Impact of Genetic Targets on Therapy in Head and Neck Squamous Cell Carcinoma

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Abstract Despite advances in surgical technique, radiation therapy and chemotherapy, the mortality from head and neck squamous cell carcinoma (HNSCC) has not improved significantly. Squamous cell carcinoma is caused by tobacco use, alcohol consumption and infection with high-risk types of human papillomavirus. It is the 6th most common cancer in the world, with upwards of 45,000 new cases reported yearly in the United States alone.

In recent years, there has been a significant increase in the understanding of the molecular and genetic pathogenesis of head and neck cancer, shedding light on the unexpected heterogeneity of the disease. Genetic analysis has led to new classification schemes for HNSCC, with different subgroups exhibiting different prognoses. In addition, multiple targets in aberrant signaling pathways have been identified using increasingly sophisticated bio-informatics tools. Advances in technology have allowed for novel delivery mechanisms to introduce genetic material into cells to produce a therapeutic effect by targeting cancer cells via a number of different approaches.

A pressing need to develop novel therapies to augment current treatment modalities has led to a number of translational studies involving gene therapy in the treatment of HNSCC. This article will focus on a review of the most recent developments in molecular biology of head and neck squamous cell carcinoma in regards to possible targets for gene therapy, as well as the array of novel therapeutic strategies directed at these targets.

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Introduction

Despite advances in surgical technique, radiation therapy and chemotherapy, the mortality from head and neck squamous cell carcinoma (HNSCC) has not improved significantly. Although the world-wide incidence of HNSCC has been steadily declining over the past 20 years, it is still the sixth most common cancer by incidence worldwide [1]. Yearly an estimated 600,000 new cases of HNSCC are reported worldwide, with upwards of 45,000 new cases reported in the United States alone [2].

Prognosis in HNSCC is largely determined by the stage at presentation. Current staging relies on the TNM (tumor, node, metastasis) system and dictates treatment planning. Although presentation at an early stage in the disease is associated with a favorable outcome, this prognosis applies to only about one third of HNSCC patients [2]. The majority of patients unfortunately present with advanced disease. The most important prognostic indicator at time of presentation is presence of lymph node metastases, which decreases long-term survival by 50% [3].

Current therapy consists of multimodality treatment. Early stage tumors, i.e. those that do not have nodal metastases, can be treated either with radiation or surgical resection alone. Advanced tumors with presence of nodal or distant metastases require a combination of surgery, chemotherapy and radiation. The most current multimodality treatments yield a 5 year survival rate of about 60% for invasive cancer of the oral cavity and pharynx according to the Surveillance Epidemiology and End Results (SEER) data [4]. Up to one third of patients experience tumor recurrence and survival rates drop dramatically with failure of first line treatment. Disease progression is the primary cause of death, causing approximately 50% of the mortality associated with HNSCC [5].

Given the continuing high mortality associated with HNSCC, new treatment innovations are imperative. Gene therapy—the introduction of RNA or DNA into diseased cells to effect genetic expression profiles—offers the option of targeting specific tissues and ideally avoiding toxic effects on surrounding healthy tissue. The recent advances in biotechnology and microarray gene analysis have given us insight into the gene expression profiles associated with HNSCC. This body of work has highlighted the unexpected heterogeneity of the disease and has also identified possible genetic targets that can be targeted using gene therapy.

Current Therapy for HNSCC

The organs involved in HNSCC effect speech, swallowing, breathing, taste and smell. Preservation of structure and function in an anatomically limited and complex space continues to be a challenge in treatment of HNSCC. Radical resection and radiation both have lasting negative effects on organ function and quality of life.

Unfortunately the majority of HNSCC patients present for treatment with locally advanced stage III or IV disease. Treatment consists of a combination of chemotherapy, radiation or surgery. Post-operative chemoradiotherapy is used after surgical resection when the patient is at an increased risk for local and distant recurrence. Pre-operative chemotherapy is considered when the risk of metastatic disease is high. Patients who present with stage I or stage II disease are often treated with either radiation or surgery alone and have an excellent prognosis.

