Charles University, Faculty of Science
Special Chemical and Biological Programmes
Molecular Biology and Biochemistry of Organisms

Viktorie Zlámalová

Important mechanisms of tumorigenesis and their role in chemoresistance of head and neck cancers

Významné mechanismy tumorigeneze a jejich role v chemorezistenci u nádorů hlavy a krku

Bachelor's thesis

Supervisor: RNDr. Milada Šírová, Ph.D.

Prague, 2017

Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracoval/a samostatně a že jsem uvedl/a všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 12.5.2016

Podpis

Poděkování:

Ráda bych poděkovala své školitelce RNDr. Miladě Šírové, Ph.D. za její trpělivost a ochotu stát se mojí školitelkou.

Abstract

Head and neck squamous cell carcinoma (HNSCC) represents the sixth most common malignancy worldwide. Despite improvements in therapeutic outcomes due to advances in surgery, radiotherapy, chemotherapy, and imaging techniques, HNSCC still has high mortality rate. For patients who are not cured with surgery and radiotherapy, there are few effective treatment options. Although HNSCC is heterogeneous in nature, current molecular classification distinguishes only human papilloma virus positive and negative tumors. HNSCC in general are characterized by considerable resistance and high rate of locoregional recurrence. Loss of p53 control pathway and numerous alterations in components of intracellular signaling pathways are consistently observed throughout the majority of HNSCC cases, supporting uncontrolled proliferation. It was proven that common mutations in the HNSCC genome play major role in tumorigenesis as well as in resistance to chemotherapy. The aim of the thesis is to describe the important mechanisms in HNSCC, which are associated with mutations in epidermal growth factor receptor and p53, and those including PI3K/Akt/mTOR and Notch signaling pathways. Association of these pathways with chemoresistance to commonly used drugs and even to advanced targeted therapeutic agents was evidenced by many experimental and clinical observations. Some mechanisms leading to resistance to conventional chemotherapeutic agents such as cisplatin or docetaxel are discussed, as well as the possibilities how to re-establish the drug sensitivity of the tumor cells.

Key words: head and neck carcinoma, tumorigenesis, chemoresistance, epidermal growth factor receptor, p53, signaling pathways

Abstrakt

Skvamózní karcinom hlavy a krku (HNSCC) představuje šestý nejčastější typ maligního onemocnění na světě. I přes mnohá zlepšení vycházející z vývoje operačních technik, radioterapie, chemoterapie a zobrazovacích technik, mají nádory hlavy a krku stále vysokou úmrtnost. Pro pacienty, kteří nebyli vyléčeni chirurgickým zákrokem či radioterapií, existuje už jen málo účinných možností léčby. Navzdory tomu, že nádory hlavy a krku tvoří velmi heterogenní skupinu, současná molekulární klasifikace je dělí pouze dle přítomnosti lidského papilomaviru (HVP) na pozitivní a negativní. Karcinomy hlavy a krku jsou charakteristické svou resistencí a vysokou mírou recidivy. Ztráta kontrolní dráhy spojené s p53 a častými změnami komponent v jeho intracelulární signální dráze, které jsou pozorovány v mnoha případech HNSCC, podporuje nekontrolovanou proliferaci. Bylo prokázáno, že běžné mutace v genomu HNSCC hrají hlavní roli v tumorigenezi, stejně jako v rezistenci vůči chemoterapii. Cílem práce je popsat důležité mechanismy u nádorů hlavy a krku, které jsou spojeny s mutacemi v receptoru epidermálního růstového faktoru a p53 nebo souvisejí se signálními drahami PI3K, Akt, mTOR a Notch. Experimentálními i klinickými pozorováními byla prokázána spojitost těchto drah s chemoresistencí k běžně užívaným lékům i k pokročilým cíleným terapeutikům. Diskutovány jsou některé mechanismy vedoucí k rezistenci vůči konvenčním chemoterapeutikům, jako je cisplatina nebo docetaxel, stejně jako možnosti obnovit citlivost nádorových buněk k léčivům.

