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Review

Preclinical models in Head and Neck Squamous Cell Carcinoma

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Simple Summary: Head and neck cancer is one of the most frequent types of cancer in the current era. Cell lines and animals are used as models to study the mechanisms of the disease and the effect of therapies, especially the efficacy and toxicity caused by drugs. In this review we discuss the different models used at the moment, the advantages and disadvantages of choosing each of them for the different research purposes, and how the future perspective looks like for the potential advances in these models that will have a positive impact in how oncologist manage the head and neck treatment, ultimately increasing the wellbeing of patients suffering this disease.

Abstract: Head and neck cancer is the sixth most frequent cancer type. Drug resistance and toxicity are common challenges of the existing therapies, making the development of reliable preclinical models essential for the study of the involved molecular mechanisms as well as for eventual intervention approaches that improve the clinical outcome. Preclinical models of head and neck squamous cell carcinoma have been traditionally based in cell lines and murine models. In this review, we will go over the most frequently used preclinical models, from immortalized-cell and primary tumour cultures in monolayer or 3D, to the currently available animal models. We will scrutinize their efficiency in mimicking the molecular and cellular complexity of head and neck squamous cell carcinoma. Finally, the challenges and opportunities of other envisaged putative approaches, as well as the potential of the preclinical models to further develop customized therapies will be discussed.

Keywords: HNSCC, preclinical model, cancer therapy

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1. Introduction

Head and neck cancer is the 6th most common malignancy worldwide with 90% of cases being head and neck squamous cell carcinoma (HNSCC)[1, 2]. HNSCC occurs frequently in elderly men exposed to long-term tobacco and alcohol consumption. More recently, human papillomavirus (HPV) has been also found as a common cause of HNSCC in countries with declining smoking habits[3]. Surgical resection and other therapies including radiation, chemotherapy and immunotherapy have improved patient's life quality[4], but local bone invasion, distant metastasis and drug resistance are usual complications of this aggressive cancer, resulting in a low survival rate[1].

To this, unfortunately, the fact is added that, compared to other malignancies, HNSCC has experienced little therapeutic development and very few drugs have been FDA-approved in the last decades. The discovery of biomarkers for improvement of tumour staging, prognosis, and customized treatment is also very slowly being incorporated to routinely clinical use mainly due to the high heterogeneity of HNSCC[5]. Thus, in order to tailor a better scenery for patients and treatment choice, it is essential to achieve a better understanding of the mechanisms involved in HNSCC development and treatment through the study of models that closely resemble the in vivo process of the disease.

Preclinical models are a requirement for the a priori testing of the therapeutic efficacy of novel therapeutic strategies. In order to achieve the optimal conditions for accurate prediction, it is important to select the most appropriate model (**Figure 1**). HNSCC cell lines have been considered the most affordable method to understand certain molecular mechanisms involved in such efficacy, where three-dimensional (3D) cultures are under promising development. Preclinical animal models, from xenograft implants to genetically modified mice, have been traditionally used to reproduce the tumour initiation and progression and to test the efficacy of drugs. Here, we will expose the advantages and disadvantages of using each of these preclinical models (**Table 1**), describe the methods that are most frequently used, and elaborate on the future perspectives in the field.

