Animal Models to Study the Mutational Landscape for Oral Cavity and Oropharyngeal Cancers

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ABSTRACT

Objectives: Cancer is likely caused by alterations in gene structure or expression. Recently, next generation sequencing has documented mutations in 106 head and neck squamous cell cancer genomes, suggesting several new candidate genes. However, it remains difficult to determine which mutations directly contributed to cancer. Here, summarize the animal models which have already validated and may test cancer causing mutations identified by next generation sequencing approaches.

Material and Methods: We reviewed the existing literature on genetically engineered mouse models and next generation sequencing (NGS), as it relates to animal models of squamous cell cancers of the head and neck (HNSCC) in PubMed.

Results: NSG has identified an average of 19 to 130 distinct mutations per HNSCC specimen. While many mutations likely had biological significance, it remains unclear which mutations were essential to, or "drive," carcinogenesis. In contrast, "passenger" mutations also exist that provide no selection advantage. The genes identified by NGS included p53, RAS, Human Papillomavirus oncogenes, as well as novel genes such as NOTCH1, DICER and SYNE1,2. Animal models of HNSCC have already validated some of these common gene mutations identified by NGS.

Conclusions: The advent of next generation sequencing will provide new leads to the genetic changes occurring in squamous cell cancers of the head and neck. Animal models will enable us to validate these new leads in order to better elucidate the biology of squamous cell cancers of the head and neck.

Keywords: head and neck neoplasms; pain, postoperative; analgesics, opioid; pain measurement; systematic review.

INTRODUCTION

While our armamentarium used to treat squamous cell cancers of the head and neck (HNSCC) has greatly expanded with the addition of chemoradiation [1], intensity modulated radiation therapy (IMRT) [2], and biological agents [3], the progression free survival rates have increased only slightly while the overall survival (OS) rates have stagnated [4]. Cancers afflicting the oral cavity (OCC) are especially prone to locoregional failure even after aggressive surgery followed by radiation and/or chemotherapy [5,6]. Aside from platinum-based chemotherapies, there has been little development of new systemic agents equipped to eradicate microscopic OCC disease. For example, a major randomized trial investigating the use of an epidermal growth factor receptor (EGFR) antagonist concurrent with radiotherapy in patients with HNSCC did not include those with OCC [3]. This lack of targeted therapies, coupled with the aggressive nature of OCC, highlights the need to identify genes that drive this malignancy in order to identify new targets.

In contrast to OCC, oropharyngeal cancers (OPC) behave more favourably and also frequently demonstrate a distinct gene expression signature. In general, cure rates using standard therapeutic regimens are 10% higher for OPC than for OCC [4]. This difference may partially be explained by the impact of the Human Papillomavirus (HPV), which has been responsible for an OPC epidemic [7]. Those with HPV-positive OPC have a 5-year OS of 70 - 90%, while those with HPV-negative OPC have a 5-year OS of less than 50% [8]. Furthermore, HPV-negative HNSCCs often have mutations in p53, a major tumour suppressor protein controlling genomic integrity, and a correspondingly worse prognosis [9]. Given the more favourable outcomes of patients with HPV-positive OPC, there is a push to de-escalate their treatment [10]. In addition, the unique molecular signature of HPV may enable better ways to target this disease specifically. Therefore, in contrast to OCCs, understanding the distinct mutational landscapes in OPCs may enable us to identify new molecular targets and, therefore, to de-escalate the toxicities associated with the current, non-targeted cytotoxic chemotherapies.

Much of our previous knowledge regarding the molecular characteristics of HNSCC was derived from expression microarrays or other assays quantifying gene expression [11-15]. With half of the studied cancers derived from the oral cavity or oropharynx, these studies demonstrated at least four unique expression patterns in HNSCC, including: an EGFR, a mesenchymal, an epithelial, and an anti-oxidant expression pattern [11].

Most tumours with the EGFR expression pattern recurred within 2 years, while the majority with the epithelial or anti-oxidant pattern never recurred. Furthermore, these expression signatures could differentiate between HPV-positive and HPV-negative cancers, as well as prognosticate responses to therapy [13,15,16]. Nevertheless, these microarray expression patterns could only implicate a large set of genes involved in HNSCC and have difficulty pinpointing the exact genes driving this disease.