Klíčová slova: nádory hlavy a krku, tumorigeneze, chemoresistence, receptor pro epidermální růstový faktor, p53, signální dráhy

OUTLINE

1 Introduction ..... - 1 -

2 Treatment of HNSCC ..... - 2 -
2.1 Chemotherapy ..... - 2 -
2.2 Targeted therapy ..... - 3 -

3 Epidermal growth factor receptor ..... - 3 -
3.1 EGFR overexpression ..... - 4 -
3.2 Therapeutic targeting EGFR-mediated signaling ..... - 5 -
3.3 Resistance to EGFR-inhibitors ..... - 6 -
3.4 Drug resistance related to EGFR ..... - 7 -

4 PI3K/Akt/mTOR pathway ..... - 7 -
4.1 Alterations in PI3K/Akt/mTOR pathway ..... - 8 -
4.2 Drug resistance related to PI3K/Akt/mTOR pathway ..... - 9 -

5 Tumor suppressor p53 ..... - 9 -
5.1 p53 in HPV^+ ..... - 10 -
5.2 p53 v HPV^- ..... - 11 -
5.3 Drug resistance related to p53 ..... - 12 -
5.4 Therapeutic targeting of p53 ..... - 12 -

6 Notch signaling pathway ..... - 13 -
6.1 Notch in keratinocytes ..... - 14 -
6.2 Notch and EMT ..... - 15 -
6.3 Notch and CSCs ..... - 15 -
6.4 Drug resistance related to the Notch pathway ..... - 16 -

7 miRNAs ..... - 16 -
7.1 miR-34 ..... - 17 -
7.2 miR-1 and miR-206 ..... - 17 -

8 Conclusion ..... - 18 -

9 Abbreviations ..... - 20 -

10 Literature ..... - 22 -

1 Introduction

Cancers in head and neck area usually arise from the squamous cells that line the mucosal surfaces. These squamous cell cancers are called head and neck squamous cell carcinomas (HNSCCs).[1] Around 680 000 new cases of HNSCC are diagnosed each year.[2] HNSCCs begin in different regions of head and neck as depicted in Figure 1 and are categorized by the area of origin.[1]

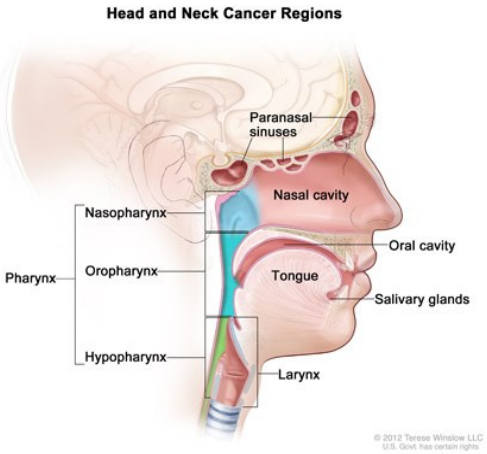

Figure 1: Regions of HNSCCs. (Adopted from www.cancer.gov)

HNSCC is very heterogeneous group of cancers but it can be divided according to the presence of human papilloma virus (HPV) into two groups, the HPV-positive (HPV^+) and the HPV-negative (HVP^-).[3] The HPV infection, mostly of serotype 16, is an established cause of oropharyngeal cancer. In the United States, it accounts for about 60% to 70% of all oropharyngeal cancers. The incidence of HPV^+ HNSCC varies depending on the geographic area, however, the incidence of HPV-related HNSCC is rising especially in economically developed countries comparing to the less developed, where the HPV^+ incidence is up to 20%. The reason for this increase might be also related to changing sexual behavior including oral HPV exposure. Consistent with this hypothesis, the increase in HPV-related cancers is more significant in people younger than 60 years.[4]

The HPV^- cancer is associated with other risk factors. The most common is alcohol and tobacco consumption which accounts for 75% of all HNSCC.[1] HPV^- HNSCCs differ in important characteristics from HPV^+ HNSCCs including molecular-genetic alterations, morphology, and clinical behaviour.[3] The division on HPV^+ and HPV^- is key to our understanding of the biology and mutational landscape of these tumors, also predicts response to treatment and survival outcomes.[1] Globally, HPV^+ tumors are less mutated than HPV^- tumours.[5] According to current knowledge, clinical experience, and clinical trials, both types of HNSCC are treated differently. In the case of HPV^+, standard chemoradiation approach is less intense. HPV^+ oropharyngeal tumors seem to have a better prognosis.[1] On the other hand, in HPV^- patients, additional therapy including targeted agents is applied to the standard approach in order to improve overall survival.[3]