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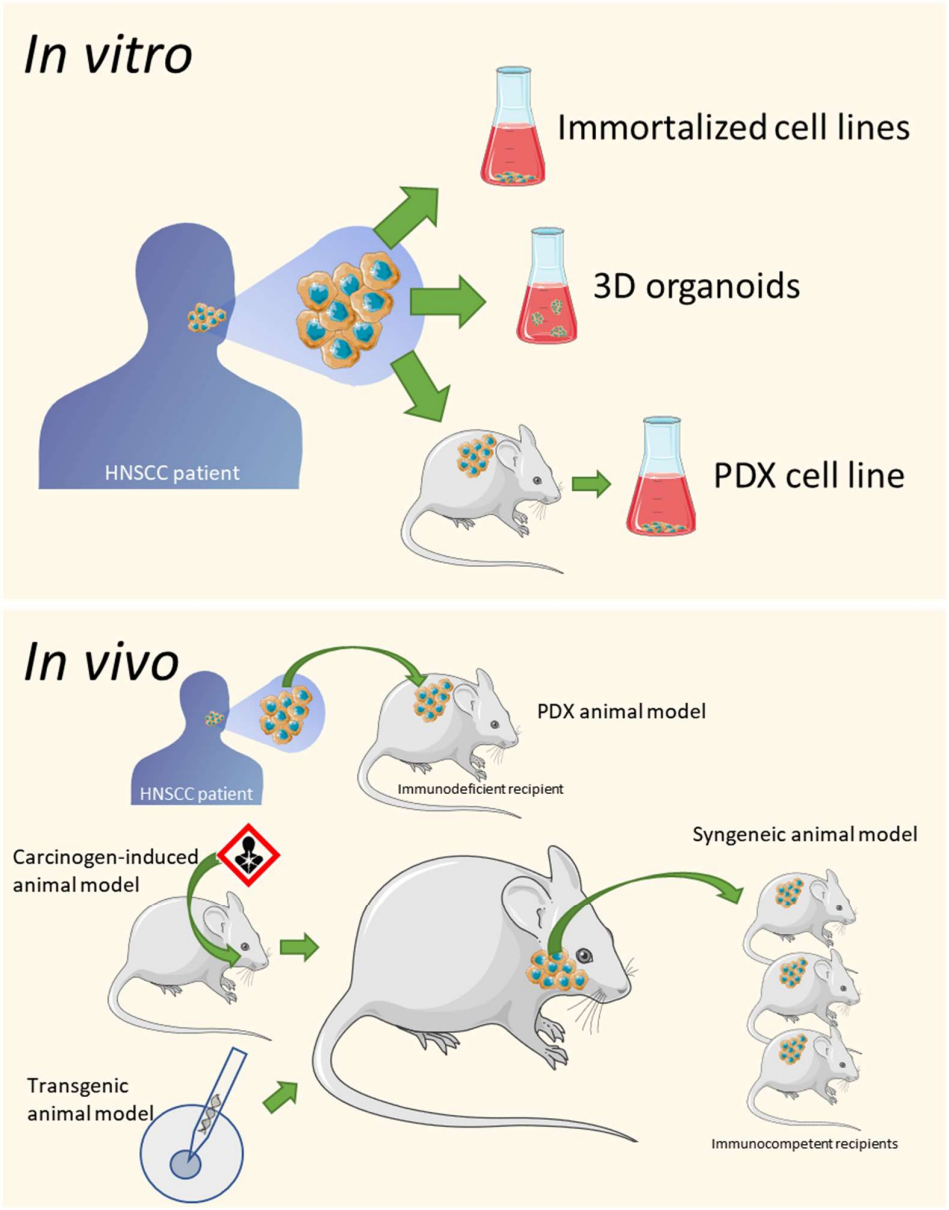

Figure 1. *In vitro* and *in vivo* approaches for generating the main preclinical models in HNSCC research.

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Table 1. Advantages and disadvantages of preclinical models in HNSCC

Preclinical model		Advantages	Disadvantages	Ref
In vitro	General	Cell diversity is kept Useful for Mechanism studies Unexpensive and fast	No tumour microenvironment. Genetic instability. Poor predictor for clinical benefits	[6, 7]
	Immortalized cell lines	Unexpensive Easy maintenance Allows genetic modifications	Genetic instability Clonal selection Lack of reproducibility	[8-10]
	PDXs	Keeps genetic heterogeneity Allows genetic modifications	Expensive and longer to develop Mouse microenvironment	[11, 12]
	3D Spheroids and Organoids	Reproduces tumour structure Keeps genetic heterogeneity	Still under development Lack of ECM effect	[13-15]
In vivo	General	Tumour microenvironment Resemblance with human disease	Long term experiments No appropriate model for all purposes	[16, 17]
	PDX	Invasion and metastasis development Maintains tumour histological and genetic features	No immune-tumour interaction. Time consuming and expensive	[18, 19]
	Carcinogen induced	Resembles tumour initiation Keeps genetic heterogeneity Immune system is kept intact	Long term for lesion development Only for accessible body regions	[20, 21]
	Transgenic	Control over gene expression Tumour development mechanism similar to human Allows study from initiation till progression	Time consuming and expensive Gene expression is altered. Unpredictability over tumour formation	[20, 22]

2. HNSCC *in vitro* models

History and origin of HNSCC cell lines

Over the years, different *in vitro* cancer models have been developed to better understand the molecular pathways governing HNSCC tumours and, thus, uncover potential novel treatments. In HNSCC context, they should be representative of all anatomic sites in the head and neck region such as larynx, oral cavity, oropharynx and hypopharynx, and also, HPV status. The initial techniques for *in vitro* culturing of HNSCC cells were reported many decades ago and departed from tumour tissue at the time of surgery. Since then, many HNSCC cell lines collections created from primary or metastatic

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tumours have been established worldwide after resolving some challenges, such as fibroblast proliferation and reliance on feeder layers[6]. HNSCC tumour cell lines including Hep2, Hep3, and KB were among the first to be created, and permanent culture of HNSCC cell lines with increasing viability was achieved over time, with more than 300 cell lines existing now[7, 23].

However, the genetic and epigenetic differences between established cell lines and the original tumour constitute an important obstacle for the faithful characterization of the disease and the drug efficacy. In this context, *in vitro* cell cultures derived from fresh patient biopsies or patient-derived-xenografts (PDXs) models may be a superior way to investigate cancer biology and evaluate drug sensitivity, since they preserve the molecular properties of patient cancers. Finally, the limitations of monolayer cell cultures (2D models) regarding the resemblance to the *in vivo* pathophysiology are being addressed with the recent emergence of 3D cultures, which depict more closely the tumour tissue architecture and cellular milieu[24].

