
<td>Larynx</td>
<td>splice-site</td>
<td></td>
<td>Disruptive</td>
</tr>
</tbody>
</table>

Wildtype | No. Wild-type |
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>44 - 46</td>
<td>Hypopharynx</td>
</tr>
<tr>
<td>47 - 53</td>
<td>Larynx</td>
</tr>
<tr>
<td>54 - 69</td>
<td>Oral Cavity</td>
</tr>
<tr>
<td>70- 74</td>
<td>Oropharynx</td>
</tr>
</tbody>
</table>

Excluded Patients
- 1 maxillary sinus A161T excluded site
- 2 maxillary sinus wt excluded site
- 3 lip wt excluded site
- 4 skin indel excluded site
- 5 skin G155stop excluded site
- 6 oral cavity wild-type received chemotherapy
- 7 oral cavity wild-type received chemotherapy
- 8 pyriform wild-type received chemotherapy
- 9 tongue wild-type received chemotherapy
- 10 oral cavity wild-type received chemotherapy
- 11 hypopharynx Inadequate sequence
- 12 hard palate Inadequate sequence
- 13 oral cavity Inadequate sequence
- 14 Oral cavity Inadequate sequence
margins after surgical resection. Table-2 summarizes the clinical and demographic characteristics of the patients.

**Table-2.** Patient characteristics and distribution of TP53 mutations.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No. (%)</th>
<th>Wild-type TP53</th>
<th>Mutant TP53</th>
<th>p-value</th>
<th>Disruptive</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>no. (%)</td>
<td>no. (%)</td>
<td>% mutant p53</td>
<td>no. (%)</td>
<td>% disruptive</td>
</tr>
<tr>
<td><strong>All Patients</strong></td>
<td>74</td>
<td>31 (42%)</td>
<td>43 (58%)</td>
<td>16 (22%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Age (median = 57 years, range 34 — 82 years)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;55 yrs</td>
<td>33 (45%)</td>
<td>14 (42%)</td>
<td>19 (58%)</td>
<td>p = 0.62</td>
<td>9 (27%)</td>
<td>p = 0.41</td>
</tr>
<tr>
<td>&gt;55 - 65 yrs</td>
<td>23 (31%)</td>
<td>8 (35%)</td>
<td>15 (65%)</td>
<td></td>
<td>5 (22%)</td>
<td></td>
</tr>
<tr>
<td>&gt;65 yrs</td>
<td>18 (24%)</td>
<td>9 (50%)</td>
<td>9 (50%)</td>
<td></td>
<td>2 (11%)</td>
<td></td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>50 (68%)</td>
<td>23 (46%)</td>
<td>27 (54%)</td>
<td>p = 0.43</td>
<td>9 (18%)</td>
<td>p = 0.43</td>
</tr>
<tr>
<td>Female</td>
<td>24 (32%)</td>
<td>8 (33%)</td>
<td>16 (67%)</td>
<td></td>
<td>7 (20%)</td>
<td></td>
</tr>
<tr>
<td><strong>Site</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oral Cavity</td>
<td>32 (43%)</td>
<td>16 (50%)</td>
<td>16 (50%)</td>
<td>p = 0.015*</td>
<td>7 (22%)</td>
<td>p = 1.00*</td>
</tr>
<tr>
<td>Hypopharynx</td>
<td>18 (24%)</td>
<td>3 (17%)</td>
<td>15 (83%)</td>
<td></td>
<td>6 (33%)</td>
<td></td>
</tr>
<tr>
<td>Larynx</td>
<td>16 (22%)</td>
<td>7 (44%)</td>
<td>9 (56%)</td>
<td></td>
<td>3 (19%)</td>
<td></td>
</tr>
<tr>
<td>Oropharynx</td>
<td>8 (11%)</td>
<td>5 (63%)</td>
<td>3 (38%)</td>
<td></td>
<td>0 (0%)</td>
<td></td>
</tr>
<tr>
<td><strong>T stage</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>4 (5%)</td>
<td>2 (50%)</td>
<td>2 (50%)</td>
<td>p = 0.37**</td>
<td>0 (0%)</td>
<td>p = 0.28**</td>
</tr>
<tr>
<td>T2</td>
<td>8 (11%)</td>
<td>4 (50%)</td>
<td>4 (50%)</td>
<td></td>
<td>1 (13%)</td>
<td></td>
</tr>
<tr>
<td>T3</td>
<td>21 (28%)</td>
<td>10 (48%)</td>
<td>11 (52%)</td>
<td></td>
<td>7 (33%)</td>
<td></td>
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<tr>
<td>T4</td>
<td>41 (55%)</td>
<td>15 (37%)</td>
<td>26 (63%)</td>
<td></td>
<td>8 (20%)</td>
<td></td>
</tr>
<tr>
<td><strong>N stage</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>12 (16%)</td>
<td>6 (50%)</td>
<td>6 (50%)</td>
<td>p = 0.09†</td>
<td>2 (17%)</td>
<td>p = 0.30†</td>
</tr>
<tr>
<td>N1-2a</td>
<td>16 (22%)</td>
<td>9 (56%)</td>
<td>7 (44%)</td>
<td></td>
<td>2 (13%)</td>
<td></td>
</tr>
<tr>
<td>≥N2b</td>
<td>46 (62%)</td>
<td>16 (35%)</td>
<td>30 (65%)</td>
<td></td>
<td>12 (26%)</td>
<td></td>
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<tr>
<td><strong>High-Risk Features</strong></td>
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<td></td>
</tr>
<tr>
<td>ECE</td>
<td>48 (65%)</td>
<td>17 (35%)</td>
<td>31 (65%)</td>
<td>p = 0.76†</td>
<td>13 (27%)</td>
<td>p = 0.06†</td>
</tr>
<tr>
<td>Pos Margins</td>
<td>13 (18%)</td>
<td>8 (62%)</td>
<td>5 (38%)</td>
<td></td>
<td>3 (23%)</td>
<td></td>
</tr>
</tbody>
</table>

