The molecular biology of head and neck cancer

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Abstract | Head and neck squamous cell carcinomas (HNSCCs) are caused by tobacco and alcohol consumption and by infection with high-risk types of human papillomavirus (HPV). Tumours often develop within preneoplastic fields of genetically altered cells. The persistence of these fields after treatment presents a major challenge, because it might lead to local recurrences and second primary tumours that are responsible for a large proportion of deaths. Aberrant signalling pathways have been identified in HNSCCs and inhibition of epidermal growth factor receptor (EGFR) has proved a successful therapeutic strategy. In this Review, we discuss the recent literature on tumour heterogeneity, field cancerization, molecular pathogenesis and the underlying causative cancer genes that can be exploited for novel and personalized treatments of patients with HNSCC.

Chemoradiation
Combined treatment with chemotherapy (usually cisplatin) and radiation.

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Head and neck squamous cell carcinoma (HNSCC) arises in the oral cavity, oropharynx, larynx or hypopharynx, and is the sixth leading cancer by incidence worldwide[1]. It is likely that approximately 600,000 cases will arise this year worldwide, and that only 40–50% of patients with HNSCC will survive for 5 years.

The most important risk factors so far identified are tobacco use and alcohol consumption, which seem to have a synergistic effect. A subgroup of HNSCCs, particularly those of the oropharynx, is caused by infection with high-risk types of human papillomavirus (HPV). In the western world, the incidence of HNSCC in specific sites has been slowly declining during the past decade, which can be attributed to a decrease in the prevalence of the more traditional risk factors, most notably smoking. However, oral tongue and particularly oropharyngeal cancers are becoming more prevalent, which may be related to an increase in oral and oropharyngeal HPV infections. HPV-positive and HPV-negative tumours represent different clinicopathological and molecular entities (as discussed below and detailed in TABLE 1). Besides the above mentioned exogenous risk factors, certain inherited disorders, such as Fanconi anaemia, and also a more general genetic susceptibility predispose to HNSCC[2–4].

The prognosis for patients with HNSCC is largely determined by the stage at presentation. The extent of the tumour, as well as the presence of lymph-node metastases and distant metastases, determines the stage. Staging of HNSCC is by clinical examination, imaging, cytology of lymph nodes and definite histopathology after surgery (such as radicality and extranodal spread). Recently, HPV status and tobacco use have also been shown to be of significant prognostic importance, possibly outweighing the traditional tumour, node, metastasis (TNM) staging system in oropharyngeal tumours[5]. About one-third of patients presents with early-stage disease, whereas the typical case presents with advanced cancer with lymph node metastases. Early-stage tumours are treated with surgery or radiotherapy and have a favourable prognosis. The mainstays of treatment for advanced tumours are surgery combined with postoperative radiotherapy. In the past decade, the role of organ-preservation protocols, with combined chemoradiation and surgery for salvage, has increased. These protocols are particularly effective for patients with moderately advanced cancers of the larynx and pharynx who are less than 70 years old and have a good performance status. Although there are no randomized studies, it is assumed that during the past two decades the quality of life of patients with HNSCC has increased as a result of the use of more advanced surgical[6] and radiotherapeutic[7] techniques, as well as organ-preservation protocols[8]. Recently, the use of targeted drugs has entered the field, most notably the application of the epidermal growth factor receptor (EGFR)-specific antibody cetuximab combined with radiotherapy.

Disappointingly, survival has not markedly improved in recent decades because patients still frequently develop locoregional recurrences, distant metastases and second primary tumours. The limited information available on the molecular carcinogenesis of HNSCC, and the genetic and biological heterogeneity of the disease has hampered

At a glance

- Head and neck squamous cell carcinomas (HNSCCs) develop in the mucosal linings of the upper aerodigestive tract and are the sixth leading cause of cancer worldwide. Risk factors are exposure to carcinogens, most notably tobacco smoking and alcohol consumption, infection with high-risk types of human papillomavirus (HPV) and genetic predisposition.
- HNSCC is a heterogeneous disease. At least two genetic subclasses can be distinguished: HPV-positive and HPV-negative tumours. Preliminary data suggest that further subclassification is likely to follow.
- A key issue in HNSCC pathogenesis is that carcinomas develop within large preneoplastic fields of mucosal epithelium made up of genetically altered cells that are clonally related to the carcinoma and often extend into the surgical margins when tumours are excised, and can cause local recurrences and second primary tumours.
- Limitless replicative potential of head and neck cancer cells is caused by abrogation of the p53 and retinoblastoma (RB) pathways that perturb cell cycle regulation, probably in the context of telomerase reverse transcriptase (TERT) expression.
- A subgroup of HNSCCs becomes independent from growth factors owing to somatic changes in the epidermal growth factor receptor (EGFR) signalling pathway.
- Some, if not all, HNSCCs escape from the growth inhibitory transforming growth factor-β (TGFβ) pathway by somatic mutation or chromosome loss of key genes. This pathway seems to be interconnected to the nuclear factor-κB (NF-κB) pathway.
- Somatic mutations and genetic changes indicate that the PI3K–PTEN–AKT pathway is frequently activated in HNSCC.
- Metastatic dissemination of HNSCC is initially to the lymph nodes in the neck. Expression profiles predict lymph node metastasis, but causative cancer genes have not yet been identified.
- The unravelling of the biological characteristics of HNSCC will lead to novel and personalized therapies in the near future.

the development of new therapeutic strategies. The first successful targeted therapy (EGFR-specific antibodies) demonstrates that improved understanding of the molecular pathways underlying HNSCC will yield valuable new treatment protocols. It is hoped that further unravelling of the molecular carcinogenesis of HNSCC will lead to novel therapies and improved tailoring of existing treatment modalities for the individual patient.

This Review discusses recent insight into the molecular pathogenesis of HNSCC and describes the evidence for the existence of molecularly different subgroups. The process of multi-step carcinogenesis and the role of field cancerization is addressed, and signalling pathways that are altered during carcinogenesis are highlighted in line with the cancer-associated phenotypes as defined by Hanahan and Weinberg[9]. The criteria for designating a gene as a candidate or established cancer gene in HNSCC are presented, and used to weigh up the published evidence on specific genes. Finally, a comprehensive model of HNSCC development, stratified for genetic subclasses, is proposed.

Molecular heterogeneity of HNSCC

The fact that more than 95% of head and neck cancers are squamous cell carcinomas suggests that it is a relatively homogeneous disease when compared with other tumour types. However, recent insight has revealed that HNSCC is, in fact, unexpectedly heterogeneous, hampering accurate prognostication, treatment planning and, from the biological perspective, identification of the causative cancer genes. Various subclasses of HNSCCs can be distinguished at the histological level[10], but RNA and DNA profiling studies in particular have highlighted the molecular heterogeneity of the disease. By making use of expression profiling, Chung *et al.*[11] identified four subgroups of HNSCC with different prognoses. Intriguingly, one particular subgroup with an EGFR-associated expression profile exhibited a relatively poor prognosis. A more specific recurrence-associated profile was reported by these authors a few years later[12].

Genetic analyses also convincingly demonstrate the existence of additional subclasses of HNSCCs. The first and most prominent distinction is the difference between tumours that are caused by infection with high-risk types of HPV, and those that do not contain HPV. More details are discussed below and can be found in TABLE 1. In addition, it was noted by karyotyping and ploidy analysis that subgroups of tumours are diploid or near-diploid, and most are aneuploid[13,14]. A recent investigation using array comparative genomic hybridization (CGH) by Smeets *et al.*[15] confirmed these findings by showing that even at 1 megabase resolution about 20% of HNSCC cases that are not caused by HPV seem to have only a few copy-number alterations, suggesting a near-normal chromosome number. These independent studies point to a separate group of tumours with a seemingly near-normal genome. Nonetheless, on the basis of the few studies that address this issue, these data should be considered preliminary, and future DNA profiling studies will hopefully shed more light on this intriguing subgroup. How these genetically classified subgroups are connected to the molecular classification on the basis of the expression profiles proposed by Chung *et al.*[11] also remains to be determined. Notwithstanding, these data clearly underline that HNSCC is a heterogeneous disease, both at the molecular level and clinical level. There is now more or less consensus that HPV-infected HNSCC in particular should be considered as a specific subclass of HNSCCs, and the arguments that led to this consensus are summarized below.