HNSCC cell lines

Immortalized cell lines

Immortalized cell lines have long been employed as *in vitro* models for cancer biology and pharmacogenomics research. Immortality is achieved by blocking the cell cycle checkpoint pathways using different protocols such as ectopic expression of telomerase, telomerase reverse transcriptase (TERT) or p53 and pRb mutated genes[8].

As mentioned above, there should be cell lines from all primary sites of HNSCC. In this regard, of all HNSCC cell lines, oral cavity corresponds to 60% because surgical resection is the primary treatment for oral cavity tumours. Cell lines immortalised from pharyngeal tumours constitute 12%, larynx 18% and nasal septum just 3%[9]. Of note, there are only six HPV-positive (HPV+) cell lines since HPV negative (HPV-) HNSCC tumours are largely pharyngeal with lower rates of primary surgery[8].

Different repositories with HNSCC cell line characteristics are currently available (**Table 2**). Barrentina et al, founded The Cancer Cell Line Encyclopedia (CCLE), a comprehensive database of human cancer diversity which also includes the molecular characterization of 947 human cancer cell lines from 36 different tissue types, including 32 HNSCC cell lines. In addition, Garnett et al, systematically profiled the genome and pharmacologic response of more than 300 HNSCC cell lines to identify markers of drug sensitivity. This study and others show that *TP53, CDKN2A, CDKN2a(p14), SMAD4* or *PIK3CA* are the most common mutated genes in 39 HNSCC cell lines corresponding to 106 human tumours[9, 10, 25].

Table 2. HNSCC Cell Lines Databases

HNSCC Cell Line Database ID	Source	Ref
The Cancer Cell Line Encyclopedia (CCLE)	https://sites.broadinstitute.org/ccle/	[25] [10]
The National Cancer Institute (NCI)	https://www.cancer.gov	[9]
Hamon Cancer Center (HCC)	https://www.hamon-center.com	
American Type Culture Collection (ATCC)	https://www.atcc.org	[23] [26]
University of Michigan	https://techtransfer.umich.	[27]

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As a material resource for researchers worldwide, The National Cancer Institute (NCI) and the Hamon Cancer Center (HCC) have a large series of HNSCC cell lines available through the American Type Culture Collection (ATCC, Manassas, VA, USA)[23]. Other worldwide available HNSCC libraries are the University of Michigan squamous cell carcinoma (UM-SCC) cell lines library with more than 120 HNSCC cells lines, including some HPV positive ones[27].

Although the technical sophistication of permanent culture of HNSCC cell lines has increased substantially, immortalized cell lines still present important limitations that impair their experimental reproducibility and utility as models: **i)** they do not properly reflect the histological nature of the tumour, **ii)** There are selective survival pressures in culture conditions that induce clonal selection and **iii)** genetic and epigenetic differences from the original tumours reduce their biological fidelity[23, 24].

PDXs cell lines

PDXs cell lines were also first described many decades ago, but only recently their use has been widespread in research. PDXs constitute an effective preclinical approach in clinical translational research because, unlike cell lines that *grow in vitro* under non-physiological conditions, they grow *in vivo* and closely resemble the heterogeneity and genetic characteristics of patients' tumours. Another advantage is the fact that PDXs are generated in a 3D microenvironment and are fully provided by nutrients, oxygen and communication within non-tumour cells such as host stromal and immune cells. These seem to contribute to a higher genomic fidelity and prediction of therapeutic responses. Indeed, engrafted PDXs in immunocompromised mice show good correlation with HSNCC aggressiveness and related survival. Moreover, utility concerning the identification of drug targets has been proven, as in the study of Karamboulas et al. which demonstrated that CDK4 and CDK6 inhibitors may constitute an important therapeutic strategy for some HNSCC patients[12, 28].

Some limitations of 2D cultures of PDXs are: i) they are time-consuming and a more expensive approach because the use of humanized mice is essential to avoid the replacement of human stroma and immune cells by mouse cells after serial passages[24], **ii)** in practice, PDXs experiments are limited to less than 10 passages to avoid major genetic and epigenetic differences[12] and **iii)** even though they show a better resemblance to the in vivo situation, they do not always accurately predict patient response, and most PDXs are sourced from HPV- tumours. Few groups have investigated the HPV status in PDXs, such as Kimple et al, that identified 6 HPV+ PDXs with p16 staining in 22 HSNCC PDXs[29].

***In vitro* 3D models**

Drug screening and molecular biology assays on monolayer cell cultures are still popular methods in cancer research. However, 3D cell culture strategies reflect better tumour tissue architecture. They include spheroids and organoids. While spheroids are cultured free-floating aggregates derived from cancer cells, organoids are miniature versions of organs derived from stem cells.