*Proportions of patients with TP53 mutation or disruptive mutation among patients with hypopharyngeal primary tumors were compared to those arising from the other sites

**Patients with stage T1 or T2 disease were compared to those with stage T3 and T4 disease.

†Patients with stage N0 – N2a disease were compared to those with N2b or greater disease.

‡Patients with ECS or Positive surgical margins were compared to those who did not have these factors.
The follow-up period for this group of patients ranged from 6.65 years to 22.20 years, with a median follow-up of 13.05 years. At the last review, 60 patients had died (range of time to death, 32 days to 13.7 years; median overall survival was 2.73 years), 35 had no evidence of disease (NED) at last contact or death, 26 had LRR or persistent disease, and 20 developed DM.

TP53 mutation and clinical-pathologic features

Forty-seven mutations in TP53 were identified in 43 patients (57%). There were 18 disruptive, 18 non-disruptive mutations, 7 indel, and 4 splice site mutations. The majority of missense mutations were within the DNA-binding domain (Figure-3), with several located in known TP53 hotspot sites (eg. codons 175, 245, 273). A list of the mutations identified is provided in Table-1.

TP53 mutations were found in tumors from 26 (63%) of the patients with T4 disease, 30 (65%) of the patients with N2b or greater disease, and 31 (65%) of patients with ECE. The proportions of TP53 mutations and disruptive mutations among the patients studied are presented in Table-2. Only hypopharyngeal primary site was associated with a significantly increased proportion of TP53 mutations (p=0.015), but the proportion of disruptive TP53 mutations did not appear enriched in this group. There did appear to be a higher proportion of disruptive mutations among the group of patients with either ECE or positive surgical margins, but this difference did not reach statistical significance (p= 0.06). Among the 43 patients with tumors harboring mutant TP53, 13 (30%) developed LRR, and 16 (37%)
developed DM, compared to 12 (39%) and 6 (19%), respectively in the 31 patients with p53 wildtype tumors. Twelve of 16 patients (75%) with tumors with disruptive mutations developed relapse: eight patients (50%) developed LRR, and eight (50%) developed DM (4 of these patients developed both LRR and DM).