In addition, others have used cytogenetic approaches to identify structural changes in chromosomes [16-20]. These studies support the model whereby HNSCC carcinogenesis begins through two distinct pathways: one caused by chemical carcinogens and the other by HPV oncogenes [20]. Later, these pathways share common chromosomal alterations during progression to invasive cancer. However, because these chromosomal changes are only detected on a megabase-pair level, the changes affecting the exact genes that drive the development of HNSCCs remain largely unknown. Therefore, much of our knowledge of genes that drive HNSCC remains limited to p53 mutations, HPV oncogenes, and the EGFR pathway. There may exist additional undiscovered driving mutations that may one day serve as new targets for novel therapies.

The goal of this article is to review recent trends in identifying HNSCC "driver" mutations, especially those occurring in OCCs and OPCs. We define "driver" mutations as mutations in genes that confer a selective advantage to a clone enabling it to better survive or proliferate. This contrasts with "passenger" mutations that have little if any advantageous effect. We will first discuss the use of next generation sequencing (NGS) to catalogue point mutations prevalent in HNSCC. Next, we will review how some of these mutant genes have already been validated in genetically engineered mouse models (GEMM). Finally, we will discuss how GEMMs may complement NGS by testing novel mutations identified by NGS as well as identify pathways observed in NGS analysis. Thus, this review will examine recent trends in the identification and validation of novel targets, which may revolutionize our understanding of HNSCC biology and usher in innovative treatment strategies.

MATERIAL AND METHODS
Literature Search

In the present article, the authors discuss ways to identify genetically engineered mouse models that supported the recent identification of mutant genes which likely acted as "driver" mutations in HNSCCs. We searched

relevant articles on PubMed (www.ncbi.nlm.nih.gov) regarding next generation sequencing and genetically engineered mouse models for head and neck cancer from 1990 to present. First, to identify mutant genes identified by NGS in HNSCC samples we performed two searches. We queried (1) "genome sequencing" head and neck cancer and (2) "exome sequencing" head and neck cancer which returned 10 and 4 results, respectively. We selected two articles that specifically described NGS (specifically whole exome sequencing) in HNSCC [21,22]. From these articles, we compiled a list of commonly mutated genes and searched whether each gene has been described in an autochthonous head and neck cancer model using the query terms "transgenic mice head and neck cancer." The specific genes searched and the resulting citations are: TP53, 55 citations; TP63, 7 citations; SYNE1,2, 0 citations; NOTCH, 4 citations; HPV, 21 citations; PI3KCA, 0 citations, PTEN, 11 citations; RAS, 53 citations; pRB, 13 citations; FBXW7, 0 citations, RIPK4, 0 citations; DICER, 0 citations. In order to identify additional GEMMs for oral cavity/oropharynx cancer, we also searched all citations using the terms "transgenic mice head and neck cancer" that resulted in 392 total articles. We reviewed all article abstracts and selected articles describing GEMMs targeting genes subsequently identified in NGS of HNSCCs. We also selected articles describing GEMMs of genes not identified by NGS to discuss pathways important in cancer but possibly missed by this approach. Given the breadth of genomic analysis in HNSCCs, we regret any omissions of GEMMs for oral cavity/oropharynx cancer.

RESULTS
Next generation sequencing (NGS) for oral cavity and oropharynx cancers (OCCs and OPCs)

Conventional or "low throughput" DNA sequencing provides one sequence read per DNA sample. With this technique, the DNA sample requires a homogeneous DNA template to decipher a maximum sequence of approximately 1000 base-pairs (bp) long. Given that the human genome contains 3.5 billion bp encoding 10 to 30,000 genes, it is not surprising that a single genome once required approximately 13 years and three billion dollars to sequence [23]. NGS can complete the same task in days, at a cost of approaching a few thousand dollars. Its efficiency has revolutionized the sequencing of entire genomes or, more commonly, "exomes" (genomic libraries limited to a cell's expressed sequences) [24]. This has allowed investigators to catalogue mutations in over twenty malignancies, including brain, breast, and prostate cancers (The Cancer Genome Atlas:

http://cancergenome.nih.gov/).
NGS is essentially multiple "low throughput" DNA sequencing reactions run in parallel in a single sample. First, DNA or mRNA is isolated from a tumour or tissue. Since the genomic or exomic sequences can be very long, the DNA or cDNA is cut or sheared by mechanical means to generate many smaller fragments. Each individual DNA molecule is then amplified in order to enhance subsequent DNA sequencing detection. These amplified DNA clones are then sequenced in a massively-parralled fashion to generate multiple short DNA sequences or "reads". These shorter reads are then aligned using computer software in order to determine the longer genomic or exomic sequence.