Radiation therapy continues to be the mainstay of treatment for oropharyngeal, advanced hypopharyngeal and laryngeal cancer. Recent advances have focused on fractionation schedules and the use of intensity-modulated radiation therapy to increase delivery of radiation to target areas while sparing surrounding structures and tissues in the hopes of preserving the function of vital structures. Chemotherapy, often administered concurrently with radiation therapy, is an integral part of treatment of locally advanced disease. Compared to radiation therapy alone, chemoradiotherapy provides an 8% absolute benefit [6]. Recent agents such as cetuximab appear to show promise and will be discussed later in this review. Additionally, surgical and reconstructive techniques continue to advance and improve preservation of structure and function.

HPV-Positive HNSCC

HNSCC affects mucosal surfaces of the oral cavity, oropharynx, hypopharynx and larynx. Traditionally, the most notable risk factors identified for HNSCC are alcohol consumption and various forms of tobacco use, the combination of the two having a significantly synergistic effect on carcinogenesis. More recently, HNSCC caused by exposure to human papillomavirus (HPV) has been increasing in incidence and has been identified as an independent subgroup in HNSCC [7].

The pathogenesis and clinical course of HPV-positive tumors is appreciably different from that of non-HPV infected HNSCC. HPV-positive HNSCC more commonly affects the oropharynx, specifically tonsils and base of tongue. A meta-analysis has shown that HPV genomic DNA was detected in 26% of all HNSCC by polymerase chain reaction [8]. Oropharyngeal cancers have a higher incidence, with 36% shown to be positive for HPV [8]. Patients with HPV-positive HNSCC are generally younger and have better performance status as compared to those with HPV-negative HNSCC. They are also less likely to have a history significant for alcohol and tobacco use. However, history of multiple sexual partners is a major risk factor for HPV-positive HNSCC and is consistent with the known transmission pattern for HPV.

HPV is a double stranded DNA virus that is most notorious for causing cervical cancer. There are multiple subtypes of HPV, with HPV type 16 and 18 being the most virulent. HPV 16 has been shown to be present in over 90% of HPV-positive tumors of the head and neck [8]. Individuals who are sero-positive for HPV-16 are at an increased risk (15-fold) for developing HNSCC. Progression through phases of mitosis is necessary for HPV infection and gene expression [9]. HPV DNA codes for expression of two oncogenic proteins, E6 and E7, which affect cell cycle regulation in infected cells.

E6 inactivates the tumor suppressor p53 while E7 inhibits pRb (retinoblastoma). E6 induces the ubiquitination of p53 and targets it for degradation. Absence of p53 causes deregulation of cell cycle checkpoints and DNA repair mechanisms in the face of DNA damage, leading to genomic instability and inhibition of apoptosis, thus allowing the virus to replicate. E7 ubiquitinates pRb and targets it for degradation. Absence of pRb releases E2F transcription factors and allows for S phase to proceed unchecked. Studies have shown that inhibition of E6 and E7 induces apoptosis and decreases cell viability, thus indicating that their expression is necessary for tumor survival [10]. Nonetheless, expression of E6 an E7 alone is not enough to cause malignant transformation and other genetic alterations may be required.

In addition to having a different molecular pathogenesis from that of non-HPV related HNSCC, HPV-positive HNSCC has been shown to have more favorable clinical outcomes [11]. These tumors appear to respond to chemoradiation therapy better than their non-HPV counterparts. Patients have better overall and progression-free survival and less locoregional recurrence. The reasons for this are unclear, but several potential lines for further investigation have been identified. Overall, HPV-positive tumors have less genome wide DNA copy number alterations and less genome-wide hypomethylation than HPV-negative tumors. Another proposal involves the tumor suppressor p53, which is involved in the pathogenesis of both HPV-positive and HPV-negative HNSCC. Although p53 is inhibited by E6 as part of the HPV mechanism of transformation, tumors that express E6 and/or E7 have wild type TP53 [12]. HPV-negative HNSCC, in contrast, frequently has TP53 mutations, and mutations in TP53 have been shown to be associated with a poor prognosis [13].

Given the difference in clinical outcomes as well as the pathophysiology responsible for disease in HPV-positive and HPV-negative HNSCC, it is prudent that HPV infection be identifiable in studies that evaluate the efficacies of new treatment modalities as it may serve as a confounder. Additionally, the presence or absence of HPV infection should serve to tailor future treatment planning. The gold standard for identifying HPV infection is based on identifying expression of E6 and E7 proteins. Immunohistochemical staining of tumors for p16 expression has also been shown to be a reliable surrogate for identifying HPV infection. A mediator of senesce and differentiation, p16 normally inhibits cyclins and cyclin-dependent kinases which regulate cell cycle progression. A hallmark of HPV infection is over-expression of p16 secondary to the effects of E7.