Figure-3. (A.) Frequency of point mutations in TP53. (B.) Proportions of patients with mutation in TP53 and proportion of mutation types identified. * Indicates position of disruptive mutations identified.
Survival analysis and risk models

Univariate analysis showed that several clinico-pathologic factors were associated with the endpoints of OS, DFS, and DM (Table-3). Stage T3 or T4 disease, N2b or greater nodal burden, and ECE and positive surgical margins were significantly associated with reduced OS and DFS. N2b or greater nodal disease was associated with DM.

Table-3. Univariate Analysis – factors associated with overall survival, disease-free survival, and the development of distant metastasis.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Overall Survival</th>
<th>Disease-Free Survival</th>
<th>Distant Metastasis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>HR</td>
<td>95% CI</td>
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<tr>
<td>All</td>
<td>74</td>
<td>0.20</td>
<td>0.07 - 0.55</td>
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<tr>
<td>T1 or T2</td>
<td>12</td>
<td>0.20</td>
<td>0.07 - 0.56</td>
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<tr>
<td>T3 or T4</td>
<td>50</td>
<td>0.50</td>
<td>1.00 - 14.08</td>
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<tr>
<td>N2b</td>
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<td>0.58</td>
<td>0.30 - 0.93</td>
</tr>
<tr>
<td>N2b or greater</td>
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<td>0.61</td>
<td>0.33 - 1.24</td>
</tr>
<tr>
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<td>0.68</td>
<td>0.35 - 1.31</td>
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<tr>
<td>Pos Marg</td>
<td>13</td>
<td>0.16</td>
<td>0.09 - 0.27</td>
</tr>
<tr>
<td>No ECE</td>
<td>47</td>
<td>0.72</td>
<td>0.96 - 3.03</td>
</tr>
<tr>
<td>ECE</td>
<td>48</td>
<td>0.74</td>
<td>0.96 - 3.03</td>
</tr>
<tr>
<td>Pos Marg or ECE</td>
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<td>0.50</td>
<td>0.26 - 0.94</td>
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<td>Neg Marg / No ECE</td>
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<td>LVI</td>
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<td>No PNI</td>
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<td>0.91 - 2.27</td>
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<td>0.30 - 0.95</td>
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<td>WT-Splc-Indel</td>
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<td>0.58</td>
<td>0.39 - 0.99</td>
</tr>
</tbody>
</table>

*indicates significance of Log Rank p <0.05; Abbreviations: Neg Marg (negative surgical margins), Pos Marg (positive surgical margins), ECE (extracapsular spread of regional disease), LVI (lymphovascular invasion), PNI (perineural invasion), WT (wildtype TP53), WT-Other Mutations (wildtype T53, splice-site, insertion-deletion, and non-disruptive mutations), WT-Splc-Indel (wildtype TP53, splice site, or insertion-deletion mutation)
Kaplan-Meier survival estimates showed that TP53 mutation was associated with decreased DFS at 5 years (31.0% vs. 43.4%, p53 mutant group vs. the wild-type p53 group, respectively, Log rank p = 0.049) as shown in Figure-4. When TP53 mutations were classified into point mutations vs. splice-site or indel mutations and wildtype p53, point mutations were associated with worse outcomes for all three endpoints (Figure-5; Table-3). Kaplan-Meier plots displaying outcomes after further division of TP53 point mutations into non-disruptive and disruptive mutations showed that the patients with tumors bearing disruptive TP53 mutations had the worst outcome for all three endpoints (Figure-6 and Figure-7). As an independent factor, disruptive mutation was strongly associated with decreased DFS (HR 2.24, 95% CI 1.22 – 4.12; Log Rank p = 0.03) and DM (HR 3.62, 95% CI 1.49 – 8.85, Log Rank p = 0.009).

Figure-4. Overall (A.) and disease-free survival (B.) for patients with HNSCC tumors carrying wildtype TP53 versus mutant TP53.
In multivariate analysis using Cox proportional hazards models (Table-4), Stage T3 or T4 disease remained a significant factor associated with OS and DFS, and N2b or greater disease was associated with decreased OS and DM. Point mutation in TP53 remained a significant factor associated with poor outcome for all three endpoints after adjustment for other significant factors. When models using disruptive mutation were examined, disruptive mutation was strongly associated with DM (HR 4.0, 95%CI 1.61 – 9.90, p = 0.003).