Spheroids are formed by spontaneous aggregation of different cells that can have different sources, giving rise to multicellular tumour spheroids (MCTS) or Tumour-derived spheroids (TDS). MCTS only use tumour cells, are clonal and grow easily into large cultures, but they do not resemble the histology of the original tumour. In contrast, TDS are not only enriched with differentiated tumour cells but also with stem cancer cells, providing a heterogeneous tumour environment[13]. Spheroids may be obtained using different protocols that are classified as those who need scaffolds or microbeads to evoke cell aggregation, more used in regenerative medicine, or those that do not need a scaffold. They may use suspension cultures, ultra-low attachment plates, hanging drop, and micro-technology platforms, and are far more economic and simpler[30, 31].

In a recent study, Kochanek et al used HNSCC MCTS from 5 human HNSCC cell lines to evaluate the effects of 19 cancer agents. Among them, 5 chemotherapy drugs approved for head and neck cancer: methotrexate, 5-FU, bleomycin, cisplatin, and docetaxel, the EGFR tyrosine kinase inhibitors gefitinib and erlotinib were used as surrogates for cetuximab, or dactolisib,a dual PI3K and mTOR inhibitor. To assess the performance advantage of MCTS versus 2D cultures, they tested the set of 19 drugs with established 2D monolayer GI assays using the same HNSCC cell lines. MCTS drug responses could be stratified into high-, intermediate-, and low-impact tiers using a cumulative multiparameter drug impact score, maximizing the usefulness of these more physiologically relevant tumour cultures to establish models of resistance[15].

On the other hand, the main achievement of the organoids technology is the preservation of the in vivo 3D architecture and the proliferation of heterogeneous cell types of the tumour microenvironment. Therefore, they are postulated as novel *in vitro* models for drug testing, correlated with patients' response to therapy. They were first developed by Köpf-Maier and colleagues and may be formed by pluripotent embryonic or induced stem cells (PSC) or by adult stem cells (ASC)[14]. Embryonic or induced stem cells may differentiate in several cell subsets to generate the mesenchymal, epithelial and endothelial cells distributions that provide the model with sufficient tissue-specific biological processes. However, the generation process of these organoids is complex, typically slow and requires several differentiation factors. In the case of ASC organoids, adult stem cells from tissue compartments with regenerative capacity are used. Also, they are simpler and more homogenous for long term expansion. In both cases, single cells usually require a supporting 3D Matrigel deposited large droplets[13].

Sawant et al generated a panel of 31 HNSCC organoids that were well established, proving that tongue-tumorigenesis can be developed and reproduced *in vitro*. Also, they identified cancer-associated fibroblasts as a key population for mimicking HNSCC pathogenesis and for regulation of epithelial thickness, cell proliferation, differentiation, and maintenance of junctions in *in vitro* grown tissues[32].

Despite different establishment success rated among organoids, there is a consensus of the promising potential for *in vitro* drug testing. Indeed, several studies demonstrated that in oncological drug testing, spheroid models derived from human tumours outperform the *in vitro* gold standard[33].

In particular, HNSCC organoids from oral mucosal or malignant tongue tissue recapitulate the disease genetically, histologically and functionally[32, 34].

Even though 3D cultures have improved the *in vivo* resemblance, they are conceptually limited to the process inherent to the tumour microenvironment, but cannot mimic the complete physiological regulation that also influences pathogenesis and drug efficiency, particularly for novel generation drugs that involve remote cells such as those from the immunological system, e.g. immunotherapy. The limitations of the *in vitro* models gave rise to the development of specific *in vivo* models for the study of HNSCC.

3. HNSCC *in vivo* models

Although *in vitro* models have demonstrated to be very useful in HNSCC preclinical research, *in vivo* animal models are essential to fully understand the mechanisms and molecular events happening during HNSCC initiation and progression in their specific environment. To this purpose, different approaches have been developed, including carcinogen-induced HNSCC models, transgenic animals and transplantable xenograft models.

The range of animal model species that have been subject of study for preclinical models of head and neck cancer cover from spontaneous cancers in domestic animals like cats, because of the similarities with human HNSCC[16], to carcinogenesis induced in the

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cheek pouch of hamsters, which has been a historical model for chemoprevention studies[35].

Compared to other animal models, mice are the most suitable choice because of the standardized, controlled and extended use in research, so they will be the focus of this section.

PDX animal models

This animal model, firstly described in 1969[36], is generated by implanting a xenograft from a tumour cell line or a patient biopsy, normally subcutaneously, in a recipient mouse[37].

The success rate of engraftment may vary depending on tumour histology, collection methods or mice choice. The development of immune-deficient mouse strains to use as PDXs recipients has facilitated the success of these implants. The NOD/SCID/IL-2Rγ−/− (NSG) mouse, which lacks mature T and B cells, is the most commonly used immunodeficient mouse to produce PDXs, as researchers have found a high rate of engraftment, tumour growth, and tumour regression in minimized with this strain[12].

In order to minimize the use of invasive techniques such as tissue biopsy, some groups have tried to generate PDXs from circulating tumour cells (CTCs). However, the low amount of CTCs and the variability in primary site and stage, makes generating HNSCC PDXs from CTCs still technically challenging[38].