In regards to HNSCC, the Cancer Genome Atlas contains 312 separately sequenced HNSCC genomes at the writing of this article. Furthermore, two recent publications by Agrawal et al. [21] and Stransky et al. [22] have also detailed common mutations in 106 HNSCC samples. Here, the authors performed whole exome sequencing that can identify coding as well as splice-site mutations. However, this approach is limited because cancer causing mutations can occur in non-protein coding regions that affect the regulation of gene expression, and may cause altered expression of a normal gene in a tissue/cell type or at a temporal point in time that is different than in normal cells. Of note, in the study by Stransky et al. [22], 50 of 77 samples were of OCC or OPC. Both publications compared sequences from tumour specimens and those of autologous tissue controls, and samples were micro-dissected to minimize contamination by adjacent stroma. The authors called mutations in tumours if the genetic changes were detected in the tumour but not the autologous control tissues. Subsequently, many of these mutations were further validated in separate cohorts or by additional sequencing and mass spectrometric genotyping analysis. Interestingly, the average number of mutations differed by almost ten-fold (19 [21] vs. 130 [22]) with each group reporting a variety of mutations per tumour. This variation in mutations may reflect technical differences, such as differences in sample size (32 samples vs. 74 samples), as well as the degree of equence coverage for each study (44 to 77-fold vs. 150-fold) which can affect sequence accuracy. In addition, Agrawal et al. [21] analyzed tumours harvested prior to chemotherapy or radiation, which can select for additional mutational events [25,26] and also account for the lower average number of mutations in their study. Finally, these differences may reflect differences in the accumulation of mutations due to cancer progression, additional DNA damage or different stages of cancer. Nevertheless, it remains unclear which mutations were driving carcinogenesis.

NGS has generated unexpected insights. While the finding that tumours arising from smokers had more mutations than those of non-smokers was expected, two tumours from non-smokers had the highest number of mutations in one study, suggesting genomic instability in HNSCC may not be entirely tobacco dependent [22]. Certain germline genetic conditions, such as Fanconi Anemia, can affect DNA repair pathways and predispose individuals to HNSCC. Therefore, these instances of increased mutations and genomic instability may be due to various Mendelian cancer syndromes in addition to carcinogen exposure.

Consistent with epidemiologic studies suggestive of biological differences based on HPV status, HPV positive tumours contained approximately half the number of mutations as HPV negative tumours, independent of smoking status. In addition, TP53 mutations were inversely correlated with HPV positivity and found in up to 78% of HPV-negative tumours [21]. In fact, Westra et al. [27] has shown an inverse relationship between p53 mutations and HPV positivity in HNSCC. Compared to 25% of HPV-positive tumours, 52% of HPV-negative tumours had p53 mutations. Furthermore, only HPV-negative tumours had mutations that disrupted p53 function suggesting that most p53 mutations resulting in a functional significance were exclusive of HPV. Therefore, NGS will continue to identify potential genes that are advantageous for HNSCCs and further elucidate those already known such as HPV oncogenes or mutations in p53.

Overall, genes previously implicated in HNSCC and confirmed by whole-exome sequencing include TP53, CDKN2A, HRAS, PTEN, PI3KCA and RB. In multiple studies, the most commonly dysregulated gene by far was TP53 [21,22]. In addition, NGS has identified new mutations in genes that regulate epithelial differentiation in up to 30% of tumours. This includes newly discovered mutations in NOTCH1, IRF6, TP63 and FBXW7. Inactivating point mutations in NOTCH1 are particularly noteworthy; in one study, point mutations affecting this gene occurred in 11% of the HNSCC tumours and focal deletions were seen in two additional tumours [22]. Importantly, the identification of Notch genes and others may represent the first new targets implicated in the genesis, as well as treatment, of HNSCC.

While advancements in sequencing may further pinpoint the structural changes causing head and neck cancer, these techniques, like previous technologies, fail to separate those changes that drive HNSCC and those passenger mutations that provide no selection advantage. Validation of driver mutations requires additional *in vivo* and *in vitro* models to confirm and to understand their importance in the biology of this disease.