HPV-infected HNSCC

Since the discovery of HPV type-16 (HPV-16) in the 1970s, the role of HPV in human malignancies has become convincingly established. HPV is a strictly epitheliotropic, circular double-stranded DNA virus that is known to be the primary cause of cervical cancer[16,17]. There are more than 100 subtypes of HPV, some of which are involved in cervical carcinogenesis[18] and have been designated as high-risk HPVs. The virus contains two oncogenes, *E6* and *E7*, the expression of which inactivates p53 and retinoblastoma (RB), respectively, causing perturbation of cell cycle regulation in the infected cells (FIG. 1), which is considered to be the onset of HPV-mediated carcinogenesis[17]. The virus is not easily cultured, therefore the involvement of the virus in tumours is usually determined by detection of the viral DNA genome or expression of the viral genes using PCR methods.

The putative role of HPV in HNSCC has been studied since the 1980s, and it seemed that the viral oncogenes *E6* and *E7* that have a crucial role in cervical cancer were also involved in HPV-mediated carcinogenesis of the upper aerodigestive tract[19,20]. Later studies revealed

Ploidy
The number of chromosomes in a cell. Normal human cells are diploid, having a DNA index of 2c, a state also referred to as euploid. Cancer cells are often tetraploid, with a DNA index of 4c, or aneuploid, with a DNA index somewhere between 2c and 4c. The DNA index reflects the number of numerical genetic changes: the losses and gains of chromosomes or parts of chromosomes.

Comparative genomic hybridization
(CGH). A method to visualize the presence or absence of chromosomes or parts of chromosomes in a tumour sample by fluorescence microscopy. Array CGH is comparable to CGH, except that the labelled DNAs are not hybridized to metaphase spreads but to DNA molecules on a glass slide, which increases the resolution.

Table 1 | **Different clinical and biological characteristics of HPV-negative and HPV-positive HNSCC**

Feature	HPV-negative HNSCC	HPV-positive HNSCC	Refs
Incidence	Decreasing	Increasing	138,139
Aetiology	Smoking, excessive alcohol use	Oral sex	31
Age	Above 60 years	Under 60 years	138
Field cancerization	Yes	Unknown	49,136
TP53 mutations	Frequent	Infrequent	26,27,140
Predilection site	None	Oropharynx	21,141
Prognosis	Poor	Favourable	5,142

HNSCC, head and neck squamous cell carcinoma; HPV, human papillomavirus.

that HPV-16 in particular is involved in HNSCC, the presence of the virus is particularly common in oropharyngeal tumours, and that tumours with HPV were associated with a more favourable clinical outcome[21].

One observation that was a little confusing in these initial studies was that the presence of the virus was not strongly inversely correlated with the mutation status of *TP53* (which encodes p53). HPV-16 E6 inactivates p53 (FIG. 1) and mutations in *TP53* are therefore rarely present in, for example, cervical carcinomas. By contrast, *TP53* is mutated in 60–80% of HNSCC cases, so it was expected that the HPV-infected tumours were among the 20–40% of *TP53* wild-type tumours, but this seemed not to be the case. One explanation might be a 'hit and run' mechanism of HPV-mediated oncogenesis, but it is more likely that the HPV DNA PCR assays that are generally used are too sensitive. These assays can detect only a few copies of viral DNA, and may detect not only the oncogenic infections, but also productive infections, virions or laboratory artefacts, problems well known from cervical cancer screening by HPV testing[22,23]. Indeed, detection of viral *E6* and *E7* transcripts seemed to be a more reliable assay for the detection of an oncogenic HPV infection in HNSCC than PCR amplification of HPV DNA[24], and when using *E6* and *E7* expression as a 'gold standard', all *E6*- and *E7*-positive cases were *TP53* wild type as expected[24,25]. In addition, it became clear that, when stratified according to viral oncogene expression, HNSCCs have different genetic profiles, particularly in relation to the early markers of progression[26], as well as differentially expressed genes[27]. This indicates that HPV infection is an early, and probably initiating, oncogenic event. In addition, these data suggested that these tumours form a distinct molecular entity within HNSCC[28].

Over the years, it became more and more apparent that the testing of tumours for oncogenic HPV infection has remained a problem, and, consequently, also influences the reliability of the reported prevalence rates and prognostic data. Although PCR amplification of HPV DNA may cause false-positive results, a problem with the more reliable reverse transcriptase (RT)-PCR assays for *E6* and *E7* transcripts is that these do not work well on archival formalin-fixed paraffin-embedded (FFPE) tissue specimens. It was shown that an algorithm of $p16^{INK4A}$ (also known as CDKN2A) immunostaining (a surrogate marker for HPV infection) combined with HPV DNA PCR on the $p16^{INK4A}$-positive cases gave reliable results on archival FFPE specimens[29], and this combined method is now more widely advocated[30].

Notwithstanding these insights from recent studies, some questions remain. There are large differences in the reported prevalence rates of HPV-infected tumours, even when results are stratified for tumour site and assays with a comparable performance are used. Clearly, there is a large regional and time-trend variation in prevalence rates[26,31,139]. A second issue relates to the more favourable prognosis of HPV-positive tumours[5,32]. HPV-positive tumours are typically *TP53* wild type and it has been shown that the presence and type of *TP53* mutation is also of prognostic relevance[33]. Therefore, it remains to be determined whether HPV-positive tumours have a relatively favourable prognosis, or whether tumours with *TP53* mutations (or a specific subtype of mutations) have a relatively poor prognosis[34], or both. It will probably be both, but the biological basis of the difference in prognosis between HPV-positive and HPV-negative tumours remains elusive.

All in all, it seems that HPV-positive tumours form a distinct group within HNSCCs. The aetiological factor differs, the tumours are different at the molecular level and the clinical outcome is different, in general HPV-infected HNSCCs have a more favourable prognosis[32] (TABLE 1). In prognostic research, HPV involvement has become an important factor, and in studies with outcome parameters as the end point, HPV status should always be included as a possible confounding factor and tested in multivariate models[5]. On the basis of HPV status and high or low chromosome instability (CIN) DNA profiles, an initial genetic classification model is proposed in FIG. 2.

Pathogenesis

Precursor lesions in the mucosal linings. By far the most knowledge on the pathogenesis of squamous cell carcinomas has been obtained from oral cancers, probably because oral precursor lesions are the most frequently diagnosed of these cancers, and specimens are available for research. Oral leukoplakia, a white lesion in the mucosa of the oral cavity, is the most common precursor lesion of oral squamous cell carcinoma and its prevalence varies between 0.1% and 0.5%[35,36]. The reported proportion of oral leukoplakia that develops into cancer depends on various factors such as the study population, the definition of leukoplakia

Oral leukoplakia
The most common premalignant lesion of HNSCC, defined as a white plaque in the mucosal linings that indicates questionable risk after the exclusion of other known diseases or disorders that carry no increased risk of cancer.