Non-HPV HNSCC

The most significant risk factors for non-HPV related HNSCC are tobacco use and alcohol consumption. Although the incidence of non-HPV related HNSCC has been decreasing, the disease carries an overall worse prognosis as compared to HPV-positive HNSCC. Non-HPV related HNSCC patients are generally older, have a worse performance status, and respond less well to treatment modalities.

It has been well documented that HNSCC progresses through a series of well-defined clinical and histopathological stages and that it typically arises in areas of preneoplastic change. In 1953, a seminal article by Slaughter et al. introduced the term 'field cancerization,' a concept describing the presence of histologically abnormal tissue surrounding oral squamous cell carcinoma [14]. Field cancerization was intended to explain several key observations made by the authors. It was noted that oral cancer develops in areas of pre-cancerous change and that similar histologically abnormal tissue surrounds the tumor. Additionally, abnormal tissue was noted to be present after tumor resection. It was thus postulated that the histological abnormalities present in the area surrounding tumor contributed to the high rate of second primary tumors as well as to local tumor recurrence.

Studies have shown that not all significant stages of the disease process in HNSCC are reflected in the histopathology. At the level of the genome, there appear to be an accumulation of genetic changes that are responsible for transforming normal squamous cell epithelium to invasive squamous cell carcinoma. Several key molecular biology concepts were used to recognize and organize these genetic changes into a meaningful model of disease progression. Loss of heterozygosity (LOH) is a genetic alteration that results in the loss or change in an allele of a gene whose other allele has already undergone a change or a loss of function. LOH can happen via multiple mechanisms including gene deletion, point mutation, chromosome loss, mitotic recombination and promoter methylation. LOH generally leads to inactivation of a gene, typically a tumor suppressor, such as TP53. In many tumor types, including HNSCC, p53 inactivation occurs in the transition from the preinvasive to invasive state [15]. To map the progression to LOH in key genes, microsatellites- or tandem repeat sequences were used as gene markers and analyzed by PCR.

Using the above concepts, recent studies have elucidated the multistep process of epithelial carcinogenesis in respect to the spectrum of chromosomal loss and alteration that propels benign hyperplasia to dysplasia, and eventually to invasive carcinoma and metastases. Califano et al. used microsatellite marker analysis to delineate a genetic progression model for HNSCC based on frequency of genetic changes in pre-invasive lesions and invasive tumors [16]. Several genetic events are required for this process. Alteration of chromosomal region 9p21 appears to be an early event and is found in 70–80% of cases of squamous cell dysplasia in HNSCC, making it the most prevalent genetic change [17]. In the Califano model, LOH at chromosomal region 9p21 is responsible for transition of normal mucosa to hyperplastic mucosa, and it is found in 30% of squamous cell hyperplasia [16]. The gene locus at 9p21 encodes for proteins p16 and p14, which are responsible for G1 cell cycle regulation and MDM2 mediated degradation of p53. Inactivation of p16 leads to an increase in phosphorylation of Rb, and thus progression from G1 to S phase of the cell cycle. In the progression from hyperplasia to dysplasia, it appears that LOH at 3p is the next most common genetic alteration, although the specific gene responsible is not yet known. LOH at 17p13 is responsible for inactivation of the tumor suppressor p53. Mutations in TP53 are common in HNSCC, with one study demonstrating that mutations are present in greater than 50% of tumors [13]. Alterations in tumor suppressor p53 are believed to be early events in transformation, with evidence

of these alterations being present in histologically normal mucosa adjacent to tumors. Notably, studies have shown that patients with p53 mutations have a poorer response to chemotherapy and an overall worse prognosis [13]. The next step in progression of HNSCC is transformation of dysplastic epithelium to carcinoma in situ, and this has been associated with amplification of 11q13 and over-expression of cyclin D1. This is noted in 30–60% of HNSCC and has been associated with poor prognosis, including an increased rate of lymph node metastases [18]. Cyclin D1 is responsible for phosphorylation of Rb and progression of cell cycle to S phase. The altered cells resulting from these numerous genetic changes carry a survival advantage and eventually displace or replace surrounding mucosa, leading to clonal expansion and field cancerization.