Interestingly, the presence of positive surgical margins and/or ECE were not significant factors for any of the multivariate models examining this patient cohort.

**Figure-5.** Kaplan-Meier curves for Overall survival (A.), disease-free survival (B.), and distant metastasis (C.) for patients with tumors carrying wildtype TP53, splice-site or insertion/deletion (splice-indel) TP53 mutations, or TP53 point mutations (defined as either nondisruptive or disruptive).
**Figure-6.** Overall (A.) and disease-free survival (B.) for patients with HNSCC tumors carrying wildtype TP53, splice-site or insertion/deletion (splice-indel) TP53 mutations, nondisruptive TP53 mutations, or disruptive TP53 mutations.

**Figure-7.** Distant metastasis in patients with tumors carrying wildtype TP53 vs. mutant TP53 (A.) and in patients with tumors carrying wildtype TP53, splice-site or insertion/deletion (splice-indel) TP53 mutations, nondisruptive TP53 mutations, or disruptive TP53 mutation (B.).
Table 4. Multivariate analysis - factors associated with overall survival, disease-free survival, and distant metastasis. Two models were reviewed - one using p53 disruptive mutation as a prognostic factor, and a second model using TP53 point mutation (combined disruptive and -nondisruptive mutation vs. other patients) as a prognostic factor.

<table>
<thead>
<tr>
<th>Model</th>
<th>Overall Survival</th>
<th>Disease Free Survival</th>
<th>Distant Metastasis</th>
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</thead>
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<tr>
<td>Disruptive Mutation</td>
<td>Hazard Ratio (95% CI)</td>
<td>p value</td>
<td>Hazard Ratio (95% CI)</td>
</tr>
<tr>
<td>T1/T2 vs. T3/T4</td>
<td>5.18 (1.86 - 14.45)</td>
<td>0.002 *</td>
<td>4.49 (1.59 - 12.7)</td>
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<tr>
<td>N2b or greater</td>
<td>1.96 (1.10 - 3.49)</td>
<td>0.022</td>
<td>NS</td>
</tr>
<tr>
<td>Pos Marg or ECE</td>
<td>1.57 (0.80 - 3.097)</td>
<td>0.194</td>
<td>NS</td>
</tr>
<tr>
<td>Disruptive TP53</td>
<td>NS</td>
<td>1.76 (0.95 - 3.26)</td>
<td>0.073</td>
</tr>
</tbody>
</table>

| Disruptive Mutation       | Hazard Ratio (95% CI) | p value  | Hazard Ratio (95% CI) | p value |
| T1/T2 vs. T3/T4           | 5.08 (1.82 - 14.21)  | 0.002 *              | 5.40 (1.92 - 15.16) | 0.001 * | N/A                  | N/A     |
| Point Mutation            | 1.78 (1.04 - 3.03)   | 0.04               | 1.87 (1.10 - 3.19)  | 0.02     | 2.78 (1.14 - 5.77) | 0.03 *  |
| N2b or greater            | 1.97 (1.10 - 3.51)   | 0.02               | 1.98 (1.11 - 3.51)  | 0.02     | 3.14 (1.04 - 9.46) | 0.04 *  |

*indicates significance <0.05; Abbreviations: Pos Marg (positive surgical margins), ECE (extracapsular spread of regional disease), NS = not a significant factor in the model, N/A = not applicable (no patients with T1/T2 disease developed distant metastasis)

Specific Aim #2: To assess the ability of the AmpliChip® p53 test to identify TP53 mutation from formalin-fixed, paraffin-embedded specimens.

Patient Characteristics

Nineteen patients were evaluated with both TP53 AmpliChip® and Sanger Sequencing. The median follow-up for this group of patients was 28.16 months. The majority of tumors (17 of 19) were from the oral cavity. Thirteen patients had a significant smoking history and one patient had a history of chewing betel quid. The characteristics of this sample set are summarized in Table 5. Among these patients, 10 developed disease recurrence and 5 patients died during the follow-up period.