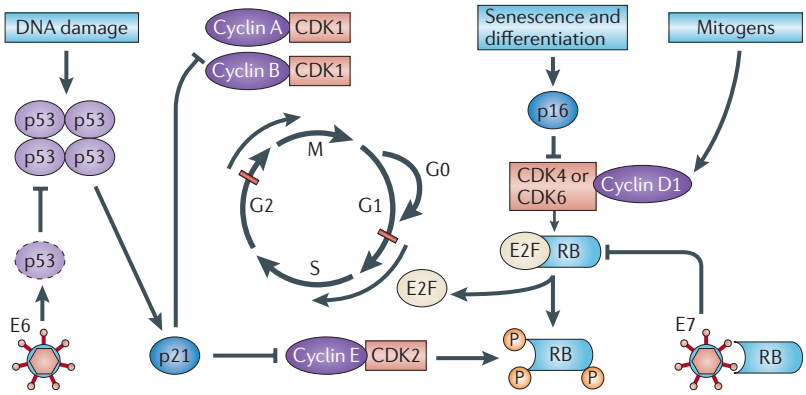

Figure 1 | **Cell cycle deregulation by human papilloma virus.** The cell cycle is regulated by complexes of cyclins and cyclin-dependent kinases (CDKs), some of which are indicated. In addition, there are various important inhibitors of these cyclin–CDK complexes. To allow cell cycle progression, cells have to pass the G1 restriction point (red bar) that is controlled by the retinoblastoma pocket proteins, RB, p107 (also known as RBL1) and p130 (also known as RBL2). Only RB is indicated, but the other pocket proteins have similar activities. These normally bind to and inactivate the E2F transcription factors, which induce the expression of S phase genes. In response to a mitogenic signal, the cyclin D1–CDK4 and cyclin D1–CDK6 complexes are activated. These phosphorylate the Rb pocket proteins, causing release (and therefore activation) of E2Fs. Induction of cyclin E by E2F and subsequent additional phosphorylation of RB by the cyclin E–CDK2 complex initiates entry into S phase. The inhibitor for the cyclin D1–CDK4 and cyclin D1–CDK6 complexes is $p16^{INK4A}$, which is encoded by *CDKN2A*, a gene in the *INK4A* locus at chromosome 9p21. The expression of $p16^{INK4A}$ mediates senescence and differentiation. The interplay between the cyclins, CDKs and their inhibitors determines whether the restriction point can be passed, and a growth factor stimulus is usually required. A second important control mechanism of the cell cycle occurs during G2 phase, when the DNA has been replicated and replication errors are repaired. The key protein involved in the response to replication errors and other DNA damage is p53, which is usually maintained at low concentrations by MDM2-mediated degradation (not shown). DNA-damage sensors, including ataxia-telangiectasia (ATM) and ataxia-telangiectasia and Rad3-related (ATR), phosphorylate the checkpoint kinases CHK1 and CHK2, leading to increased p53 activity by phosphorylation of various downstream molecules, including p53 itself (not shown). The p53 tetramers act as a stress-induced transcription factor and induce the expression of $p21^{CIP}$ (also known as CDKN1A), which inhibits several cyclin–CDK complexes and halts the cell cycle. Besides its crucial role in cell cycle control, p53 is also a master regulator of apoptosis and many other stress-associated cellular functions, and is therefore one of the main targets for inactivation in many cancers. The human papillomavirus (HPV) genome contains various early and late open reading frames and encodes two viral oncoproteins: E6 and E7. The E6 protein binds p53 and targets the protein for degradation, whereas the E7 protein binds and inactivates the Rb pocket proteins. The molecular consequence of the expression of these viral oncoproteins is cell cycle entry and inhibition of p53-mediated apoptosis, which allows the virus to replicate. In a 'productive infection' the expression of E6 and E7 is confined to the differentiating layers of the squamous epithelium of the cervix and virions are produced. An oncogenic infection is associated with *E6* and *E7* expression in the basal layer (where the stem cells reside) and causes abrogation of the cell cycle checkpoints.

used and the length of observation time, but an annual transformation rate of 1–2% per year is a reasonable assumption[35,36]. Risk factors for progression are female gender, size of lesion and the presence and grade of dysplasia[35]. Although criteria have been defined by the World Health Organization, it is difficult to make an objective categorization of dysplasia owing to a high inter-observer and intra-observer variation in assessment.

It is considered appropriate to actively treat leukoplakia, irrespective of the presence of dysplasia[36]. Unfortunately, there is no scientific evidence that any type of treatment is able to prevent squamous cell carcinoma in these patients[37]. Factors that may explain this are that the leukoplakia recurs despite removal or that cancer develops outside the visible lesion[38]. Although chemoprevention may cause the regression of leukoplakia lesions, a decrease in cancer incidence has rarely been observed[39].

The problems with histological grading and treatment of leukoplakia have fuelled molecular studies to assess the risk for progression and to identify targets for treatment. Several studies have shown that the presence and number of cancer-associated genetic changes can be used to discriminate leukoplakias with a low risk from those with a high risk of malignant transformation[35,38,40–43]. To avoid painful biopsies, saliva and exfoliated cells can be obtained as a source for biomarker-based risk assessment[44–46].

Field cancerization. Oral leukoplakias are visible precursor lesions that are macroscopically recognized. However, there are several histological and clinical indications that many precursor changes in the oral mucosa are not visible to the naked eye. In 1953, the term 'field cancerization' was proposed to explain the high propensity to develop local recurrences after treatment of HNSCC and the high likelihood that multiple independent tumours will develop in the head and neck mucosa. Slaughter *et al.*[47] carefully studied oral cancer specimens and linked the frequent observation of dysplastic changes surrounding these tumours with the occurrence of local recurrences and multiple primary tumours. Owing to the developments in molecular research during the past two decades, the process of field cancerization has now been defined in molecular terms. In 1996, the first genetic multi-step progression model for HNSCC was postulated on the basis of the genetic characterization of morphological changes in the squamous epithelium[48]. Loss of heterozygosity at chromosomes 3p, 9p and 17p seemed to occur in dysplasia, apparently reflecting early carcinogenesis, whereas other alterations at chromosomes 11q, 4q and of chromosome 8 were typically present in carcinomas, probably corresponding to a relatively late phase in carcinogenesis.

Using these genetic markers combined with *TP53* mutations, it was shown that in at least 35% of the oral and oropharyngeal tumours analysed, the carcinoma was surrounded by mucosal epithelium that contains genetic changes[49]. This epithelium has a macroscopically normal appearance, but may be histologically dysplastic[50], confirming the older studies by Willis[51] and Slaughter[52]. This tumour-adjacent mucosal epithelium characterized by genetic changes has also been termed 'field'[49,53], in line with the earlier study[47]. Importantly, these fields are often found in the surgical margins when the tumour is excised, meaning that they can remain in the patient[49]. In retrospective studies, it was shown that these unresected fields are an important source of the local recurrences and the second primary tumours that are so often seen in patients treated for HNSCC[54–56]. The important role of field cancerization in HNSCC pathogenesis and its consequences for patient management is depicted in FIG. 3. Comparison of the genetic profiles of carcinomas and their surrounding fields often indicates a clonal relationship[49], and this

Squamous epithelium
Multilayered epithelium covering the linings of the upper aerodigestive tract.

Loss of heterozygosity
(LOH). A genetic change that describes the loss of one allele of a gene for which the other allele is already inactivated.

idea formed the basis of the hypothesis that such a field of contiguous preneoplastic cells precedes the development of an invasive carcinoma[57].

There is some information on what seems to precede the development of fields. van Houten *et al.*[58] reported small, p53-positive focal patches in tumour-adjacent mucosal epithelium. Some were sequenced and showed a mutation in *TP53*, but this mutation was not identical to the one in the corresponding tumours, indicating that these patches are not clonally related to the tumour. These mutated p53-positive patches were considered equivalent to the 'clones' or 'clonal units' defined as a family of cells from a common progenitor cell or adult stem cell that comprises the squamous epithelium[59] and that have now become detectable by mutation of p53. These p53-mutated clonal units are considered to represent the first oncogenic changes in the mucosa and, together with the genetically defined fields, form the basis of a hypothetical patch–field–tumour–metastasis progression model for HNSCC development. This model may also be valid for other tumour types[60], and was the basis of identification of the molecular changes found in HNSCC discussed below (FIG. 4).

Changes in signalling pathways

In general, cancer arises through the accumulation of genetic and epigenetic changes in genes acting in cancer-associated signalling pathways, causing the acquisition of cancer-related phenotypes that have been well summarized by Hanahan and Weinberg[9], including limitless replicative potential, self-sufficiency in growth signals, insensitivity to anti-growth signals, ability to evade apoptosis, invasion and metastasis, and angiogenesis. Recently, this summary has been updated by others[61].

A plethora of studies has been published on the identification of candidate cancer genes in HNSCC. It is not possible to cover these comprehensively, and several other reviews have been published on this topic[62–64]. A list of frequently found genetic changes in HNSCC and the cancer genes at these loci is provided in TABLE 2. It is worth noting that these changes have not been grouped according to the suggested classification scheme (FIG. 2), because this has not been investigated.

The decision of whether enough experimental evidence has been obtained to accept a gene as an 'established cancer gene' in HNSCC or whether to consider it as a 'candidate' is arbitrary, and we decided to formulate criteria to define cancer genes in line with the levels of evidence for aetiological factors[65] (BOX 1).

Below, we discuss the acquired cancer-associated phenotypes according to the Hanahan and Weinberg model[9,61] and describe the relevant cancer genes according to the defined levels of evidence. Important pathways are indicated in FIG. 1 and FIG. 5, and molecular carcinogenesis has been summarized in FIG. 4.