2 Treatment of HNSCC

The choice of treatment depends on the location and the TNM* stage of tumor. The early stages (I or II) are usually treated with single-modality therapy such as radiation or surgery, and are potentially curable. Although, surgery might be complicated depending on the location of tumor and may cause loss of certain organ, sense or function. Unfortunately, more than half of patients usually come with advanced stages (III and IV) already having lymph node metastases. These patients require the multi-modality treatment meaning a combination of radiation, surgery, and chemotherapy.[3]

2.1 Chemotherapy

Chemotherapy in general is administration of synthetic drugs or derivatives obtained from plants or molds. However, the term chemotherapy is most frequently used for treatment of neoplastic diseases. In this sense, the drugs can be cytotoxic (poisonous for cells), or cytostatic (stopping the growth and propagation of cells). They usually target DNA or structures necessary for cell division. Most of the drugs affect proliferating cells, that obviously applies to tumor cells but also to cells of normal tissues.[6] There are three ways to combine chemotherapy with radiation and surgery. One is induction or neoadjuvant chemotherapy, which is applied in several courses before surgery or radiation. Second way is the concomitant administration of chemotherapy and radiation. Lastly, the adjuvant chemotherapy is applied in addition to the primary surgery or radiation to maximize its effectiveness.[3] The addition of chemotherapy to local treatment benefits in all HNSCC locations, however, a comprehensive meta-analysis of clinical trials performed between 1965 and 2000 shows a significant benefit only for concomitant administration with radiation.[7] There are many drugs used in chemotherapy treatment of HNSCC, among the well-known belong methotrexate, cisplatin, fluorouracil, and docetaxel.[1]

The effectiveness of chemotherapy is compromised by the presence of different types of resistance. Drug resistance in tumors involves many mechanisms, including reduced drug accumulation, decreased apoptosis, and increased DNA damage repair. Alterations in tumorigenesis pathways may lead to one of these effects and therefore support the

* *Classification of Malignant Tumours: T describes the size of the original tumour, N lymph nodes that are involved, M distant metastasis (spread of cancer from one part of the body to another)*

chemoresistance.[8] Generally, the primary (intrinsic) resistance to the chemotherapeutics (chemoresistance) is insensitivity of cancer cells to a certain cytostatic drug. The secondary (acquired) chemoresistance develops during the cytostatic treatment.[9]

2.2 Targeted therapy

There is a great effort to find new agents that would either help to increase the cancer's sensitivity to chemotherapy and radiotherapy, or to target specific molecules and pathways expressed exclusively or predominantly in cancer cells. They usually affect pathways that are associated with tumorigenesis and thus represent the main drivers of the tumor growth. One of these alternative drugs are the monoclonal antibodies cetuximab and panitumumab which inhibit the function of epidermal growth factor receptor (EGFR).[10] The frequently clinically used cetuximab has been approved for the treatment of HNSCC in combination with radiotherapy, as well as the panitumumab.[10] The overall survival in locally advanced HNSCC was improved by adding concomitant administration of cetuximab to radiotherapy comparing to radiotherapy alone.[11] In contrast, addition of cetuximab to cisplatin with radiotherapy did not show a significant improvement over cisplatin with radiotherapy alone.[12] Incorporation of these monoclonal antibodies also seems to increase the acute toxicity of the treatment.[13] Another targeted therapeutic agents are low-molecular-weight tyrosine kinase inhibitors that prevent activation of EGFR, such as gefitinib and erlotinib.[14] Other agents which target the Notch and PIK3CA-mTOR signaling pathways are under investigation. To date, there has been no breakthrough in the targeted therapy in HNSCC.[3]

3 Epidermal growth factor receptor

EGF is located in the cell membrane and binding of the ligand initiates many processes such as proliferation, angiogenesis, or cell migration. The receptor consists of an extracellular ligand-binding domain, a single transmembrane domain and an intracellular tyrosine kinase domain.[15] (Figure 2) The ligands of EGFR are multiple; mainly the epidermal growth factor (EGF) and transforming growth factor-α (TGF-α). Once the ligand binds to the receptor, the dimerization of EGFR occurs and its cytoplasmic tyrosine kinase domain (TK) is auto-phosphorylated and activates the mitogen activated protein kinase (MAPK), signal transducer and activator of transcription 3 (STAT3) and phosphatidylinositol-3-kinase (PI3K)/Akt signaling pathways, which together modulate cellular adhesion, proliferation,

angiogenesis and migration.[15] Alterations in EGFR can have a key role in resistance of the tumor cells to drugs, radiation, or in induction of apoptosis.[16]