Table 5. Patient Characteristics

<table>
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<tr>
<th>N =</th>
<th>19</th>
</tr>
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<td>&lt;10 pack years</td>
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</tr>
<tr>
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<td>T1 or T2</td>
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<tr>
<td>IV</td>
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TP53 sequencing results

Sanger sequencing identified 12 mutations in 12 patients (41% of cohort). There were 5 missense mutations, 4 nonsense mutations, 1 splice mutations, and 2 deletions using this sequencing technique. TP53 AmpliChip® results were obtained from a single paraffin-embedded slide in all 19 cases. The average area of tumor from which DNA was isolated was 28.14 mm² (range 15 mm² - 84 mm²), calculated from the tumor area multiplied by the number of sections per slide. This method identified 17 mutations in 15 patients (79% of cohort). There were twelve missense mutations, 3 nonsense mutations, 1 splice mutation, and 1 deletion (frameshift) mutation using the AmpliChip®. The two sequencing techniques agreed in 11 of 19 patients (58%). The AmpliChip® identified 5 missense, 3 nonsense, 1 splice site, and 1 frameshift mutation (1bp deletion) that were also detected with Sanger sequencing, and the AmpliChip® identified an additional 7 missense mutations. The Sanger method identified 1 nonsense mutation and 1 two bp deletion that were not detected with the AmpliChip®. The two methods agreed upon 1 wildtype call. These results are presented in Table-6.
**Table-6.** Comparison of sequencing results of standard Sanger sequencing versus AmpliChip® p53 Test from HNSCC specimens. Specimen characteristics provided.

<table>
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<tr>
<th>Patient ID</th>
<th>% tumor total specimen</th>
<th>% tumor dense region</th>
<th>Resection to p53 Eval [months]</th>
<th>Paraffinization to p53 Eval [months]</th>
<th>Section thickness (um)</th>
<th>Tumor area (mm²)</th>
<th># of sections on slide</th>
<th>TP53 AmpliChip®</th>
<th>Sanger Sequencing</th>
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<td>G&gt;T GTT&gt;TGT</td>
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</tr>
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<td>2</td>
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<td>56</td>
<td>24</td>
<td>2</td>
<td>10</td>
<td>8</td>
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<td>Exon 8 Missense</td>
</tr>
</tbody>
</table>
Survival Analysis

Disease-free survival was calculated for the patient cohort comparing the outcome in patients with wildtype TP53 to those with mutant TP53 as determined by the two sequencing methods. When results from the Sanger sequencing method were used to stratify patients, the median disease-free survival for the wildtype TP53 group was 15.42 months, versus 33.03 months in the TP53 mutant group. When the TP53 AmpliChip® was used, the median disease-free survival was 35.08 months in the TP53 wildtype group versus 33.02 months for the TP53 mutant group. In both cases, a significant difference was not observed between the wildtype and mutant groups when survival curves were compared using the Log Rank test (data not shown).
Discussion

Several reports have linked TP53 mutation to poor outcome in HNSCC. The work by Poeta, et al. provides strong evidence that TP53 mutation, particularly disruptive TP53 mutation, is associated with decreased overall and progression-free survival(57). In their study, 131 patients received surgery alone and 203 patients received additional treatment. Seventy-eight patients received salvage surgical resection, suggesting surgery was not the primary modality of treatment in at least the majority of this subset. The variability in treatments provided to the patients in this study makes it difficult to determine for which patients TP53 mutation is most relevant. In addition, the pattern of relapse was not reported. An earlier study by Erber, et al., classified TP53 mutation into contact mutations (those that disrupted the core DNA-binding region) and structural mutations (those that alter the conformation of p53), a system defined according to the crystal structure of the p53 molecule(62). TP53 contact mutations were associated with poor outcome among 86 patients with HNSCC(45). Sixty-six of 86 (77%) of the patients in this study received PORT. A similar paper by Koch, et al., examined 110 patients who received either primary or adjuvant radiation therapy, and concluded that local-regional failure was associated with mutation in TP53(44). Most recently, Lindenbergh, et al. evaluated TP53 mutation and HPV-status among 141 patients with oral cavity or oropharyngeal squamous carcinoma who received either surgery or surgery and radiation(61), and determined that truncating mutations (defined as nonsense mutations, splice mutations, or frameshift mutations) were associated with the poorest outcome. This group found that the truncation categorization was
more discriminating than several other classification systems, including the disruptive/nondisruptive system supported by Poeta, et al(57) and by our study. The evidence to date has suggested that TP53 mutation may be a predictor for poor outcome among patients treated with PORT, however, there is debate as to which TP53 mutation classification system is most prognostic.