This system allows the direct study of the human tumour, which as such maintains its original heterogeneity and molecular identity in the implant. This is very useful for the evaluation of the response to therapies, proving to have, therefore, a high value for the drug screenings in the clinic[18, 39]. Schuh et al. have recently published a systematic review on the translational applicability of this model[19].

However, this system has drawbacks. PDX establishment and maintenance are time consuming, expensive and laborious. In addition, because the clones selected and grown in the mouse may not totally resemble the behaviour of the patient´s tumour of origin, the observations derived from these preclinical studies are often not reproduced in human disease. Regarding the response to therapy, there is evidence that some drugs may have significant antitumour effects in xenograft models, but they show no benefit when used in humans[40]. The relevance of the immune implications in mouse PDX models are amply discussed by Rossa et al.[41].

Another relevant issue is the requirement to use immunodeficient mouse strains in order to ensure implant success, which has obvious limitations is achieving a resemblance with the human pathologic environment, as it oversees the important involvement of the immune system in the tumorigenesis, tumour progression and therapy response in HNSCC. There are current and promising advances regarding this issue, as the generation of humanized mice is being incorporated to these studies. To generate this model, human hematopoietic stem cells are transplanted into immunodeficient mice in order to restore a "humanized" immune system in the recipient mouse before the PDX transplantation. Recently, a protocol for this method has been published by Fu et al., where patient's bone marrow cells are used to humanize the mouse recipients while avoiding the risk for HLA mismatch, that were then successfully transplanted with HLA-A2+ HNSCC tumours, and human T cells were found infiltrated afterwards [42, 43].

Finally, other caveat of this system is, in line with the scarcity of cell lines derived from HPV+ HNSCC tumours, the lack of standardized protocols for engraftment of these specific tumours, which so far is largely unsuccessful. Moreover, Epstein-Barr virus-positive lymphoma contamination result in a lower rate of success[44]. Consequently, most PDXs are sourced from HPV- human oral squamous carcinoma such as tongue, soft palate or floor of the mouth, and, less frequently, from oropharynx or hypopharynx[29]. Addressing this limitation should be prioritized given recent rise of HNSCC cases associated with HPV infection.

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Syngeneic models

To generate syngeneic mouse models, tumour tissues or cells from mice are transplanted into another mouse with similar genetic background, allowing to keep the receptor's full immune capabilities. Therefore, the normal immunologic behaviour against the tumour can be reproduced, and the observed mechanisms better resemble the human setting. This model is more appropriate for the study of those processes requiring the interaction between the tumour and the host environment, not only immune responses, but also stromal signalling, angiogenesis and metastasis.

Various studies have generated syngeneic models by orthotopic transplantation in the floor of the mouse mouth [45], but this reproduces the destructive nature of the disease to such degree that these syngeneic tumour cells are preferably placed subcutaneously in the flank of mice. The model that has been most frequently applied consists in the subcutaneous implantation of SCC VII/SF cell line in C3H/HeJ mice[17] and it has been used for a broad spectrum of assays, especially for the evaluation of new chemo- and immunotherapies[46], but this system offers a poor ability to replicate the human disease.

Carcinogen induced models

HNSCC carcinogen models may be the best approach to mimic the human clinical disease, which is normally originated by the long-term exposure to low doses of carcinogens. In chemically induced cancer models, lesions are generated by the application of a potent chemical carcinogen. Exposure to 4-Nitroquinolone oxide (4NQO) is an alternative for tobacco exposure in animal models of human oral squamous carcinogenesis and has been broadly used for investigating the effects of anti-tumour drugs[21]. This carcinogen has a high rate of success in generating multiple neoplastic lesions in mouse and rat that show histological changes and pathological behaviours that mimic oral cancer development in humans, and the method was standardized by Tang et al in 2004[47].

A different model in use is generated through the administration of dimethyl-1,2,benzanthracene (DMBA). Its use as carcinogenic chemical has been described in hamster and in mouse model[48].

This model has the advantage of allowing the observation of the tumour generation from its origin with a molecular, histological and immunological behaviour that is similar to the human clinic, which is especially useful for the study of the carcinogenic mechanisms and the immunotherapeutic evaluation of the immunotherapeutic value of newly developed therapies in the different stages of the disease.

However, this method requires a long term of study to fully evaluate the development of the effects, which may be not suitable for certain purposes.

Transgenic models

Genetically engineered transgenic mouse models (GEM) are generated to express oncogenes or tumour suppressor genes, normally through the alteration of its promoter regulation, in immunocompetent systems. Thanks to this fact, this is a more realistic model for studying the molecular mechanisms of cancer origin, and very promising in HNSCC research. The use of this method to generate models of head and neck tumours is relatively recent[17]; and include endogenous mutants through knockout or knock-in technologies, and conditional GEM models where the mutation can be induced at specific sites and times. Mutations in *K-ras* has been the most common modification for the generation of HNSCC genetically engineered models through this system. In the SL-KrasG12D mouse, K-rasG12D is overexpressed in the oral epithelium of mice, regulated through different keratin promoters[20, 49]. In case of HPV+ oral tumours, transgenic animal models can be generated by genetically engineering them to express the HPV oncogenes E6 and E7[50]. More recently, a more efficient model to induce spontaneous HPV+ tumours using plasmids encoding the HPV oncogenes and a synthetic transposable element together with a transposase that randomly integrates them in the host genome.