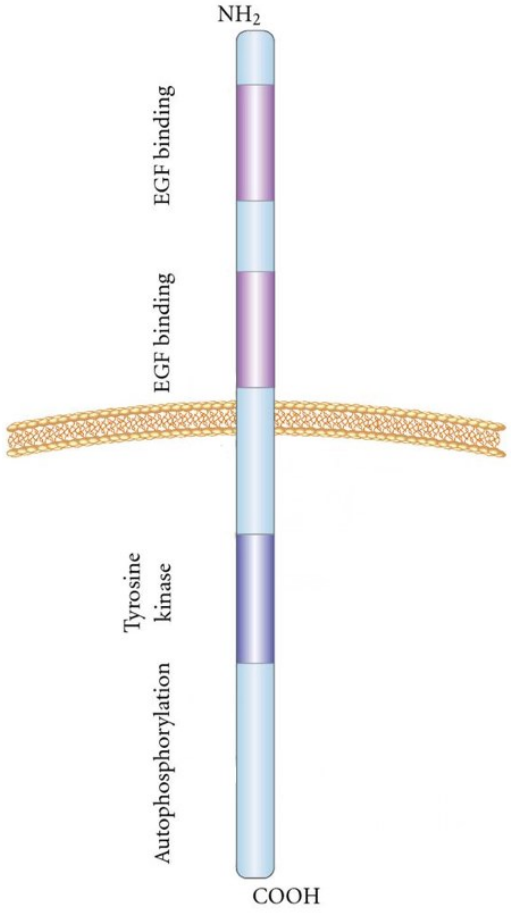

Figure 2: Structure of EGFR.
EGFR consists of extracellular, transmembrane, and intracellular domains. The extracellular domain is the least conserved among the EGFR family members and consists of 4 subdomains—two ligand-binding domains and two domains for dimerization. The cytoplasmic end of the EGFR is highly conserved and forms the tyrosine kinase domain. Activation of EGFR leads to autophosphorylation of the tyrosine residues to which then proteins with SRC homology 2 bind and which transduce the signals downstream. (Adopted from Siwak DR et al., Journal of Oncology, 2010)

3.1 EGFR overexpression

In about 80-90% of HNSCC cases, EGFR is overexpressed. The overexpression and have been related to a more aggressive phenotype of HNSCC, including increased resistance to treatment. [14] The overexpression may be a result of intensified transcription. Factors, which are responsible for increased EGFR mRNA synthesis, include EGFR amplification, polymorphisms in intron 1, and also dysregulated p53.[16] A recent study shows EGFR gene amplification in eight out of the 71 primary tumors, 11.2%. An increased EGFR gene copy number was also found in 43.7% of HNSCCs. Comparing to the normal copy number of EGFR, the increased number was significantly associated with poor survival.[17] At last, the polymorphisms in the EGFR gene has also an effect on the EGFR overexpression. It takes place in intron 1, which's sequence contains a certain number of cytosine-adenine (CA) dinucleotide repeats, usually between 9 to 21 repeats. The number of repeats happens to be crucial when it comes to gene transcription. Apparently, the larger number of CA dinucleotides the lower levels of mRNA and protein expression. The number of CA repeats is also associated with the responsiveness to anti-EGFR therapies.[16] Alterations in EGFR expression and its mutations significantly influence targeted therapeutic approaches and may result in resistance to these agents.[14]

3.2 Therapeutic targeting EGFR-mediated signaling

EGFR signaling can be pharmacologically targeted in two ways. First, the extracellular ligand-binding domain of the receptor can be blocked by a monoclonal antibody (mAb). There are several mAbs like panitumumab and zalutumumab but the most studied and clinically used is cetuximab, which, together with panitumumab, received FDA approval for the treatment of locally or regionally advanced HNSCC in combination with radiotherapy.[18]