Improved understanding of the molecular pathogenesis of HNSCC, specifically as it relates to field cancerization, has raised concerns about the management of surgical margins after tumor resection. The high rate of recurrence has propelled several recent studies into examining the genetic changes in tumor margins. The concepts of clonal expansion and field cancerization dictate that the para-neoplastic field is also genetically altered, but likely in earlier stages of cancerization as compared to tumor. Ideally, the ability to identify these changes at surgical resection would allow for identification of patients who are at higher risk for recurrence and perhaps result in tailored treatment plans. Molecular analysis of surgical margins based on LOH showed that greater than one-third of lesions sampled contained genetic abnormalities associated with tumor progression in surgical margins that appeared to be histopathologically normal [19]. Additionally, in almost all cases, the genetic alterations between tumor and genetically altered mucosa left in the patient contained similarities [19]. This has raised significant concern for ability to provide clean margins at time of resection. Studies addressed the detection of genotypically abnormal cells at the surgical margins as this information can be used to identify cohorts with high-risk disease. Retrospective studies have shown that remaining surgical fields are a prevalent source of local recurrences and secondary primaries in HNSCC patients [20]. Brennan et al., using a sensitive TP53 plaque assay, demonstrated the presence of p53 mutations in 13 of 25 patients who appeared to have complete tumor resection on the basis of microscopically negative surgical margins [21]. Five of the 13 patients developed local recurrences whereas none of the 12 patients without mutations had recurrence of their tumor. More recently Van Houten et al. showed that p53 mutations were present in surgical margins regarded as negative by histopathology in 50 of 76 patients [22]. Nine of the patients developed recurrence and four patients developed locoregional disease. However, in the group of patients without p53 mutations, none developed recurrence and only one developed regional disease [22]. In a study published in 2009, Poeta et al. used TP53 LigAmp to assay for residual disease in tumor margins in 95 patients with HSNCC and identified TP53 mutations in an attempt to identify high-risk patients and predict recurrence [23]. Unfortunately due to a small cohort and limited number of recurrences, no survival benefit was identified in this study. Nonetheless, using molecular analysis of tumor margins to identify disease during the surgical procedure is ideal, and with future advances in technology and possibly additional biomarkers, this technique would serve as welcome adjunct to the treatment of HNSCC patients.

Epigenetics

Epigenetics, or the study of heritable changes in gene expression caused by mechanisms other than changes in the underlying genetic code, has greatly contributed to our understanding of cancer biology in recent years. Heritable changes to the DNA molecule occur through several mechanisms that alter gene expression, mainly DNA methylation and post-translational modification of histone proteins. Hypermethylation of promoters is a method primarily associated with inactivation of tumor suppressor genes by gene silencing, and combined with quantitative PCR it is a powerful tool in gene profiling. Recently, these tools have been evaluated in several studies for their diagnostic and therapeutic uses. Given that prognosis in HNSCC is poor when disease is diagnosed at advance stages, HNSCC screening modalities have been investigated. Carvalho et al. designed a study to evaluate for epigenetic changes that may be useful in identifying patients with early disease [24]. Using quantitative PCR, they evaluated promoter hypermethylation in a panel of 21 candidate tumor suppressor genes in a cohort of 211 HNSCC patients compared to normal controls. They designed several panels with a range of specificities and sensitivities that were able to improve rates of identification of early disease as compared to single marker analyses [24]. In another study, Goldenberg et al. used rapid quantitative methylation-specific PCR to analyze tumor margins intraoperatively in 13 patients with HNSCC [25]. The tumors underwent molecular analysis at time of biopsy for promoter hypermethylation of two target genes, and qualified patients underwent intraoperative tumor margin analysis by rapid PCR. The analysis required on average 5 h, which is an acceptable time period for complex tumor resection and reconstruction, and was as sensitive as standard PCR analysis.

Impact of the Tumor Microenvironment and Cancer Stem Cells

Stem cells play a major role in the biology of HNSCC. Unlike embryonic stem cells, adult stem cells are undifferentiated cells with a limited capacity for regeneration. They play a major role in tissue homeostasis and regeneration, however they have recently also been shown to play a major role in the biology of cancer [26]. Cancer stem cells are defined as a subset of tumor cells that exhibit the ability of self-renewal and multi-potency, serving as progenitor cancer cells [27].