Our report is, to our knowledge, the first to focus on a group of HNSCC patients with locally-advanced disease treated uniformly with surgery and PORT. We have demonstrated that p53 point mutation is associated with decreased OS and DFS. In addition, thorough review of patterns of failure in our patient set enabled us to detect that disruptive mutation was strongly associated with the occurrence of distant metastasis.

Our study suggests that patients with HNSCC that carry TP53 point mutation, especially disruptive mutations, may require additional treatment to PORT in order to improve outcome. Multiple studies have shown the benefit of CCR for enhancing local-regional control and OS in advanced HNSCC in both the adjuvant and definitive treatment settings(6, 13, 14, 69). Similarly, induction chemotherapy may significantly reduce the rate of distant metastasis(69, 70). Presently, the efficacy and potential toxicity of induction chemotherapy followed by concomitant chemoradiotherapy is under study for patients with regionally advanced disease(71). More information is needed regarding the significance of TP53 as a biomarker in patients undergoing sequential induction chemotherapy and radiation or concomitant chemoradiotherapy. TP53 as a biomarker of virulent disease may
become an important selection factor for patients being considered for treatment with these combined therapeutic options.

Several studies have shown that HNSCC tumors that retain wild-type p53 are more sensitive to cisplatin-based chemotherapy regimens, whereas loss of p53 function is associated with resistance to cisplatin(72-74). Studies in ovarian cancer have shown that addition of taxane therapy to cisplatin can improve responses among patients with tumors carrying a mutation in TP53(75, 76). A recent phase II trial, RTOG 0024, reported promising results incorporating early post-operative paclitaxel followed by concurrent paclitaxel-cisplatin with radiation in patients with advanced, resectable HNSCC as compared to historical controls treated with concurrent cisplatin alone and post-operative radiation(77). Future prospective trials incorporating evaluation of TP53 mutation are necessary to determine if alternative drug regimens such as these can improve local-regional and distant control in the setting of tumors with mutations in TP53 treated with surgery and PORT.

Our study design did not aim at exploring the mechanism by which TP53 mutation affects tumor biology, thus we can only speculate as to why TP53 point mutation and disruptive mutations were associated with poor outcome. It is possible that PORT had a reduced effect in patients whose tumors had TP53 point mutations/disruptive mutations as compared to those with wild-type p53 or splice-site/indel mutations in our series. P53 is known to be a key regulator of the response to DNA damage, influencing several pathways leading to cell cycle arrest, DNA repair, autophagy, senescence, and apoptosis(36). Disruption of p53 leads to
altered regulation of each of these responses, which likely contributes to resistance to radiation therapy in tumor cells(78). Additionally, expression of mutant p53 can have gain-of-function activity, which has been reported to increase genetic instability and alter the response to DNA damage(79, 80). Preclinical evaluation of TP53 mutations and the response of HNSCC cells to radiation have led to mixed conclusions(81), and remains to be further elucidated.

Another interesting observation is the association of TP53 point and disruptive mutations with the development of DM in our study. Among the largest studies to evaluate TP53 in HNSCC, several have found an association between specific TP53 mutations and OS and DFS in HNSCC(45, 57, 61), but only one study examined DM, specifically(44). Koch, et al. did record DM in their report, but the proportions of patients who developed DM in the TP53 wildtype and the TP53 mutant groups were roughly equivalent, and no further evaluation regarding DM was reported. Two notable preclinical studies have described metastasis as a potential gain-of-function property of the of R172H-TP53 mutation in in vivo ‘knock-in’ mouse models of cutaneous(82) and pancreatic cancers(83). In our patient set, we have identified an association of TP53 point mutations and disruptive mutations with the development of DM. Whether gain-of-function TP53 mutations are contributing to metastasis in HNSCC remains to be elucidated.