Validation of next generation sequencing (NGS) with existing genetically engineered mouse models (GEMMs)

Using information gleaned from NGS, we may better understand the physiological significance and molecular mechanisms of several candidate genes driving the development of HNSCCs. Previous mouse models of HNSCC relied mainly on chemical carcinogens such as coal tar, cigarette smoke, 9,10-dimethyl-1,2-benzanthracene (DMBA), and 4-nitroquinoline 1-oxide (4NQO) [28,29]. Over the last 20 years, GEMM have been developed to study how changes in the structure or expression of specific genes impact HNSCC development *in vivo*. These mice have been further engineered to express these altered genes in a tissue-specific and temporal manner. Below, we will describe some of these previously known mutations identified in NGS that also cause HNSCCs in GEMMs. These observations indicate that GEMMs can be used to test whether novel mutations identified by NGS "drive" HNSCCs.

Oncogene P53

TP53 is one of the most frequently mutated genes in human cancers, including OCC [9]. Loss of TP53 function may be nearly universal event in the development of HNSCC by mutation, deletion, amplification of MDM2, deletion of CDKN2A or expression of HPV oncogenes [30]. In addition to p53 deletion, the more common way of inactivating p53 in OCC is by mutation, which induces both dominant negative, as well as lesser understood gain-of-function mutations [31]. With gain-of-function mutations, the tumour suppressive activities of p53 are inhibited, while other potentially progrowth functions of p53 are maintained. In fact, these gain-of-function mutations in p53 predict for worse outcome in HNSCCs [9].

The loss of p53 has been shown to be an initiating event in mouse models, where its deletion predisposed *de novo* tumour formation and greatly sensitized mice to chemical carcinogens [32-34]. However, the majority of mice with p53 deletion or mutations in all tissues died rapidly due to lymphoma or other cancers before the impact of their p53 defects caused development of HNSCC or squamous cell carcinomas (SCC) at other cutaneous sites. In murine models where p53 defects successfully led to HNSCC development, mice with mutations or loss of p53 were treated with carcinogens or bred to mice with additional genetic abnormalities. HNSCCs developed in mice which possessed p53 defects in the germline or when p53 was conditionally disrupted in the basal epithelial layer of the oral cavity

and skin. To conditionally delete p53, mice expressing Cre recombinase expressed under the Keratin 5 [35] or Keratin 14 [36] promoter which is active in the basal keratinocyte layer of the epithelium were bred to knock-in mice possessing floxed p53 alleles. Since Cre recombinase was expressing in the basal keratinocyte layer, the mice possessed disrupted p53 pathways in the epithelium of their skin and upper aerodigestive tract. Transgenic mice expressing dominant negative p53 or mice with p53 haploinsufficiency in the germline experienced accelerated HNSCCs after 4-NQO treatment compared to wild type mice [37,38]. In addition, mice that lost p53 expression or had p53 gain-of-function mutations in the basal keratinocytes of the oral cavity developed invasive HNSCC when tumours also expressed a mutant KRAS gene [35,36]. These results confirmed the clinical observations where loss or mutation in p53 was an important event in at least 50% of HNSCCs.

Loss of TP53 and HPV-positivity appear to be exclusive events. In one study using whole-exome sequencing, TP53 mutations were not identified in any of the HPV associated tumours but were found in 78% of the HPV-negative tumours [21,22]. As such, investigation of HPV oncogenes will likely provide future insights into a distinct subset of tumours and will be described below.

Human Papillomavirus (HPV)

HPV-associated cancers likely arise due to the expression of the viral oncoproteins E6 and E7 [39,40]. E7 binds to and inhibits the retinoblastoma protein (Rb) enabling cells to progress through the cell cycle and to divide [41,42]. However, abnormal cell division usually activates p53, which induces cell apoptosis. HPV relies on E6 to bind p53 and to degrade it, enabling HPV infected cells to escape this safeguard [43]. Mice expressing high risk HPV16 oncogenes, E6 and E7, from the promoter of the bovine keratin 6 gene develop focal epithelial hyperplasia on the tongue by 27 weeks of age [44]. However, no tumours develop in these mice suggesting E6 and E7 alone were insufficient to drive tumourigenesis. Furthermore, epithelial cells derived from HPV16 transgenic or from HPV18 immortalized cells cannot form independent colonies in soft agar or tumours in syngeneic or immuno-compromised mice unless they are extensively cultivated *in vitro* [45]. Other available HPV transgenic mice that target expression via the αA crystallin and keratin 14 promoter have a low incidence of epithelial malignancies that develop after 15 months in only 5 - 10% of mice [46]. However, tumour development in the oral cavity has not been noted [47-50]. Taken together, these studies indicate oncogenes E6 and E7 from high-risk HPV

can immortalize epithelial cells but additional genetic events are required for transformation.