Limitless replicative potential: the p53 and RB pathways. One of the key cellular functions that is often, if not always, changed in cancer cells to overcome senescence and to obtain limitless replicative potential is the regulation of the cell cycle[66]. Crucial genes involved in the regulation of the cell cycle that are targeted by mutations in HNSCC, or alternatively by HPV oncogenes, are those encoding proteins in the p53 and RB pathways (FIG. 1). On the basis of the formulated levels of evidence described in BOX 1, we can state with confidence that *TP53* is an established cancer gene in HNSCC. Somatic mutations in *TP53* are found in 60–80% of HNSCC cases[33,58,67], and overexpression of a dominant-negative mutant of p53, in conjunction with ectopic expression of telomerase reverse transcriptase (TERT, the catalytic subunit of telomerase), as well as overexpression of cyclin D1, or a $p16^{INK4A}$-insensitive cyclin-dependent kinase 4 (CDK4) mutant, causes immortalization of cultured mucosal keratinocytes *in vitro*[68,69]. Recently, these initial studies on primary epithelial cells were extended using a conditionally immortalized model of oral keratinocytes *in vitro*[70]. An extended lifespan was conferred on oral keratinocytes by inactivation of p53, either by knock down of *TP53* with short hairpin RNA (shRNA), by expression of dominant-negative mutant $p53^{R172H}$ or by expression of the HPV16 oncoprotein E6. When combined with $p16^{INK4A}$ knockdown, ectopic cyclin D1 or HPV16 E7 expression, the cells became immortal, albeit in the context of TERT expression[70]. In summary, p53 is frequently inactivated in HNSCC: either by somatic mutation in non-HPV tumours or by HPV16 E6 in HPV-induced tumours.

The same is true for the $p16^{INK4A}$–cyclin D1–CDK4–RB or $p16^{INK4A}$–cyclin D1–CDK6–RB axis. *CDKN2A*, which encodes $p16^{INK4A}$, is located on chromosome 9p21 and is frequently inactivated in HNSCC by mutation or methylation in combination with chromosome loss or, in most cases, by homozygous deletion[71]. *CCND1*, which encodes cyclin D1, is located on chromosome 11q13, and is amplified or gained in more than 80% of cases of HPV-negative HNSCC[27]. Together with the abrogation of p53, these changes cause cellular immortalization[70]. As a result, *TP53*, *CCND1* and *CDKN2A* are established cancer genes in HPV-negative

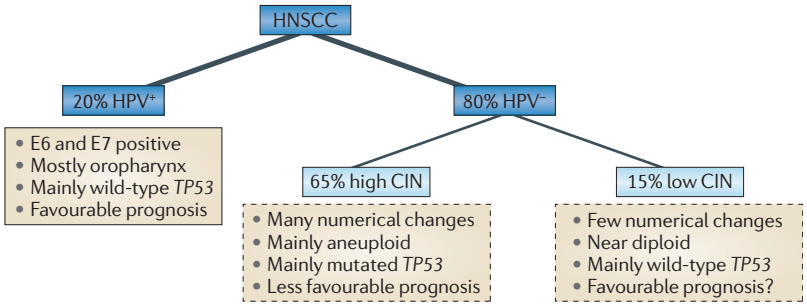

Figure 2 | **Schematic overview of the genetic classification of head and neck squamous cell carcinoma.** At least three genetic subclasses of head and neck squamous cell carcinomas (HNSCCs) can be distinguished at present: tumours that contain transcriptionally active human papillomavirus (HPV^+); tumours that are HPV-negative (HPV^-) and are characterized either by many numerical genetic changes (high chromosome instability (high CIN)) or by a low number of numerical genetic changes (low CIN). These low CIN HNSCCs might follow independent molecular routes, or show specific epigenetic changes or subtle genetic changes that are only detectable at ultrahigh resolution. Compared with the numerous studies on HPV, the low CIN and high CIN classification is preliminary because it is based on a limited number of studies. The prevalence rates indicated are estimates.

Telomere
The linear end of a chromosome. The telomere is shortened with each round of DNA replication.

HNSCC, and *TP53* and the genes encoding the Rb family (comprising *RB1, RBL1* (which encodes p107) and *RBL2* (which encodes p130)) are established cancer genes in HPV-positive HNSCC. The cancer-associated phenotype caused by inactivation of these two pathways in oral keratinocytes is at least cellular immortalization. This phenotype fits with the timing of the genetic events that occur early in the progression of HPV-negative HNSCC. The loss of chromosome 9p21 (which is where *CDKN2A* is located), and *TP53* mutations are frequently found in precursor fields[48,49,57]. In HPV-positive HNSCC, these same pathways are also the first to be inactivated by the viral *E6* and *E7* oncogenes.

It is noteable that the identification of *CCND1* as an established oncogene in HNSCC does not necessarily mean that this is the only relevant cancer gene in the chromosome 11q13 amplicon. It is possible that other genes located within this amplicon are also relevant, as has been suggested for Fas-associated via death domain (*FADD*)[72].

Although we postulate that the abrogation of p53 — either by somatic mutation or through E6 expression — is one of the first causative genetic hits, not all tumours contain mutant p53 or HPV. Approximately 60% of HNSCCs contain a mutation in *TP53* and about 20% contain transcriptionally active HPV[26]. In the remaining 20% of cases, p53 seems not to be inactivated. There is the unlikely possibility that mutations have been missed, but it is more plausible that other genes encoding proteins in the p53 pathway are targeted in selected cases[73] or that these tumours undergo p53-independent malignant progression.

Besides abrogation of cell cycle regulation by the inactivation of the p53 and RB pathways, telomere shortening probably also needs to be overcome to achieve limitless replicative potential. The activity of telomerase or TERT is detectable in 80% of HNSCC cases analysed[48]. Moreover, in most *in vitro* models, TERT seemed to be an important factor[69,74], although the data are not consistent. It has been proposed that keratinocytes may undergo alternative lengthening of telomeres (ALT), which is a TERT-independent process of telomere lengthening[68]. The exact role of *TERT* is still unclear and it should therefore be considered as a candidate cancer gene. The chromosomal location of *TERT* (5p15.33) is not known to be frequently gained or amplified in HNSCC. In HPV-positive tumours the role of increased *TERT* expression seems more important, at least in the cervix[75].

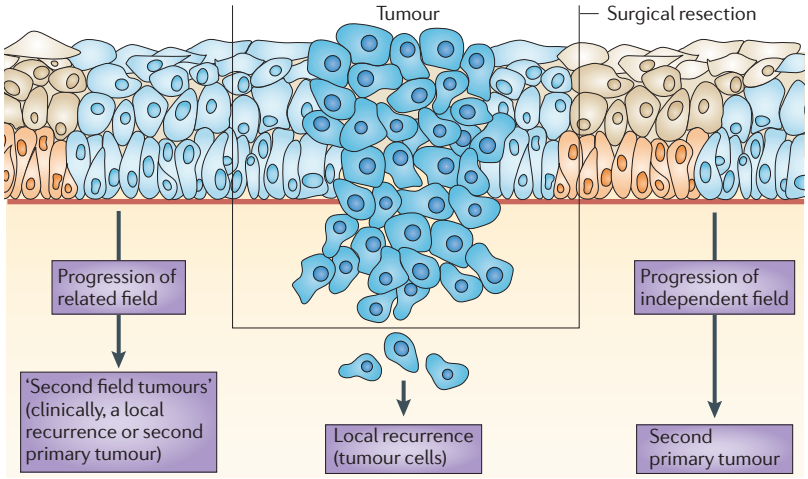

Figure 3 | **Field cancerization and local relapse.** The relationship between field cancerization and types of relapse is shown. On the basis of recent molecular findings, field cancerization is defined as the presence of one or more mucosal areas consisting of epithelial cells that have cancer-associated genetic or epigenetic alterations. A precursor field (or field; shown in light blue) is monoclonal in origin and does not show invasive growth or metastatic behaviour, which are the hallmarks of an invasive carcinoma. A field is preneoplastic by definition; it may have histological aberrations characteristic of dysplasia, but not necessarily[60,132]. A leukoplakia is the clinical manifestation of a field, but most fields are clinically invisible. At least some fields can be visualized by autofluorescence[133,134]. An important clinical implication of a field is that it may be the source of local recurrences and second primary tumours after surgical resection of the initial carcinoma. These two possibilities can be distinguished clinically on the basis of their distance from the index tumour or the time interval after which they develop (whereby a local recurrence is less than 2 cm away from or occurs within 3 years of the primary tumour; a second primary tumour is more than 2 cm from or occurs more than 3 years after the primary tumour). Additional genetic changes are needed to transform a field into a new carcinoma. The field and primary tumour share genetic alterations and should be considered as having a common clonal origin. Tumours that do arise in a non-resected field have been described as 'second field tumours' as opposed to true local recurrences (which develop from residual tumour cells) or true second primary tumours (which have an origin that is independent from that of the first tumour)[135]. This process has been summarized in an animation that can be found in the VU Medical Center website (see Further information). It is not known what specific genetic characteristics determine the risk of a field developing into cancer. Recent studies, as well as immunostaining for mutant p53, have shown that genetic changes at chromosome 9p, decreased cytokeratin 4 expression and decreased cornulin expression are promising biomarkers in this respect[56,136]. From leukoplakia studies we know that the presence and number of genetic changes, typically chromosome 9p loss, chromosome 3p loss and chromosome 17p loss, are associated with the risk of progression[38,41,42].