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This model succeeded to obtain persistent expression of HPV16 related genes and to mimic the stages in cancer progression from initiation to local invasion and metastasis rapidly[51].

However, because of the origin of these lesions, this model presents a low tumour penetrance, which causes low predictability about the outcome of the experiment. Moreover, one would expect to find the levels of gene expression altered in all the tissues of the endogenous GEM animals since development, which does not resemble the human tumour initiation mechanism, and this, together with the precautions that must be considered about the fine tune regulation of the molecular mechanisms, besides several bioethical considerations, has to be taken into account when selecting this method.

3. Concluding remarks

The biological and clinical heterogeneity of the different anatomical locations of HNSCC has made it difficult to obtain good preclinical models. Further efforts are required to generate and improve reliable preclinical models to achieve a better understanding of the molecular, cellular, and immunological mechanisms involved in initiation, treatment resistance and progression of the disease, and therefore to be able to tailor more appropriate and efficient therapeutic strategies, contributing to advance the clinical management of HNSCC.

Approaches using HNSCC cell lines have been a very affordable method to understand the molecular biology of tumours and to screen novel drugs before introduction into clinical use. However, these models become inefficient to predict the whole complex behaviour of the tumour in an in vivo system and are not appropriate for customizing the treatment to individual cancer patients. The development of technologies using 3D cultures, more expensive at the moment, could be a more promising way of achieving the complexity that resembles better the in vivo situation, while remaining under more controlled conditions and allowing high-throughput drug screening. However, these models will never reproduce completely the in vivo conditions.

Traditionally, the development and evaluation of novel therapies have relied on mouse orthotopic models, which are not robust models for the human HNSCC, in particular for the disease initiation. However, not a single murine model meets the ideal features for both the study of the HNSCC pathogenesis and the prediction of the response to the therapy. On one hand, carcinogenesis models have yielded useful data for the study of chemoprevention but have limitations for other purposes. On the other hand, genetically modified mice are useful for studies focused on genetic mechanisms but also have several limitations regarding low tumour penetrance and uncertainty. Recently, there has been a rise in studies that combine the use of carcinogens on transgenic models, to accelerate the growth of tumours, with promising advances. However, there are further technical challenges that remain to be addressed, such as modelling the metastases. These models are very scarce in the field of HNSCC. There are PDX models that develop metastasis and local bone invasion, but cannot be set in immunocompetent recipients. The future perspectives are set then in the establishment of new models able to achieve metastasis in genetically modified mice.

In silico approaches, currently in expansion, are promising technologies to study biological systems. Mathematical modelling is an inexpensive and useful tool, as it allows to virtually and preclinically evaluate outcomes in cancer progression and therapy efficacy, through the consideration of multiple parameters that could not be easily integrated through conventional wet lab techniques[52]. As drawback, the risk of overseeing determining factors is high, considering the very complex network of parameters that must be integrated to recreate the system.

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In conclusion, despite the ample battery of *in vitro* and *in vivo* models of HNSCC that are already available, the particular heterogeneity of this type of tumour and the general limitations of the current models make it still necessary to refine the current methods. The availability of models that better predict the clinical outcomes of disease and treatment would also facilitate the discovery of biomarkers of disease and response and in turn, improve the life of the patients.

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Appendix A

Abbreviations

3D	three-dimensional
4NQO	4-nitroquinolone oxide
ASC	adult stem cells
ATCC	American Type Culture Collection
CCLE	The Cancer Cell Line Encyclopedia
CTCs	circulating tumour cells
DMBA	dimethyl-1,2,benzanthracene
GEM	genetically engineered transgenic mouse models
HNSCC	head and neck squamous cell carcinoma
HPV	human papillomavirus
MCTS	multicellular tumour spheroids
NSG	NOD/SCID/IL-2Rγ−/−
PDX	patient-derived-xenograft
PSC	pluripotent stem cells
TDS	tumour- derived spheroids
TERT	telomerase reverse transcriptase