While E6 and E7 alone are not sufficient to drive tumour formation, mice that co-express mutant RAS or those exposed to chemical carcinogens are highly susceptible to the development of tumours of the oral cavity. Schreiber et al. [51] demonstrated strong synergy between the mutant HRAS and HPV16 E6/E7. In this model, mice expressing HRAS driven by the zeta-globin promoter, were crossed with transgenic mice that express HPV16-E6/E7 in epithelial tissues using a keratin 14 promoter driven. Double transgenic mice developed dysplastic squamous papillomas of the transitional epithelium that involved the mouth, eye and ear beginning around 3 months of age. Furthermore, K14-HPV-E6/E7 mice treated with 4NQO, a chemical carcinogen, developed oral SCC [52]. Importantly, these E6/E7 driven tumours resembled the molecular characteristics of human HPV-positive OCC, including overexpression of p16, a surrogate for HPV infection. In addition, minichromosome maintenance protein 7 (MCM7) was overexpressed in this model of HNSCC, verifying a previous study on human cervical cancer [53]. Although E7 may play a more prominent role than E6 with regard to long term carcinogenesis [54], the development of HNSCCs in mice likely required a synergy between E6 and E7 [55]. It is believed that E7 may be the predominant initiating oncogene whereas E6 is thought to play a more important role in the progression to malignancy. In addition, E7 likely targeted multiple RB family members to cause HNSCC as deletion of both p107 and Rb recapitulates many features of HPV-16 E7 mice after 4NQO treatment [56]. Therefore, the development of HPV-positive HNSCCs require both the inhibition of p53 pathways and RB family members by HPV E6 and E7 respectively as well as additional mutagenic events.

To study the contribution of other genes to the development of HPV-associated cancers, several reports have studied mice that express HPV oncogenes and that harbour additional defects in other cellular genes. Compared to the general population, Fanconi Anemia (FA) patients who reach 50 years of age are more likely to develop a solid tumour [57-60] where the majority of these tumours are squamous cell cancers (SCCs) involving the head and neck [59]. In a study by Kutler et al. [61], 84% of SCCs in FA patients, of which the majority had HNSCCs, tested positive for HPV and none of these SCCs had p53 mutations. By contrast, van Zeeberg et al. [62] did not detect HPV signatures in HNSCCs but did demonstrate that two-thirds of anogenital cancers contained HPV DNA. Although the aetiology of HNSCCs in FA patients remains a hotly debated topic, it is likely that HPV plays an important

role in this process. This relationship was demonstrated by Park et al. [63] who showed that mice expressing HPV16-E7 and deficient in the FA gene FANCD2 developed SCCs of the tongue and oesophagus at a higher frequency than that observed in control mice. Here, the HPV oncogenes were driven by a K14 promoter and expressed in the basal epithelium of the oral cavity and skin. Therefore, HPV oncogenes may cooperate with other cellular genes to cause HNSCC and other cancers.

Mutant RAS oncogene

Although mutations in RAS are present in only 4 - 5% of HNSCC [21,22], alterations of in RAS signalling occurs frequently in cancer. This often includes amplification of chromosome 7p11, the locus for EGFR and a downstream mediator of RAS [64]. In addition, promoter methylation of RASSF1A, a negative regulator or Ras, is frequently observed in OCC [65,66]. Parallel to these observations, mice expressing a G12D mutation in KRAS developed benign squamous papillomas of the oral mucosa, tongue and palate by 16 - 24 weeks [67]. Although highly proliferative, these papillomas never progressed to malignancy suggesting a role for KRAS in the initiation, but not progression to SCC. In another study, mice expressing mutant KRAS in the basal epithelium developed papillomas exclusively located within the oral mucosa [36]. Again, these papillomas failed to progress to carcinoma. However, mice possessing KRAS and mutant p53 did progress to invasive SCCs. This indicates that RAS requires other factors to increase genomic instability and that this ultimately can lead to the development of frank malignancy. Several groups have used these models to study novel therapies for HNSCCs. For example, rapamycin prevented tumour progression of benign or malignant tumours in mice possessing mutant KRAS, with or without loss of p53, respectively [36]. This preclinical model parallels recent finding using this small molecule inhibitor in HNSCC patients [68]. Similarly, Samuel et al. [69] showed that deletion of RAC1 prevented oral papilloma development in mutant KRAS mice, providing another possible therapeutic target for mutant KRAS mice. Studies such as these with mutant KRAS mice demonstrate that genetically engineered mice can be used to identify novel targets and therapeutic regimens for HNSCCs.