Changes in growth factor signalling: the EGFR pathway. One of the most studied groups of receptor tyrosine kinases is the Erbb family. After ligand binding or other activating interactions, the four Erbb receptors form homodimers or heterodimers, and initiate a signalling cascade. EGFR seems to be crucial in squamous cells and signals through the Ras–MAPK, PI3K–PTEN–AKT and phospholipase C pathways[76]. Most intriguingly, EGF-bound EGFR is also able to translocate to the nucleus and it functions as a transcription factor or co-activator of other transcription factors, such as signal transducer and activator of transcription (STAT) proteins[77,78]. One of the genes induced by intranuclear EGFR is *CCND1*, directly linking cell cycle progression to mitogen stimulation[77]. Therefore, the intracellular effects of these activated receptors can be pleiotropic and influence cellular homeostasis at various levels. Whether all of these relay systems are activated in keratinocytes or HNSCC cells remains to be discovered. Nevertheless, ectopic expression of *EGFR* has been implicated in the transformation of oral keratinocytes *in vitro*[79].

In 1986, it was claimed that *EGFR* is overexpressed in many cases of HNSCC[80]. This was later confirmed by a multitude of studies[81,82], finally resulting in a clinical trial that showed increased efficacy of radiotherapy when it was combined with EGFR-specific antibodies

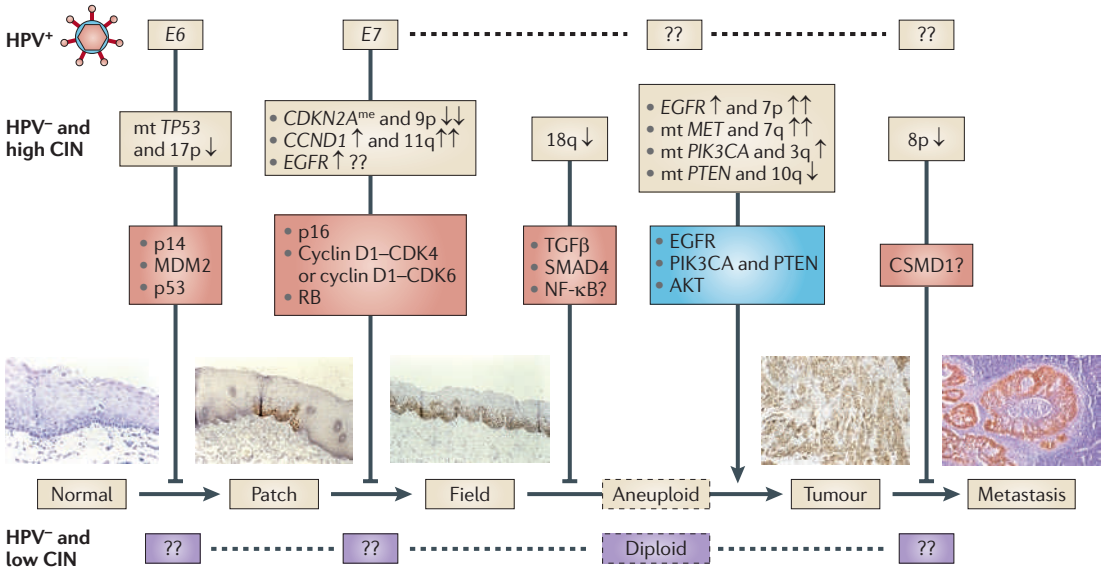

Figure 4 | **Proposal of an integrated model of molecular carcinogenesis for head and neck squamous cell carcinoma.** A hypothetical model of head and neck squamous cell carcinoma (HNSCC) development in which the genes and pathways involved are depicted. Most information has been deciphered from oral carcinogenesis, there are fewer data on the other subsites of HNSCC. A progenitor or adult stem cell acquires one (or more) genetic alterations, including a mutation in *TP53*, and forms a patch containing genetically altered daughter cells that can be detected by immunostaining for mutant p53. By escaping normal growth control and/or gaining growth advantage, this patch or clonal unit develops into an expanding field, laterally replacing the normal mucosal epithelium. Eventually, a subclone in the field evolves into an invasive cancer, and progresses to metastasis. Three critical steps can be discriminated in this model: the conversion of a single mutated stem cell in a patch into a group of stem cells without proper growth control (field); the eventual transforming event, which turns a field into an overt carcinoma showing invasive growth and metastasis; and the development of metastasis. Both aneuploidy and the accumulation of cancer-associated genetic changes in fields are linked to the risk of malignant progression. The established signalling pathways involved in HNSCC are depicted, stratified for all three genetic subtypes of tumours listed in FIG. 2 — human papilloma virus (HPV)-induced HNSCC (HPV-positive (HPV^+)), as well as tumours not caused by HPV (HPV^-) that have many numerical genetic changes (high chromosome instability (high CIN)) or HPV^- with few genetic changes (low CIN). For HPV^- low CIN tumours no detailed molecular data are available at present. Reliable assays are now available to assess the HPV status of a tumour and, therefore, to enable incorporation of the proposed genetic classification in future studies. In addition, the discrimination between low CIN and high CIN HNSCCs — by means of ploidy determination — is fairly straightforward. Genetic and chromosome alterations are indicated in yellow boxes, oncogenic pathways are depicted in the blue box, tumour-suppressive pathways are shown in the orange boxes. The order of p53 or retinoblastoma (Rb) pathway abrogation has not been definitively established and might not be relevant. Epidermal growth factor receptor (*EGFR*) and *MET* are amplified and mutated in tumours and may relay signals through Ras–MAPK, PI3K–PTEN–AKT and other pathways. Our choice to place *EGFR* and *MET* in the PI3K–AKT route is arbitrary and requires more detailed investigation. ↑ indicates overexpression or gain; ↑↑ indicates high-level amplification; ↓ indicates loss; and ↓↓ indicates homozygous loss. *CCND1*, cyclin D1; CDK, cyclin-dependent kinase; CDKN2A, cyclin-dependent kinase inhibitor 2A; me, methylated; mt, mutated; NF-κB, nuclear factor-κB; PIK3CA, phosphoinositide-3 kinase subunit-α; TGFβ, transforming growth factor-β.

to treat patients with HNSCC[83]. Together, these data argue strongly for an important role of *EGFR* in HNSCC carcinogenesis.

Notwithstanding this more or less clear and successful story, there are still many open questions and perhaps even concerns. Reports of EGFR overexpression are often based on immunostaining studies, which have a large variation in antibodies and antigen-retrieval protocols, and sometimes lack controls (usually normal tissue). As a result, the reported prevalence rates of expression and overexpression vary widely. In addition, it has been shown that only a small proportion of HNSCCs that have *EGFR* overexpression also have receptor cross-phosphorylation, suggesting an active autocrine loop[11,82]. Moreover, there are multiple (at least 13) tyrosine phosphorylation sites in EGFR that mediate interactions with different partner proteins, causing different downstream effects[84]. Therefore, the exact role of EGFR in HNSCC remains elusive.