In HNSCC, evidence suggests the existence of a small group of cells with distinct tumorogenic potential. Prince et al. showed that CL44 expression discriminates a sub-population of progenitor cells [28]. In a follow up study, they showed that aldehyde dehydrogenase activity also identifies a group of highly tumorogenic cells [29]. The combination of both markers revealed that 1–3% of cells from primary human HNSCC are capable of generating tumors [30]. Given that such a specific subpopulation of cells exists, the ability to identify cancer stem cells should lead to more specific and targeted cancer treatment.

Epithelial-to-mesenchymal (EMT) transition is the process that allows a polarized cell to assume a phenotype characterized by enhanced motility and invasiveness.

This process plays a role in embryogenesis and is involved in several pathologies, including cancer. Recent studies suggest that EMT is involved in the acquisition of cancer stem cell properties [31]. The loss of cell polarity is a critical step in EMT and is mediated by the loss of E-cadherin, which appears to be correlated with tumor progression [32]. The tumor microenvironment appears to contribute to the survival of stem cells. In head and neck cancer, the stem cells are found in close proximity to blood vessels, suggesting the existence of a perivascular niche [30]. Studies demonstrate that this niche is functionally relevant in HNSCC, suggesting that disruption of these communications may be used in the treatment.

Gene Expression Profiles in HNSCC

Gene profiling uses high throughput techniques such as cDNA microarrays and comparative genomic hybridization to perform genome wide analysis of specimens. These techniques allow us to compare the genetic profiles of a multitude of tumors or patients in attempts to identify differences in expression patterns. Correlating this information to clinical outcomes can set up paradigms that can be used to guide treatment selection.

Two studies have attempted to identify a group of genes that can be used to predict clinical outcome. A study by Chung et al. used a 75 gene profile to identify two clusters of patients with significant difference in recurrence free survival [33]. Additionally, they noted that genes involved in epithelial-to-mesenchymal transition were associated with high-risk tumors. Alternatively, Mendez et al. used an expression profile of 108 genes that were selected based on differences in expression between oral squamous cell carcinoma and normal epithelium, and identified a cluster of genes that correlated with a poor disease-specific and overall survival [34].

Gene expression profiling has also been used to predict lymph node metastasis and distant metastasis. In 2005, Roepman et al. reported a set of 102 genes that offered predictive value for lymph node metastases [35]. The series included 82 oral and oropharyngeal carcinomas that were separated based on absence or presence of lymph node metastases in lymph node dissection specimens. The predictive value of this profile was better than the clinical diagnosis and has been validated in an independent series.

Angiogenesis in HNSCC

Angiogenesis is the process that leads to the formation of new blood vessels and is the hallmark of tumor progression. The role of angiogenesis has been studied in many cancers and many agents are available and useful in the treatment of certain cancers. In the treatment of HNSCC however few trials have shown promising results.

Vascular endothelial growth factor A (VEGF-A) is the most well known agent associated with induction of angiogenesis. It is part of the platelet derived growth factor (PDGF) super family and its expression is induced by hypoxemia and mediated via hypoxia-inducible factor (HIF-1a) [36]. The VEGF family signals through several surface receptor tyrosine kinases, known as VEGFR. Through these receptors VEGF exerts its mitogenic, chemotactic and vascular permeabilizing effects on endothelial cells.

Angiogenesis has been shown play an important role in HNSCC. Expression of VEGF in HNSCC is associated with more advanced disease, increased resistance to cytotoxic agents, and poor prognosis [37]. A meta-analysis of 12 studies revealed that VEGF expression was associated with a twofold higher risk of death at 2 years [38].

There are at least two different molecular pathways for inducing angiogenesis in HNSCC [39]. Studies have focused on targeting these specific molecular pathways in attempts to block angiogenesis and consequently tumor growth. The best results against tumor growth were obtained with triple combination of two drugs such as an anti-EGFR agent (cetuximab), anti-VEGFR agent (sunitinib) and radiotherapy [40]. Other in-vivo studies have suggested that inhibition of angiogenesis alone is not enough to suppress growth of HNSCC and these treatments need to be integrated with different approaches [41].