Using genetically engineered mouse models (GEMMs) to test for "Driver" mutations identified by next generation sequencing (NGS)

The significant amount of next NGS data provides a starting point to develop novel *in vivo* models for HNSCC in order to better understand the biology and treatment of this disease. Table 1 compares the genes involved in HNSCC identified with NGS and/or GEMMs. As mentioned above, NGS identified inactivating mutations in the NOTCH gene family in 22% of the samples. Originally described in Drosophila, NOTCH family members are transmembrane proteins that regulate cell-cell communication and differentiation. NOTCH mutations consistently mapped to the transactivating C-terminal ankyrin repeat domain. The predicted effect of this mutation is a truncation resulting in a loss-of-function mutant [21,22]. Additional mutations occurred in the extracellular ligand binding domain and splice junctions that were also likely inactivating in nature. These mutations are similar to those recently described for myeloid leukaemia [70] but contrast sharply with NOTCH activating mutations observed in other lymphocytic leukaemia's and lymphomas [71,72]. These results suggest that NOTCH mutations may be context dependent whereby NOTCH inhibition may promote some cancers while inhibiting others.

Along these lines, a clinical trial using a NOTCH inhibitor was stopped due to an unanticipated consequence of increased cutaneous cancers [73]. Similarly, mice with a tissue specific deletion of NOTCH1 in the skin resulted in corneal hyperplasia and skin tumours as early as 8 months post inactivation [74]. Furthermore, DMBA treatment accelerated tumour formation and frequency. This group suggested that loss of NOTCH1 drove skin cancers by elevating β-catenin possibly resulting in de-differentiation of epithelial cells. As no respective GEMMs exist for Notch driven OCC, the precise role of the NOTCH gene family remains unclear in HNSCC and may be context-dependent.

Using NGS approaches, Stransky et al. [22] confirmed previous observations that Cyclin D1 was also amplified in 22% of HNSCC samples. The Cyclin D family promotes cellular proliferation by enabling cells to enter the S phase of the cell cycle in order to synthesize DNA and prepare for cell division. Amplifications or overexpression of Cyclin D1 frequently occurs in SCC leading to dysregulation of the cell cycle [75]. In transgenic mice, expression of Cyclin D1 was directed to the oral-oesophageal squamous epithelium using part of the Epstein-Barr virus ED-L2 promoter (L2-CD1). Expression of Cyclin D1 caused hyperplasia of the basal and suprabasal epithelia of the tongue, oesophagus and forestomach [76,77]. These mice were treated with 20 to 50 ppm of 4NQO for 8 weeks and then observed for an additional 16 weeks. Half of the L2-CD1 mice treated with 50 ppm of 4NQO, exhibited SCC of the tongue and oesophagus by 16 weeks.

Table 1. Non-chemically induced transgenic mice models of oral cavity cancers (OCC)