With respect to *EGFR* alterations, the data become more consistent. There are few activating *EGFR* mutations found in HNSCC. *EGFR* point mutations are reported in only 1% of the Caucasian and 7% of the Asian population[85,86]. Of the 7% reported in the Asian population, only a subgroup seemed to be activating and therefore oncogenic[82]. Besides these somatic point mutations, a specific mutant form of EGFR that is found more frequently has also been described in HNSCC. This mutant, EGFRvIII, was originally discovered in glioblastomas and typically occurs in *EGFR*-amplified regions[87]. It was found in 42% of the HNSCCs analysed and showed biological activity, causing enhanced

Table 2 | **Cancer genes at frequently altered chromosome locations in HNSCC***

Chromosomal location	Gene	Cancer gene status	Refs
Tumour-suppressor genes			
3p14	*FHIT*	Candidate	123
3p21	*RASSF1A*	Candidate	143
8p23	*CSMD1*	Candidate	112
9p21	*CDKN2A*	Established	70,71
9p23	*PTPRD*	Candidate	144
10q23	*PTEN*	Established	107
17p13	*TP53*	Established	70,145,146
18q21	*SMAD4*	Established	98
Oncogenes			
3q25	*CCNL1*	Candidate	108
3q25	*PARP1*	Candidate	147
3q26	*PIK3CA*	Established	106,108
3q26	*TP63*	Candidate	124,125
3q26	*DCUN1D1*	Candidate	148
7p11	*EGFR*	Established	91
7q31	*MET*	Established	93
8q24	*MYC*	Candidate	79,126,149
8q24	*PTK2*	Candidate	150
11q13	*CCND1*	Established	68–70,151,152
11q13	*CTTN*	Candidate	152
11q13	*FADD*	Candidate	72

CCN, cyclin; *CDKN2A*, cyclin-dependent kinase inhibitor 2A; *CTTN*, cortactin (also known as *EMS1*); *DCUN1D1*, defective in cullin neddylation 1, domain-containing 1; *EGFR*, epidermal growth factor receptor; *FADD*, FAS-associated via death domain; *FHIT*, fragile histidine triad gene; *PARP1*, poly (ADP-ribose) polymerase 1; *PIK3CA*, phosphoinositide-3 kinase subunit-α; *PTK2*, protein tyrosine kinase 2; *PTPRD*, protein tyrosine phosphatase, receptor type, D. *The genes included in the table have been limited to those located in frequently changed (>50%) chromosome locations. In addition, we focused on genes that have been reported in multiple studies, that display mutations and/or homozygous deletions and/or that functional studies indicate that they have a role in oncogenesis. Many more chromosome regions have been reported than are listed in the table, including numerical losses at 1p, 4, 5q, 6q, 11q and 21, and gains at 5p, 8p, 9q, 17q, 19 and 20 (REFS 27,149,153), and allelic losses at 2q, 4p, 4q, 5q, 6p, 9q, 10q, 11q, 13q, 14q, 15q and 19q (REFS 48,154). The only chromosomes that do not seem to be involved in head and neck squamous cell carcinoma (HNSCC) are chromosomes 12 and 16.

proliferation[88]. This mutant form therefore has an effect on the intracellular signalling network and it has also been shown to decrease the effect of anti-EGFR treatment[88]. It is noteworthy that a recent study by Hama *et al.*[82] did not find expression of EGFRvIII, which might be because of the population studied, but obviously causes confusion.

Amplification is an alternative method by which *EGFR* can be oncogenically activated and was first reported in 1986 (REF. 89). Although the frequency of amplification varies between studies, it is generally 10–30%[90,91]. In a study by Sheu *et al.*[91] using high-resolution 250 kb single-nucleotide polymorphism arrays, 31% of the 29 oral cancers tested showed amplification at 7p11.2, which correlated with EGFR overexpression both at the RNA and protein levels. This was confirmed in an independent set of 128 tumours analysed by fluorescence *in situ* hybridization and immunostaining[91], and probably reflects the actual situation.

When evaluating the plethora of data against the levels of evidence to define an established cancer gene (BOX 1), we may conclude that *EGFR* is an established oncogene in HNSCC. Mutations and gene amplifications have been reported, albeit at relatively low frequencies. Ectopic expression of *EGFR* effects the transformation of oral keratinocytes. As a result, *EGFR* seems to play a part in at least a subgroup of tumours. However, only specific signalling functions of EGFR might be hijacked by a specific tumour cell. One tumour might exploit EGFR to activate the AKT pathway, another tumour to induce the Ras–MAPK pathway, and yet another tumour to induce *CCND1* expression. This pleiotropy may influence both the role of EGFR in a tumour cell and anti-EGFR drug responsiveness. Often, but not always, there is an association between EGFR overexpression and a poor clinical outcome. In general, 60% of the studies show an association between EGFR overexpression and poor outcome, whereas 40% do not.

The molecular circuitry around EGFR becomes even more complex as another growth factor receptor, MET (the receptor for hepatocyte growth factor (HGF); also known as scatter factor), has recently been

Box 1 | **How to define a cancer gene as candidate or established**

We scanned the literature but were not able to find working definitions of 'candidate' cancer genes or 'established' cancer genes. To simplify the discussion and to allow decisions on whether a gene can be considered a candidate or an established cancer gene in head and neck squamous cell carcinoma (HNSCC), we used several levels of evidence for cancer genes that relate to the assessment of aetiological factors[65]. The levels we used were:

Level 5. Somatic genetic or epigenetic changes at the chromosome locus where the gene resides are found in HNSCC.

Level 4. Somatic point mutations in the gene are found in HNSCC, potentially pathogenic mutations in the gene are found in HNSCC-prone families, or the function of the gene product is changed by a virus.

Level 3. Manipulation of the gene leads to a cancer-associated phenotype in established HNSCC cell lines that corresponds to the expected oncogenic or tumour-suppressing function of the gene.

Level 2. The gene fulfils the criteria for at least two of the levels 5–3, and manipulation of the gene leads to a cancer-associated phenotype in an *in vitro* carcinogenesis model of mucosal keratinocytes or in a mouse squamous cell carcinoma model, in line with the expected oncogenic or tumour-suppressing function.

Level 1. The gene fulfils the criteria for at least three of the levels 5–2 and shows proven interactions in a signalling pathway with other established cancer genes.

We call genes that fulfil the criteria defined in levels 5 and/or 4 and/or 3 candidate cancer genes and those fulfilling the criteria of levels 1 and/or 2 established cancer genes in HNSCC. Notably, we decided not to include associations with clinical outcome as a level of evidence. Outcome association is highly relevant for clinical applications but is biologically undefined and an outcome association might not be causative.

shown to be important for HNSCC[92]. This receptor tyrosine kinase, encoded by *MET* on chromosome 7q31, also activates the AKT and Ras pathways, thus interconnecting with EGFR-mediated signalling. Both mutations and gene amplifications of *MET* have been reported in HNSCC[93], and on the basis of the formulated levels of evidence, *MET* can be described as an established cancer gene in HNSCC that influences cell growth, motility and angiogenesis[93,94]. There are, therefore, still many issues to be resolved around the molecular networks of the receptor tyrosine kinases involved in HNSCC. Furthermore, biomarkers that would enable an accurate prediction of tumour response, when applying anti-EGFR or anti-MET treatment, are urgently awaited.

Changes in growth factor signalling: the TGFβ pathway. Another important, but inhibitory, growth factor pathway associated with HNSCC is the transforming growth factor-β (TGFβ) pathway. TGFβ1 signals through the TGFβ receptors and these transduce the signal by phosphorylating SMAD2 and SMAD3, which, together with SMAD4, regulate the transcription of target genes (FIG. 5a). There are several lines of evidence implicating TGFβ in HNSCC. Downregulation of TGFβ receptors is often found in tumours[95,96]. This might be linked to the frequent loss of chromosome 18q, which contains the *SMAD2*, *SMAD3*, *SMAD4* and TGFβ receptor II (*TGBRII*) genes. Mutations in *SMAD2* and *SMAD4* have also been reported in two of eight HNSCC cell lines[97]. Moreover, it has very recently been shown that conditional knock out of *Smad4* in the oral mucosa causes HNSCC in mice[98]. Taken together, these data strongly indicate the relevance of this signalling pathway in HNSCC; at least *SMAD4* seems to be an established cancer gene in HNSCC, fulfilling all criteria of evidence.