Second way to target the EGFR is using low-molecular-weight tyrosine kinase inhibitors (TKIs) that prevent activation of the cytoplasmic tyrosine kinase of EGFR.[14] Several TKIs have been developed to competitively bind to the ATP spot of EGFR leading to the inhibition of phosphorylation and activation of the receptor's tyrosine kinase. The most known TKIs are gefitinib and erlotinib, which both selectively and reversibly inhibit tyrosine kinase activity.[17] The number of CA repeats in intron 1 has been related to the response to EGFR TKIs in HNSCC. Patients with a low number of CA dinucleotides happened to have higher prevalence of rash when treated with one of the above mentioned drugs.[16] The somatic mutations in the tyrosine kinase domain of the EGFR gene, which are common in non–small cell lung carcinomas, have low incidence in HNSCC. Interestingly, these mutations have been demonstrated in a small percentage of cases in Asian populations (7%), while in individuals of Caucasian origin in very few cases (1%).[14] Both of these strategies, mAbs and TKIs, are used as part of combination therapies with radiation; their efficacy is not as promising when used as a single agent.[14] However, many cancers have been shown to develop resistance to anti-EGFR mAbs.

3.3 Resistance to EGFR-inhibitors

In Table 1, main mechanisms of resistance to EGFR-targeted therapies are listed. A major oncogenic mutation of the extracellular domain leads to so called EGFRvIII variant that has been detected in many types of malignancies. However, until now just few studies have described EGFRvIII in HNSCC. One of these studies[14] shows that 42% of HNSCC tumors were found to be expressing this mutated protein; another study reports 21%.[17] This mutation is actually caused by deletion of exons 2 to 7, changing the protein to its truncated version of 150kDa, which causes constitutive phosphorylation and activation of EGFR, independent of ligand binding. Ligand-independent activation of EGFRvIII explains the resistance to mAbs like cetuximab which bind to the extracellular ligand-binding domain of EGFR in competition with its ligand and therefore block the activation. The overexpression of this mutated variant of EGFR is linked to a poor response to cetuximab, by comparison with a parental type that overexpresses a wild-type variant.[14]

Table 1: Mechanisms of resistance to EGFR-targeted therapies

Mechanisms of resistance	Examples
EGFR mutations	Extracellular domain (EGFRvIII)
	TK domain
Epithelial-mesenchymal transition	Increased vimentin expression
	Decreased E-Cadherin expression
Activation of alternative and/or downstream pathways	Cyclin D1 upregulation
	PTEN mutations
	PI3KCA mutations
	Akt Amplification

(Adopted from Chen L et al, Clinical Cancer Research, 2010)

The epithelial-to-mesenchymal transition (EMT) is a process by which epithelial cells undergo morphologic changes to gain the mesenchymal phenotype. The epithelial cells lose the actin organization and their cell-to-cell connections; on the other hand, the expression of mesenchymal markers and the activity of matrix metalloproteinases rise. In various cancers including HNSCC, EMT leads to increased motility and invasion of the cancer cells and most likely is associated with metastasizing and drug resistance.[19] One of the markers of EMT, cortactin, has related to gefitinib resistance. Also the E-cadherin repressor delta-crystallin enhancer binding factor 1, another of EMT markers, is associated with erlotinib resistance in HNSCC.[18]

3.4 Drug resistance related to EGFR

EGFR-signaling pathway plays a role in hyaluronan-CD44–mediated chemotherapy resistance through its targets mitogen-activated protein kinases ERK1 and ERK2 (extracellular signal–regulated kinase). Hyaluronan (HA) is a glycosaminoglycan which is the primary ligand for the transmembrane receptor CD44. The interaction between HA and CD44 influences tumor cell progression, abnormal adhesion, migration, and invasion. The chemoresistance to methotrexate and doxorubicin was found in HNSCC cell lines.[8] The HA treatment caused the recruitment of a significant amount of EGFR into the CD44 complex proving the association between HA-CD44 and EGFR-mediated signaling in tumor progression and chemotherapy resistance.[8] It was also proved that HA can promote CD44-dependent resistance to cisplatin through Ca^{2+} mobilization.[20]