A potential flaw of our study is that 8 patients (11%) had oropharyngeal cancer, and the HPV status of these tumors is not known. Notably, only 5 of these patients retained wildtype TP53. Five of the oropharyngeal cancer patients had no evidence of disease at last follow up, and two of these patients who did not recur
had tumors with mutations in TP53. Thus, there may have been HPV positive

tumors among our patient set, however the impact these patients may have had on

the survival outcomes evaluated in our study is likely negligible. This is in contrast
to previous reports linking TP53 mutation to poor outcome, in which substantial

numbers of patients with oropharyngeal cancer were evaluated (45, 57, 61). The

study by Lindenbergh, et al. (61) did evaluate HPV DNA and E6/E7 expression,

identifying HPV-related disease in 12 cases. The cohort in this study were all
treated with surgery or surgery and radiotherapy. As primary surgery is not the
standard recommendation for oropharyngeal cancer in the large majority of centers
in the United States, it is difficult to interpret the results regarding the 43 patients
with oropharyngeal cancer that were evaluated in this study. TP53 mutations in
patients with HPV-negative oropharyngeal squamous cell carcinoma have been
evaluated by Fallai, et al., and disruptive TP53 mutation did not appear to be
associated with poor outcome in this study (64), however further evaluation of this
patient population is necessary.

The evidence to date has supported TP53 as a potentially useful marker in
the management of HNSCC. If TP53 sequencing is to become part of the routine
diagnostic regimen, the question arises as to what methodology would best identify
TP53 mutations in tumor specimens when a patient is initially diagnosed with
HNSCC. Our pilot study of the TP53 AmpliChip® has demonstrated the utility of
this method. As the AmpliChip® identified mutations in 5 additional patients who
were called as wildtype with standard sequencing methods, it would appear that the
AmpliChip® is a more sensitive technique for identifying mutations, however
additional testing of these samples with a third chemistry is necessary to determine whether the results from the Sanger method or AmpliChip® are true in the discordant cases. One could argue that the Sanger method would have been more accurate if microdissection were performed on these samples, but the tumor volume necessary to obtain adequate DNA for this technique would required time-consuming microdissection of several slides given the small area of tumor present in these samples. Typical biopsy samples are small, and often contaminated with a significant amount of normal stroma or lymphoid tissue. Furthermore, initial biopsy material is often formalin-fixed and paraffin embedded prior to ultimate referral to the head and neck oncology team. Thus, the TP53 AmpliChip® appears to be a superior method given the ease of processing from readily available materials, as well as concerning the seemingly high accuracy of this method despite fixation and embedding of the tissue and the ability to call mutations in a significant background of wildtype contamination.

The AmpliChip® offers other potential applications, including TP53 sequencing from lymph node micrometastases, which often have a high degree of contaminating normal surrounding lymphocytes and stroma, and sequencing from fine needle aspiration samples. Testing of this method in these settings has not been undertaken and is a potential area of further study.

One could argue that the additional mutations identified in this pilot study represent false-positive mutation calls. This would seem unlikely given the identical calls made in the 11 concordant samples, but a third chemistry sequencing analysis (4-5-4 sequencing) from the DNA isolated after microdissection is currently
underway in order to determine if additional mutations called by AmpliChip® or the Sanger method were true positive calls. The fact that there was considerable discrepancy between the two methodologies used to determine TP53 status in our pilot study suggests that all studies that have examined TP53 in HNSCC should be reviewed with some caution and skepticism. Differing techniques in tissue processing, variability and inaccuracy in pathologic review to determine percentage of tumor, extent of microdissection, and methodologies used to detect TP53 mutations can each alter the proportion of false-negative TP53 mutation results, and has surely contributed to the variation in conclusions drawn from the existing literature studying TP53 in HNSCC. Our pilot study comparing the AmpliChip® and standard sequencing highlights this fact, and further comparative studies between TP53 mutation detection methods should be undertaken in order to arrive at a consensus as to the best method to obtain the most reliable TP53 sequence results in a cost-efficient manner.