Pathway/ Gene family	Gene	Function	Freq. in NGS	Epithelial mouse model genotype	Tumour type	Reference
p53 family	TP53	Tumour suppressor involved in apoptosis, activates DNA repair proteins, cell cycle regulation at G_1/S checkpoint	50 - 78%	p53$^{-/-}$ or mutant p53 mice with chemical carcinogens or mutant KRAS	Various HNSCC	Acin, Raimondi [35,36]
	TP63	Involved in development and regulation of apoptosis	8%	Not described	-	n/a
Nesprins	SYNE1,2	Found in the outer nuclear membrane and bind to actin filaments	24%	Not described	-	n/a
Notch	NOTCH1,2,3	Transmembrane proteins that are involved in development by controlling cell fate decisions by regulating interactions between adjacent cells.	22%	NOTCH1$^{-/-}$	Basel Cell Carcinoma	Nicolas [74], Agrawal [21]
HPV Oncogenes	E6	Degrades p53 through ubiquitination	15%	HPV-E6/E7 with chemical carcinogens or mutant Hras	Papillomas of the lip	Schreiber [51]
	E7	Binds pRb to free the transcription factor E2F				
PI3K/AKT/ mTOR	PIK3CA	Oncogene	8%	Not described	-	n/a
	PTEN	Tumor suppressor that regulates AKT	8%	myrAKT PTEN$^{-/-}$	Dysplastic lesions in the palate, cheeks, and lips	Bian, Moral [80,82]
TGF-β pathway	TGF-β	Regulates proliferation and differentiation, angiogenesis, and serves as an immune modulator	Not described	TGFBR1$^{-/-}$	OCC, ears, periorbital, perianal	Bian [81]
	SMAD4	Downstream transcription factor that activates apoptosis	Not described	SMAD4$^{-/-}$	OCC, lymph node mets	Bornstein [83]
	TGFB2	Encodes transmembrane Ser/Thr protein kinase that is activated by TGF-β, amongst other signalling molecules	Not described	Not described	-	Lu [82]
RAS/RAF/ MEK/MAPK	RASSF1A	Tumour suppressor involved in DNA repair and cell cycle arrest; negative regulator of RAS	Not described	G12D mutant KRAS	Benign squamous papillomas of oral mucosa, tongue and palate	Caulin [67]
	EGFR	Epidermal growth factor receptor; downstream mediator of RAS	Not described	Not described	-	n/a
Cyclins	Cyclin D1	Promotes cellular proliferation by enabling cells to enter S phase and synthesize DNA in preparation for cell division	Not described	L2-CyclinD1	Hyperplasia of tongue, oesophagus	Mueller [76], Nakagawa [77]
Pocket protein family	RB1	Tumour suppressor that inhibits cell cycle (G_1/S) and is involved in chromatin remodelling	3%	pRb/p107-deficient pRB/p130-deficient	Head and neck Squamous cell carcinomas	Shin [56]
	RBL1	Gene product p107 is a tumour suppressor involved in cell cycle regulation		pRb/p107-deficient	Head and neck Squamous cell carcinomas	Shin [56]
	NOLC1	Gene product is p130, unclear function		pRb/p130-deficient	Head and neck Squamous cell carcinomas	Shin [56]
Interferon regulator transcription factor family	IRF6	Involved in the formation of connective tissues	Not described	Not described	-	n/a
F-box protein family	FBXW7	Binds to cyclin E and targets it for ubiquitination to prevent progression from G_1 to S phase	Not described	Not described	-	n/a
	RIPK4	Serine/threonine protein kinase that interacts with PKC-δ and can also activate NFkappaB.	3%	Not described	-	n/a
	DICER1	RNA helicase that functions as a ribonuclease in RNA interference and microRNA pathways to repress gene expression	3%	Not described	-	n/a

NGS = next generation sequencing.

By contrast, wild-type mice failed to develop SCC [78]. Furthermore, when mice both overexpressed CCND1 and were haploinsufficient for p53, invasive SCC occurred by 12 months of age [79]. Cancers were evident in the buccal mucosa (12%), tongue (25%) and upper and lower oesophagus (11 - 12%) with 25% containing metastasis to lymph nodes.

Finally, these NGS approaches showed deletion or inactivating mutations in the phosphatase and tensin homolog (PTEN) gene. PTEN functions as a tumour suppressor by regulating AKT which promotes cell survival and metabolism. Mice expressing a myrisolated, and hence, constitutively active, AKT (myrAKT) under control of a bovine Keratin 5 promoter, developed dysplastic lesions in the palate, cheeks and lips [80]. When the epithelial cells expressed myrAKT and also lost p53 expression, mice developed malignant tumours in the oral cavity, palate, tongue and lips with local metastasis to regional lymph nodes. Therefore, as shown with NOTCH1, CCND1, and PTEN, mouse models complimented and were able to confirm candidate genes that drive HNSCC as initially identified by NGS.