References

1. Bray, F., et al., *Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries.* CA Cancer J Clin, 2018. **68**(6): p. 394-424.
2. Mesia, R., et al., *SEOM clinical guidelines for the treatment of head and neck cancer (2020).* Clin Transl Oncol, 2021. **23**(5): p. 913-921.
3. Rettig, E.M. and G. D'Souza, *Epidemiology of head and neck cancer.* Surg Oncol Clin N Am, 2015. **24**(3): p. 379-96.
4. Rothschild, U., et al., *Immunotherapy in head and neck cancer - scientific rationale, current treatment options and future directions.* Swiss Med Wkly, 2018. **148**: p. w14625.
5. Budach, V. and I. Tinhofer, *Novel prognostic clinical factors and biomarkers for outcome prediction in head and neck cancer: a systematic review.* Lancet Oncol, 2019. **20**(6): p. e313-e326.
6. Tinhofer, I., D. Braunholz, and K. Klinghammer, *Preclinical models of head and neck squamous cell carcinoma for a basic understanding of cancer biology and its translation into efficient therapies.* Cancers Head Neck, 2020. **5**: p. 9.
7. Easty, D.M., et al., *Ten human carcinoma cell lines derived from squamous carcinomas of the head and neck.* Br J Cancer, 1981. **43**(6): p. 772-85.
8. Greaney-Davies, F.S.T., et al., *Essential characterisation of human papillomavirus positive head and neck cancer cell lines.* Oral Oncol, 2020. **103**: p. 104613.

 doi:10.20944/preprints202203.0391.v1

9. Li, H., et al., *Genomic analysis of head and neck squamous cell carcinoma cell lines and human tumors: a rational approach to preclinical model selection.* Mol Cancer Res, 2014. **12**(4): p. 571-82.
10. Garnett, M.J., et al., *Systematic identification of genomic markers of drug sensitivity in cancer cells.* Nature, 2012. **483**(7391): p. 570-5.
11. Klinghammer, K., et al., *A comprehensively characterized large panel of head and neck cancer patient-derived xenografts identifies the mTOR inhibitor everolimus as potential new treatment option.* Int J Cancer, 2015. **136**(12): p. 2940-8.
12. Karamboulas, C., et al., *Patient-Derived Xenografts for Prognostication and Personalized Treatment for Head and Neck Squamous Cell Carcinoma.* Cell Rep, 2018. **25**(5): p. 1318-1331 e4.
13. Gunti, S., et al., *Organoid and Spheroid Tumor Models: Techniques and Applications.* Cancers (Basel), 2021. **13**(4).
14. Kopf-Maier, P. and B. Kolon, *An organoid culture assay (OCA) for determining the drug sensitivity of human tumors.* Int J Cancer, 1992. **51**(1): p. 99-107.
15. Kochanek, S.J., et al., *Maximizing the Value of Cancer Drug Screening in Multicellular Tumor Spheroid Cultures: A Case Study in Five Head and Neck Squamous Cell Carcinoma Cell Lines.* SLAS Discov, 2020. **25**(4): p. 329-349.
16. Supsavhad, W., et al., *Animal models of head and neck squamous cell carcinoma.* Vet J, 2016. **210**: p. 7-16.
17. Smith, L.P. and G.R. Thomas, *Animal models for the study of squamous cell carcinoma of the upper aerodigestive tract: a historical perspective with review of their utility and limitations. Part A. Chemically-induced de novo cancer, syngeneic animal models of HNSCC, animal models of transplanted xenogeneic human tumors.* Int J Cancer, 2006. **118**(9): p. 2111-22.
18. Lai, Y., et al., *Current status and perspectives of patient-derived xenograft models in cancer research.* J Hematol Oncol, 2017. **10**(1): p. 106.
19. Schuch, L.F., et al., *Head and neck cancer patient-derived xenograft models - A systematic review.* Crit Rev Oncol Hematol, 2020. **155**: p. 103087.
20. Ishida, K., et al., *Current mouse models of oral squamous cell carcinoma: Genetic and chemically induced models.* Oral Oncol, 2017. **73**: p. 16-20.
21. Vitale-Cross, L., et al., *Chemical carcinogenesis models for evaluating molecular-targeted prevention and treatment of oral cancer.* Cancer Prev Res (Phila), 2009. **2**(5): p. 419-22.
22. Mery, B., et al., *Preclinical models in HNSCC: A comprehensive review.* Oral Oncol, 2017. **65**: p. 51-56.
23. Lin, C.J., et al., *Head and neck squamous cell carcinoma cell lines: established models and rationale for selection.* Head Neck, 2007. **29**(2): p. 163-88.
24. Huo, K.G., E. D'Arcangelo, and M.S. Tsao, *Patient-derived cell line, xenograft and organoid models in lung cancer therapy.* Transl Lung Cancer Res, 2020. **9**(5): p. 2214-2232.
25. Barretina, J., et al., *The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity.* Nature, 2012. **483**(7391): p. 603-7.
26. Zhao, M., et al., *Assembly and initial characterization of a panel of 85 genomically validated cell lines from diverse head and neck tumor sites.* Clin Cancer Res, 2011. **17**(23): p. 7248-64.
27. Nair, T.S., et al., *Characteristics of head and neck squamous cell carcinoma cell Lines reflect human tumor biology independent of primary etiologies and HPV status.* Transl Oncol, 2020. **13**(10): p. 100808.
28. Kang, H.N., et al., *Establishment and characterization of patient-derived xenografts as paraclinical models for head and neck cancer.* BMC Cancer, 2020. **20**(1): p. 316.
29. Kimple, R.J., et al., *Development and characterization of HPV-positive and HPV-negative head and neck squamous cell carcinoma tumorgrafts.* Clin Cancer Res, 2013. **19**(4): p. 855-64.
30. Santi, M., et al., *Production of 3D Tumor Models of Head and Neck Squamous Cell Carcinomas for Nanotheranostics Assessment.* ACS Biomater Sci Eng, 2020. **6**(9): p. 4862-4869.