4 PI3K/Akt/mTOR pathway

The phosphoinositide 3-kinase PI3K is activated in many ways. It is a major effector downstream of insulin-like growth factor receptor 1 and G-protein-coupled receptors. Additionally, PI3K interacts with stimulated receptor tyrosine kinases (RTKs) such as the EGFR described previously. Once PI3K is activated, it phosphorylates the second messenger phosphatidylinositol PIP2 to PIP3 which binds to the pleckstrin homology domain of Akt, also known as PKB, changing Akt's conformation and localizing it in the cytoplasmic membrane. There Akt can be phosphorylated by its activating kinases; mTORC2, PDK1, and CK2. Activated Akt can have many effects in apoptosis, metabolism, cell proliferation and cell growth. One of the downstream molecules of activated Akt is mTOR complex 1 (mTORC1), also frequently mutated part of PI3K/Akt/mTOR signaling pathway. Akt does not activate the mTORC1 directly but it inactivates the tuberous sclerosis complex (TSC). The TSC consists of tumor-suppressor proteins TSC2 and TSC1. Inactivation of the TSC1-TSC2 complex results in the loss of its ability to inhibit Rheb1, subsequently leading to activation of mTORC1. Akt also decreases the cytoplasmic level of p53 through mouse double minute 2 homolog (MDM2).[21] (Figure 3)

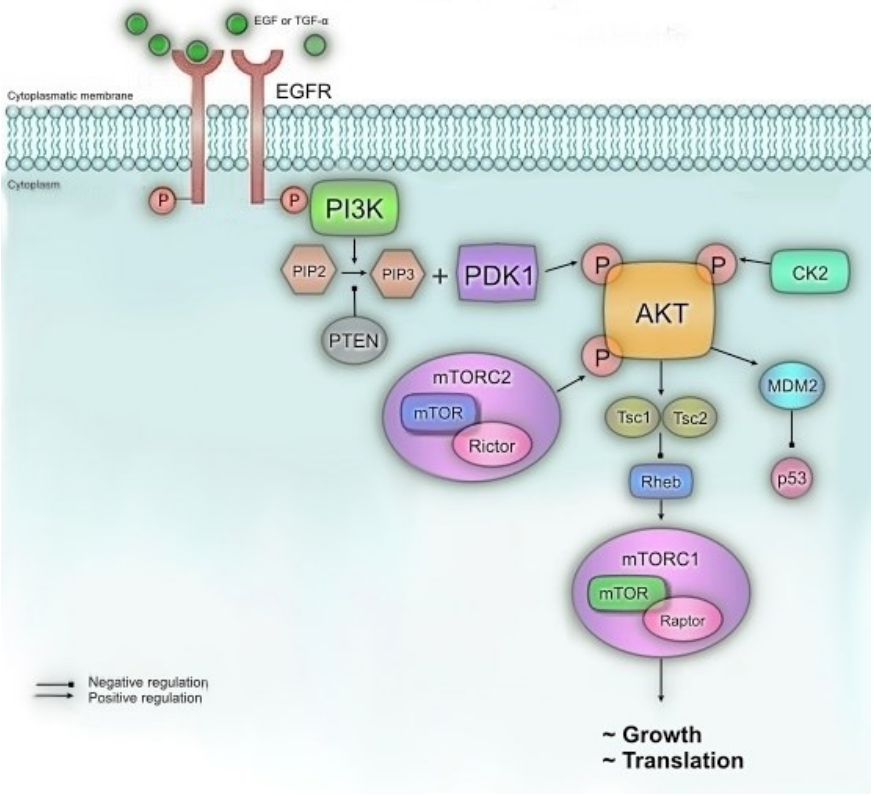

Figure 3: **PI3K/Akt/mTOR pathway** Binding of ligands (EGF or TGF-α) to EGFR activates PI3K which phosphorylates PIP2 to PIP3. Akt is activated by mTORC2, PDK1, and CK2, to pass the activation onto mTORC1.

4.1 Alterations in PI3K/Akt/mTOR pathway

Alterations of the PI3K pathway are common in HNSCC; mostly including loss-of-function mutations of phosphatase and tensin homologue (PTEN) and gain-of-function mutations of PI3KCA which lead to enhanced proliferation and cell growth.[5] The association of PI3K with human cancer was not established until the late 1990s, when it was shown that the tumor suppressor PTEN acts as a phosphatase that is specific for the lipid products of PI3K. PTEN blocks PI3K signaling by changing the PIP3 back to PIP2.[22] (Figure 3) Loss of PTEN results in unrestricted signaling by the PI3K pathway, resulting in cancer formation.[22] The transcription of PTEN, and also TSC2, is activated by the tumor suppressor p53 which leads to down-regulation of the entire PI3K signaling pathway.[23] p53 is activated in response to cellular stress signals. Regulation of the PI3K pathway represents an additional level of protection against dysregulated DNA replication in the presence of genotoxic stress. *PI3KCA* is a gene that codes for the catalytic subunit p110a of class IA PI3K. Because of its increased kinase activity and genomic amplification in tumor it was classified as an oncogene. Three most frequently found mutations in *PIK3CA* elevate its lipid kinase activity and activate the downstream Akt signaling pathway.[24] The PI3K pathway appears to be quite frequently activated in HPV^+ tumours.[25]