A connection was recently reported between the TGFβ signalling pathway and nuclear factor-κB (NF-κB)[99], a transcription factor that provides an important survival signal to cells (for reviews on NF-κB see REFS 100,101). Cohen *et al.*[102] showed that abrogation of the TGFβ pathway was associated with activation of NF-κB, and this intriguing finding suggests that decreased TGFβ signalling is linked to NF-κB activation. At present, the available data make it difficult to pinpoint the most relevant cancer genes in these signalling pathways. High-resolution array CGH combined with sequencing for mutations and downstream pathway analysis might elucidate this in more detail.

Evading apoptosis: PI3K–PTEN–AKT. Another important signalling pathway in cancer, including HNSCC, is the PI3K–PTEN–AKT pathway, which is reviewed in REF. 103 (FIG. 5b). The class Ia PI3Ks, which are most frequently associated with cancer, are heterodimers coupled to receptor tyrosine kinases such as EGFR or adaptor molecules that may become active after receptor phosphorylation. The class Ia PI3Ks consist of a 110 kDa catalytic subunit and an 85 kDa regulatory subunit. One of these catalytic subunits is p110α, which is encoded by *PIK3CA*, located on chromosome 3q26 — a locus often gained in HNSCC. Somatic mutations of *PIK3CA* have also been described and are found in about 10–20% of HNSCCs[104–106]. It has also been shown that the identified mutations caused increased kinase activity, as well as increased migration and invasion of cells transfected with these mutants[106]. Besides activating *PIK3CA* mutations, inactivating mutations or homozygous deletions of *PTEN* have also been described in approximately 10% of HNSCCs. Inactivating mutations of *PTEN* mean that, once activated, the PI3K pathway cannot be turned off[107]. High-resolution array CGH combined with mutational sequencing of *PTEN* and *PIK3CA* and other potential cancer genes in the pathway may confirm mutual exclusion of genetic changes in these genes, which could indicate that the AKT signalling pathway is frequently perturbed in HNSCC.

The evidence for the role of the PI3K–PTEN–AKT pathway in HNSCC is therefore convincing because activating mutations in *PIK3CA* as well as inactivating mutations of *PTEN* have been found, both of which cause activation of AKT. Moreover, oncogenic activation of *PIK3CA* increased lipid kinase activity and caused cancer-associated phenotypes in a cell model. In addition, chromosome 3q26 gains are frequently reported[62], and enhanced *PIK3CA* expression in the frequently gained 3q26 region might explain the cancer-associated phenotype[108,109]. On the basis of the levels of evidence, we propose that *PIK3CA* and *PTEN* are established cancer genes in HNSCC. A remaining issue is how this connects to EGFR activation, because EGFR might signal

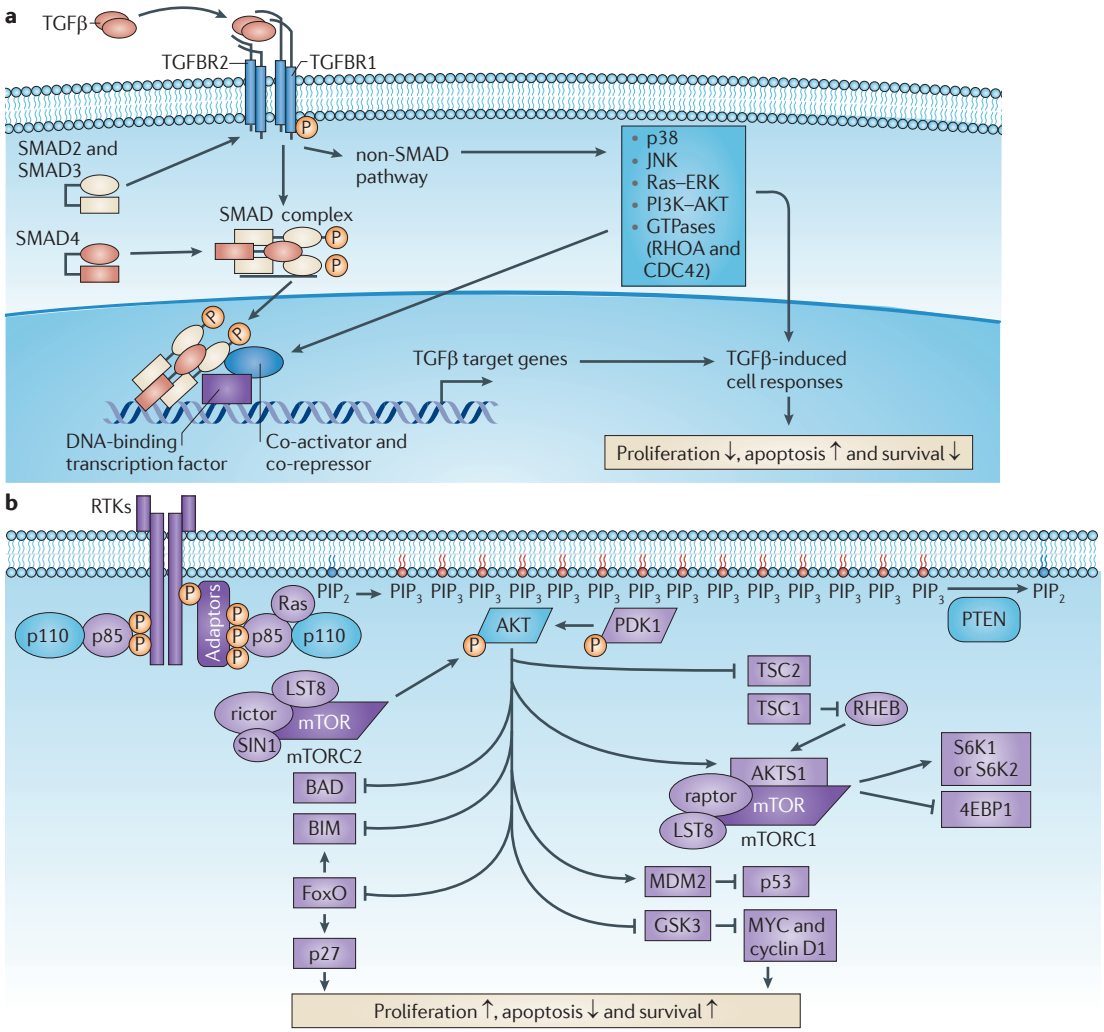

Figure 5 | **Schematic overviews of two signalling pathways that have a role in head and neck squamous cell carcinoma. a** | Depiction of the transforming growth factor-β (TGFβ) pathway. TGFβ signalling relayed through the SMAD pathway seems to be most important for head and neck squamous cell carcinoma (HNSCC). The TGFβ pathway inhibits growth. TGFβ ligands bind to the receptors TGFBR1 and TGFBR2, which phosphorylate TGFBR1 and subsequently activate SMAD2 and SMAD3. With the addition of SMAD4, the SMAD complex is formed, and this enters the nucleus and binds transcription factors, co-activators and co-repressors, which subsequently bind to the promoter regions of TGFβ target genes. Many of the target genes of the TGFβ pathway are suppressors of cell proliferation, such as the cell cycle inhibitors cyclin-dependent kinase inhibitor 2B (*CDKN2B;* which encodes $p15^{INK4B}$), *CDKN1A* (which encodes $p21^{CIP}$) and *CDKN1C* (which encodes $p57^{KIP}$)[118,137]. It has recently been shown that the loss of TGFβ signalling is associated with an increase in nuclear factor-κB (NF-κB) signalling, which promotes cell survival and immune responses. This link is not shown here because it has not yet been completely elucidated[102]. **b** | Schematic of the PI3K–PTEN–AKT pathway. There are several classes of PI3Ks, of which class Ia are most frequently associated with cancer. The class Ia PI3Ks are heterodimers coupled to receptor tyrosine kinases (RTKs) — such as epidermal growth factor receptor (EGFR) — or adaptor molecules. They consist of a 110 kDa catalytic subunit and an 85 kDa regulatory subunit. One of these catalytic subunits is p110α, which is encoded by *PIK3CA*, located at chromosome 3q26 — a locus often gained in HNSCC. There are several isoforms of these subunits, but in general the p85 subunits phosphorylate tyrosines (recognized by their SRC-homology domains) of the RTKs or adaptor proteins such as growth factor receptor-bound protein 2 (GRB2). The 110 kDa catalytic subunit subsequently phophorylates phosphatidylinositol 1,4-bisphosphate (PIP_2), creating phosphatidylinositol 1,4,5-trisphosphate (PIP_3). PIP_3 attracts proteins with pleckstrin-homology domains, including phosphoinositide-dependent protein kinase 1 (PDK1), which phosphorylates AKT. AKT is a serine/threonine kinase that, when fully activated, phosphorylates multiple downstream proteins, including transcription factors (FoxO), apoptosis inhibitors (BAD), cell cycle inhibitors and many other factors, to stimulate cell survival and proliferation. The reverse reaction from PIP_3 to PIP_2, which counteracts the activation of AKT, is mediated by the tumour suppressor PTEN. When PTEN is inactivated, the AKT pathway cannot be turned off once RTK stimulation has stopped. AKTS1, AKT substrate 1; BIM, BCL2-like protein 11; 4EBP1, eukaryotic translation-initiation factor 4E binding protein 1; GSK3, glycogen synthase kinase 3; JNK, Jun N-terminal kinase; mTORC, mTOR complex; RHEB, Ras homologue enriched in brain; S6K, ribosomal protein S6 kinase; SIN1, stress-activated map kinase-interacting protein 1; TSC, tuberous sclerosis. Part **a** modified, with permission, from REF. 118 © (2010) Macmillan Publishers Ltd. All rights reserved. Part **b** modified, with permission, from REF. 103 © (2009) Macmillan Publishers Ltd. All rights reserved.