Future Directions

In selecting patients with advanced, resectable disease who were treated uniformly with surgery and PORT, we have isolated a patient subset that is in need of new management options that improve outcome. We have shown that TP53 mutation can potentially be used as a marker to predict for a worse outcome and the development of DM, and the presence of TP53 mutation in advanced, resectable tumors may warrant escalated therapy. Validation of our findings and
those of others requires prospective evaluation of specific therapeutic interventions of site- and stage-matched patients with and without TP53 mutations. Current studies of HNSCC at UTMDACC designed to examine TP53 mutation have opened, and the author is aware of a recent European study that intends to stratify patients by TP53 mutation and examine the response to induction therapy with a taxane-based regimen.

Our work with the TP53 AmpliChip® suggests that this is a promising method that could be introduced into the clinical setting and be a powerful research tool that allows rapid and reliable evaluation of TP53 from large sets of archived FFPE samples. To further test this method, the author and supervisory professor have initiated a large retrospective study utilizing the AmpliChip® to evaluate TP53 from archived oral cavity squamous cell cancer specimens from patients treated at institutions from several regions of the world. The goal is to study both the prevalence of TP53 mutations, and the association of TP53 mutation with outcome in the setting of different risk-factors for this disease (e.g. tobacco smoke vs. alcohol use vs. betel quid use). TP53 AmpliChip® results will be validated in a subset of these patients with 4-5-4 sequencing in order to establish the error rate of this method in this patient population.

**Conclusions**

Patients with advanced, resectable HNSCC and point mutations in TP53, particularly disruptive mutations, have a poor prognosis if treated with standard
therapeutic approaches. Our findings need to be validated in prospective trials. If so, novel treatment strategies targeting the primary mechanisms of treatment failure must be developed. Consideration should be given to trials investigating the efficacy of systemic-dose level chemotherapy, either as an induction or possibly an adjuvant strategy, in an attempt to decrease the rate of distant disease recurrence in this subgroup. As support for TP53 mutation analysis as a biomarker in the management of HNSCC grows, there is a necessity for a reliable, rapid method of TP53 mutation detection from available tissue. Our pilot study examining the AmpliChip® p53 test demonstrates the potential advantages and applications of this method, particularly for HNSCC.
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Vita

Dr. Thomas J. Ow was born in Chicago, Illinois in 1978. He graduated from Lawrence University in Appleton, WI in 2000 with a Bachelor of Arts degree in Biology. He was awarded the Phi Sigma award in Biology, graduated with magna cum laude honors, and was a member of the phi beta kappa honors society. Dr. Ow went on to attend medical school at the Northwestern University Feinberg School of Medicine, where he received Alph Omega Alpha honors, graduating in 2004.

Dr. Ow completed an internship in general surgery in 2005 at Beth Israel Medical Center in New York City, and went on to complete residency training in otolaryngology-head and neck surgery at the Albert Einstein College of Medicine in 2009. He was subsequently accepted to a fellowship in advanced training in head and neck surgical oncology at the University of Texas, MD Anderson Cancer Center, where he has recently completed two years of research training under the mentorship of Dr. Jeffrey N. Myers, supported by the NIH T32 training grant. Dr. Ow's work has focused on the role of p53 mutation in head and neck squamous cell cancer. He was awarded the American Academy of Otolaryngology-Head and Neck Surgery/American Head and Neck Society Young Investigator Combined Award in 2010, receiving funding for a project entitled “TP53 gain-of-function mutation in head and neck squamous cell carcinoma”. In 2009, Dr. Ow enrolled in the Masters Degree program to study Patient Based Biologic Research at the
University of Texas, Graduate School of Biological Sciences. In 2010, Dr. Ow received his board certification in Otolaryngology-Head and Neck Surgery.

After completing his fellowship training, anticipated in June of 2012, Dr. Ow plans to care for patients as a head and neck surgical oncologist and continue pursuing his research interests in studying potential biomarkers and molecular mechanisms of tumor progression, metastasis, and treatment resistance in head and neck squamous cell carcinoma.