However, one notable gene important in HNSCCs but not covered with NGS involves the Transforming Growth Factor (TGF-β) signalling pathway. TGF-β regulates cellular proliferation and differentiation as well as angiogenesis and immune suppression. This pathway is often mutated in cancer cells so that these cells become resistant to the anti-proliferative effects of TGF-β but still benefit from its pro-angiogenic and immunosuppressive functions. Although mutations in TGF-β signalling were not found by whole-genome sequencing [21,22], tumours of the head and neck have frequent loss of chromosome 18q, which contains the SMAD2, SMAD3, SMAD4 and TBR2 genes [17,81]. In addition, TGF-β is well known to cause differentiation of epithelial cells and whole-exome sequencing identified up to 30% mutations in genes that play a role in terminal differentiation [21,22]. Therefore, future studies still require vigilance to examine candidate genes not identified by massive sequencing efforts or other high-throughput technologies.

To this end, several mouse models have shown that loss of the TGF-β signalling pathway in cancer cells resulted in HNSCCs. After chemical carcinogen treatment, mice possessing deletion of TGFBR1 in their epithelium developed SCCs of the oral cavity. These mice also developed regional and distant metastasis within one year after treatment. Furthermore, tumours exhibited enhanced proliferation, reduced apoptosis and the tumour stroma appeared highly inflamed with high levels of TGF-β. Furthermore, the TGF-β signalling pathway may also cooperate with the AKT pathway to cause HNSCCs; mice that had lost both TGFBR1

and the AKT inhibitor PTEN in their epithelia developed oral SCCs within ten weeks [82]. In addition, mice that lost other proteins involved in the TGF-β signalling pathway such as TGFBR2 [83] or the downstream effector molecules SMAD4 [84] also developed HNSCCs mainly affecting the oral cavity and regional lymphatics. Consistent with previous studies [85], tumours and stroma from mutant Smad4 mice had high levels of TGFB1 and inflammation. Thus, these mouse models may provide additional insight into genes mediating HNSCCs that were not observed using NGS and other powerful high throughput techniques.

CONCLUSIONS

As with other high throughput technologies such as expression microarrays and comparative genomic hybridization, recent advances in NGS can identify both new candidates and novel structural information regarding genes that drive HNSCCs. In addition to genes known to be involved in HNSCC such as HPV oncoproteins E6 and E7, p53 and RAS, these studies have also identified novel mutations in genes such at NOTCH1 and PTEN, among others. All of these genes have been show to accelerate the development of SCCs in genetically engineered mice. Furthermore, NGS along with other works have identified mutations in several novel pathways. For example, 22% of tumours contained mutations in spectrin repeat containing, nuclear envelope (SYNE1) which may regulate cytoskeletal regulation. In addition, these studies reported that 3% of cancers had mutations in the endoribonucelase DICER, an important player in miRNA genesis. Still, it remains unclear which candidates actually promotes SCC development as well as the mechanism by which this occurs.

GEMMs provide a novel platform to better understand and validate these novel mutations that have been identified by sequencing HNSCCs genomes. It has been shown that these GEMMs develop SCCs when mice possess mutated genes known to be involved in HNSCCs and continued study will allow the discovery and validation of novel "driver" mutations important in HNSCCs. Understanding how these novel mutations promote malignant transformation may enable us to target HNSCCs more rationally. Furthermore, these models will provide an *in vivo* platform to study the effectiveness of different strategies utilizing cytotoxic chemotherapy as well as other small molecule inhibitors. One caution with this approach centers on the extent to which regional differences in mutations contribute to tumour heterogeneity and possibly response to therapy. For example, Gerlinger et al. [86] showed that more that 60% of all somatic mutations differed among

tumour regions. Despite such heterogeneity, many subclonal populations exhibited convergent tumour evolution with distinct mutations affecting similar pathways. Furthermore, epigenetic alterations also occur in HNSCCs [87,88] and may promote tumour growth. These changes may be missed in NGS and may be difficult to study in GEMMs. Finally, mutations in mitochondrial DNA are associated increased HNSCC aggressiveness [89]. Such mutations can be studied with GEMMs but may be missed with NGS as these events turn off gene expression without causing mutations and lead to further tumour heterogeneity. Therefore, coupling NGS with GEMMs will also be essential to understand which mutations and pathways drive HNSCCs. Thus, coupling NGS approaches with GEMMs will provide important platforms to investigate the best ways to target individual candidate genes and, more generally, those pathways essential to HNSCC.

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