through the PI3K–PTEN–AKT pathway. In addition, the relationship between these genetic alterations and the genetic heterogeneity of HNSCC requires further study. Functional studies in keratinocyte transformation models are urgently awaited to support the apparent role of the PI3K–PTEN–AKT signalling pathway in HNSCC carcinogenesis.

Invasion and metastasis. HNSCC behaves more or less classically in the sense that tumours metastasize primarily to the lymph nodes[110]. Not only the presence, but also the number of lymph node metastases, the proportion in the neck and extranodal spread are important prognostic factors and predictors of distant disease and survival. Metastatic dissemination involves several steps, including degradation of the extracellular matrix as one of the initial steps. Many studies have been carried out to investigate the involvement of the matrix metalloproteinases (MMPs), which are known to be involved in the degradation of the extracellular matrix. However, strong associations have not been found and treatments targeting MMPs were not very successful[111].

The *CSMD1* gene on chromosome 8p has been intensively studied for its involvement in the invasion and metastasis of HNSCC[112]. In 1996, the 8p23 region was shown to be significantly associated with outcome in supraglottic laryngeal cancer[113]. Refined deletion mapping led to the localization of the potential tumour-suppressor gene in a small region between two microsatellite markers[114], and finally resulted in the cloning of *CSMD1*[112]. It is unfortunate that the functions reported for *CSMD1* as a complement inactivating protein do not substantially add to our understanding of the role of this protein in HNSCC at present[115]. We need to consider that the protein has additional functions or that other genes or microRNAs (miRNAs) at this locus are causally involved.

Therefore, the currently available data do not point to specific cancer genes involved in invasion and metastasis. Nevertheless, metastatic dissemination, or at least early metastatic dissemination of tumours, seems to be biology-driven in HNSCC. It has recently been shown that a certain expression profile in the primary tumour can predict the presence or absence of lymph node metastasis[116], strongly suggesting that specific genes might drive the early metastatic dissemination to the lymph nodes in the neck.

These metastasis-associated profiles contain a large number of genes that may reflect the process of epithelial-to-mesenchymal transition (EMT)[12,117]. EMT is a fundamental biological process originally discovered in embryonic morphogenesis, wherein cells can change from an epithelial phenotype to a mesenchymal phenotype, a process also frequently seen in cancer cells, and most particularly linked to invasion and metastasis. Epithelial cells do not posess the cellular plasticity for metastatic dissemination and may undergo EMT to obtain a more mesenchymal, and therefore metastatic, phenotype. Recent findings indicate that the neurotrophic receptor tyrosine kinase NTRK2 and its ligand, brain-derived neurotrophic factor (BDNF), seem to have a crucial role in this process in HNSCC[102]. In addition, the TGFβ pathway has been identified as a key player in the EMT process[118]. Although these data are preliminary, this intricate process might become an important therapeutic target in the future.

Angiogenesis. Tumours that grow to more than a few millimetres in diameter require blood vessels for nutrient and oxygen supply, as well as disposal of catabolites[119]. All solid tumours therefore exploit methods to induce neo-angiogenesis, usually by producing angiogenic factors. These growth factors induce sprouting of endothelial cells, and new vessels feeding the tumour may develop. There are many inducers of angiogenesis, but the strongest is vascular endothelial growth factor (VEGF)[120]. Many studies have linked VEGF expression (usually assessed by immunostaining) to HNSCC prognosis, and in a meta-analysis a significantly increased risk of 1.88 was reported[121]. The analysis also highlighted a trend with VEGF expression and the development of lymph node metastasis. These data suggest at least a link between VEGF expression and outcome, but adjustments for other prognostic factors could not be made. It has recently been suggested that strong conclusions on the role of VEGF expression and outcome are mystified by other relevant prognostic factors, such as HPV status[122]. There are, therefore, still open questions to be answered.

Other candidate genes. Other candidate cancer genes have been implicated in HNSCC (TABLE 2), some of which were identified some time ago, including loss of fragile histidine triad gene (*FHIT*) at chromosome 3p14 (REF. 123), gain of *TP63* (which encodes p63) at chromosome 3q28 (REFS 124,125), gain of *MYC* at chromosome 8q24 (REF. 126) and loss of deleted in colorectal carcinoma (*DCC*) at chromosome 18q (REF. 127). There is a strong case for involvement in HNSCC for some of these candidate genes, but the number of studies to support roles for them is limited, and follow-up research to generate the required levels of evidence has not yet been completed.

miRNAs. Various papers have been published on the involvement of miRNAs in HNSCC[128–130]. In these papers, miRNA profiling was used to associate miRNA expression with malignant progression and prognosis, generally including comparisons of normal and tumour samples. How this relates to the different classes of HNSCCs is not known at present, and causal relationships have also not been elucidated in detail. However, these initial data already suggest that miRNAs are involved in squamous cell carcinogenesis.

Therapeutic implications

Therapy for advanced HNSCC is continuously adjusted to current scientific knowledge. It is often said that for HNSCC there is no treatment of choice, but rather a choice of treatments. Therefore, finding markers that

MicroRNA
(miRNA). Small RNA (of 22–24 oligonucleotides) generated from larger transcripts that bind target sequences in messenger RNAs in a large complex called the RNA-induced silencing complex (RISC). Binding of miRNAs to their (usually multiple) target transcripts causes transcript degradation or inhibition of protein translation.

predict response to chemoradiation protocols, so as to personalize treatment for the individual patient, is paramount. An alternative approach is to use induction chemotherapy. Good responders would receive further non-surgical treatment, whereas non-responders would be best treated by surgery[131].

A very topical question that needs to be answered relates to the treatment of HPV-infected tumours. Because it has been established that these form a distinct entity and have a favourable prognosis compared with HPV-negative tumours of a similar stage, it has been suggested that less intensive treatment modalities should be examined in order to decrease treatment-related morbidities. Recently, Ang *et al.*[5] found that patients with oropharyngeal cancer could be grouped according to prognosis (good, poor and intermediate) on the basis of staging, tobacco use and HPV status.

The introduction of anti-EGFR therapy is the first of the novel biological treatment modalities to find its way to the clinic. Effective methods to select anti-EGFR-sensitive tumours are urgently awaited. Not all HNSCCs are addicted to EGFR, and more insight is required about the effect of treatment and the involvement of altered signalling pathways. We need to explore all genes and miRNAs that might be able to kill a tumour, either by targeting the oncogenic pathway to which the tumour is addicted or by making use of synthetic lethal interactions. Finally, more attention should be focused on the treatment of precancerous fields to prevent local recurrences and second primary tumours. Targeted therapy will increasingly demand more predictive biomarkers besides HPV, *EGFR* and the mutation status of *TP53*. It is likely that these markers need to be combined with conventional staging, tobacco use and other clinical factors for optimal personalized treatment[5].

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Competing interests statement
The authors declare no competing financial interests.

DATABASES
National Cancer Institute Drug Dictionary: http://www.cancer.gov/drugdictionary
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Pathway Interaction Database: http://pid.nci.nih.gov
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FURTHER INFORMATION
C. René Leemans's homepage: http://www.vumc.com/afdelingen/Otolaryngology/2755295/
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