Sorafenib and sunitinib are two tyrosine kinase inhibitors with activity against VEGF and PDGF receptors that have been tested in clinical trials in HNSCC. Sunitibib has been shown to have no significant activity in monotherapy against HNSCC in two studies [42, 43]. Another study showed limited activity of sunitinib at higher doses against recurrent HNSCC however this was associated with significant risk of hemorrhage [44]. Sorafenib, although well tolerated, also did not show a significant response rate, however when used in chemonaive patients, overall survival was comparable to that achieved with more toxic regimens [45]. Lastly, an anti-angiogenic agent in combination with an EGFR inhibitor showed response to therapy. This study also identified a molecular biomarker that could predict greater likelihood of response to anti-angiogenic treatment: an exciting idea that warrants further study [46].

Gene Therapy Approaches

Although several different gene therapy approaches have been investigated in attempts to augment treatment of HNSCC, the most notable and successful therapy has been aimed at epidermal growth factor receptor (EGFR). EGFR is a tyrosine kinase that signals through the Ras-MAPK, PI3K-PTEN-AKT and phospholipase G pathways. These pathways are associated with cellular proliferation, apoptosis, angiogenesis and invasion (Fig. 1). EGFR dysregulation can induce cell proliferation and block apoptosis, and it has been identified as a key component in invasion and metastases [47]. EGFR is over-expressed in more than 80% of HNSCC [48].
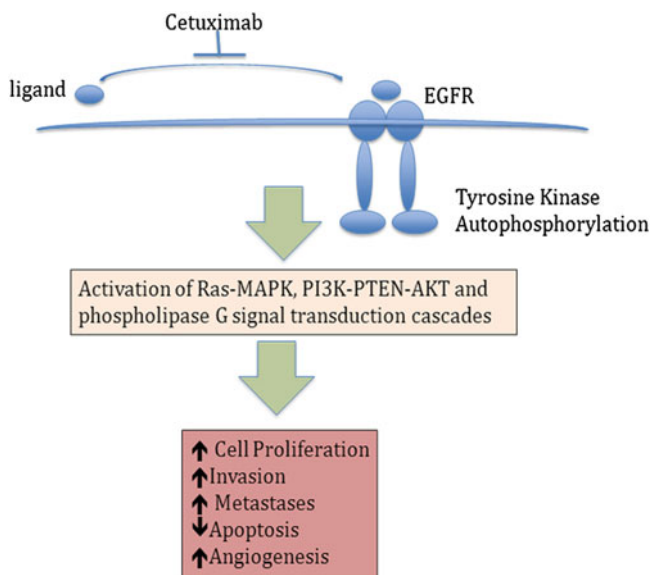
Fig. 1 Cetuximab is a monoclonal antibody that targets EGFR, a tyrosine kinase receptor which signals through the Ras-MAPK, PI3K-PTEN-AKT and phospholipase G pathways

An EGFR-associated expression profile has been noted to show a poor prognosis [49]. Mutations in the EGFR gene are rare; protein over expression is often responsible for activation of the pathway in HNSCC [50].

Cetuximab, a monoclonal EGFR specific antibody, has recently been the focus of multiple trials as a treatment modality for HNSCC. Cetuximab has been approved by the FDA for use in patients with platinum resistant disease [51]. When used as a single agent in patients with recurrent or metastatic head and neck cancer that was resistant to platinum, cetuximab resulted in a 46% rate of disease control—including complete or partial response or stable disease. As first line therapy in patients with metastatic disease or in patients with recurrent HNSCC, cetuximab plus fluorouracil and cisplatin prolonged progression free survival [51].

Cetuximab has also been approved for use in combination with radiation therapy in previously untreated patients [52]. In combination with radiation therapy, cetuximab improved locoregional control and overall and progression-free survival in patients with locally advanced disease [52]. The risk of death was decreased by 26%. The rate of metastases was unaffected [52].

Conclusion

Given the high mortality rate in HNSCC with current treatment and the associated side effects of aggressive treatment, new options for treatment are imperative. Gene therapy offers the option of targeting specific tissues and ideally avoiding toxic

effects on surrounding healthy tissue. Advances in molecular biology and biomolecular technology will help us diagnose HNSCC disease earlier and expand our scope of options for treatment.

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