31. Bialkowska, K., et al., *Spheroids as a Type of Three-Dimensional Cell Cultures-Examples of Methods of Preparation and the Most Important Application.* Int J Mol Sci, 2020. **21**(17).
32. Sawant, S., et al., *Establishment of 3D Co-Culture Models from Different Stages of Human Tongue Tumorigenesis: Utility in Understanding Neoplastic Progression.* PLoS One, 2016. **11**(8): p. e0160615.
33. Tanaka, N., et al., *Head and neck cancer organoids established by modification of the CTOS method can be used to predict in vivo drug sensitivity.* Oral Oncol, 2018. **87**: p. 49-57.
34. Driehuis, E., et al., *Oral Mucosal Organoids as a Potential Platform for Personalized Cancer Therapy.* Cancer Discov, 2019. **9**(7): p. 852-871.
35. Gimenez-Conti, I.B. and T.J. Slaga, *The hamster cheek pouch model of carcinogenesis and chemoprevention.* Adv Exp Med Biol, 1992. **320**: p. 63-7.
36. Rygaard, J. and C.O. Povsen, *Heterotransplantation of a human malignant tumour to "nude" mice. 1969.* APMIS, 2007. **115**(5): p. 604-6; discussion 607-8.
37. Karamboulas, C., J. Meens, and L. Ailles, *Establishment and Use of Patient-Derived Xenograft Models for Drug Testing in Head and Neck Squamous Cell Carcinoma.* STAR Protoc, 2020. **1**(1): p. 100024.
38. Morrow, C.J., et al., *Tumourigenic non-small-cell lung cancer mesenchymal circulating tumour cells: a clinical case study.* Ann Oncol, 2016. **27**(6): p. 1155-1160.
39. Panaccione, A., et al., *Chromosomal abnormalities and molecular landscape of metastasizing mucinous salivary adenocarcinoma.* Oral Oncol, 2017. **66**: p. 38-45.
40. Johnson, J.I., et al., *Relationships between drug activity in NCI preclinical in vitro and in vivo models and early clinical trials.* Br J Cancer, 2001. **84**(10): p. 1424-31.
41. Rossa, C., Jr. and N.J. D'Silva, *Immune-relevant aspects of murine models of head and neck cancer.* Oncogene, 2019. **38**(21): p. 3973-3988.
42. Fu, J., et al., *Autologous reconstitution of human cancer and immune system in vivo.* Oncotarget, 2017. **8**(2): p. 2053-2068.
43. Fu, J. and Y.J. Kim, *Autologously Humanized Mice for Immune-Oncologic Studies.* Curr Protoc Pharmacol, 2020. **89**(1): p. e76.
44. Facompre, N.D., et al., *Barriers to generating PDX models of HPV-related head and neck cancer.* Laryngoscope, 2017. **127**(12): p. 2777-2783.
45. Vahle, A.K., et al., *Optimization of an orthotopic murine model of head and neck squamous cell carcinoma in fully immunocompetent mice--role of toll-like-receptor 4 expressed on host cells.* Cancer Lett, 2012. **317**(2): p. 199-206.
46. Li, Q., et al., *Mouse Tumor-Bearing Models as Preclinical Study Platforms for Oral Squamous Cell Carcinoma.* Front Oncol, 2020. **10**: p. 212.
47. Tang, X.H., et al., *Oral cavity and esophageal carcinogenesis modeled in carcinogen-treated mice.* Clin Cancer Res, 2004. **10**(1 Pt 1): p. 301-13.
48. Kim, T.W., et al., *Oral mucosal carcinogenesis in SENCAR mice.* Anticancer Res, 2002. **22**(5): p. 2733-40.
49. Vitale-Cross, L., et al., *Conditional expression of K-ras in an epithelial compartment that includes the stem cells is sufficient to promote squamous cell carcinogenesis.* Cancer Res, 2004. **64**(24): p. 8804-7.
50. Riley, R.R., et al., *Dissection of human papillomavirus E6 and E7 function in transgenic mouse models of cervical carcinogenesis.* Cancer Res, 2003. **63**(16): p. 4862-71.
51. Lin, Y.H., et al., *Integration of Oncogenes via Sleeping Beauty as a Mouse Model of HPV16(+) Oral Tumors and Immunologic Control.* Cancer Immunol Res, 2018. **6**(3): p. 305-319.
52. Nazari, F., et al., *In Silico Models Accurately Predict In Vivo Response for IL6 Blockade in Head and Neck Cancer.* Cancer Res, 2020. **80**(7): p. 1451-1460.