4.2 Drug resistance related to PI3K/Akt/mTOR pathway

One of the mechanisms of resistance against EGFR inhibitors is downstream activation of the PI3K/Akt/mTOR pathway which leads to unrestricted activation of Akt.[26] The Akt activity is increased in many cancers including the HNSCC. In general, the enhanced activity supports the aggressive phenotype with decreased apoptosis and differentiation.[27] Persisting activity of Akt has already become a marker predicting resistance to EGFR inhibitors in EGFR-overexpressing cancers. However, sometimes the EGFR is poorly phosphorylated and Akt is still over-activated which leads us to an idea of EGFR-independent signaling mechanisms. It might be due to *PIK3CA* activating mutations[26] because constant Akt activation was also found in cells with mutated *PIK3CA* which were cetuximab-resistant. The sensitivity of these cells was restored after inhibition of Akt phosphorylation by a PI3K inhibitor. Thus, alternative treatment to cetuximab might be gefitinib which is able to decrease the Akt phosphorylation even though the *PIK3CA* is mutated.[28]Another way to overcome the consequences of Akt activation is through the mTOR blockade which might restore the sensitivity of the cell to EGFR-inhibitors.[26] The Akt and mTOR phosphorylation/activation is probably also responsible for the resistance against farnesyltransferase inhibitors (preventing the proper functioning of the Ras protein).[29] Akt is responsible for the anti-apoptotic character of carcinomas and also associated with induction of EMT. mTOR is thought to be a mediator of EMT. Decreased level of E-cadherin is common during EMT, the activation of Akt has been shown to down-regulate E- cadherin.[30]

Several inhibitors of PI3K/Akt/mTOR pathway have been suggested as an attractive therapeutic option. One of them is perifosine which binds to the pleckstrin homology domain of Akt competing with PIP3. In the presence of perifosin, Akt cannot be activated and cells are blocked in G1-S or G2-M phase of the cell cycle.[21,31]

5 Tumor suppressor p53

In HNSCC, the most common genetic alteration is mutation in *TP53* gene. The product of *TP53*, p53, is a tumor suppressor.[5] Its tumor-suppressive function is demonstrated by regulating transcription of many downstream target genes which are part of cell cycle arrest, apoptosis, senescence, DNA repair, and metabolism. Under normal conditions, the half-life

of p53 is very short mainly thanks to E3 ubiquitin ligase and MDM2. However, when a cell is exposed to a genotoxic stress, p53 is post-translationally stabilized by modifications such as phosphorylation or acetylation. Therefore, p53 level rises resulting in transcription and activation of genes of previously-mentioned processes. By using these activities, it is secure that a cell does not pass the DNA damage on the daughter cells. When an alteration in *TP53* causes loss of activity in p53, the cell is not able to control the DNA damage through repair or cell death, leading to genomic instability and optionally cancer.[32]

5.1 p53 in HPV$^+$

As we already know, HNSCC can have the HPV-positive or HPV-negative origin which is also associated with various alterations of p53. HPV-induced HNSCCs, often oropharyngeal and tonsil tumors, are different from HPV$^-$ tumors and characterized by the presence of virus which contains two oncogenes: E6 and E7. The *TP53* tumor suppressor gene is inactivated by the viral oncoprotein E6, and the retinoblastoma suppressor gene (*RB*) by the oncoprotein E7.[33] These viral oncoproteins are able to transform human primary keratinocytes and to disrupt the cell-cycle regulatory pathways during the progression of HNSCC.[34] (Fig. 4) Many studies have shown that HPV$^+$ patients with p53 wild-type tumors have better overall survival and progression-free statistics than HPV$^-$ patients with altered p16 or p53 HNSCC.[35]

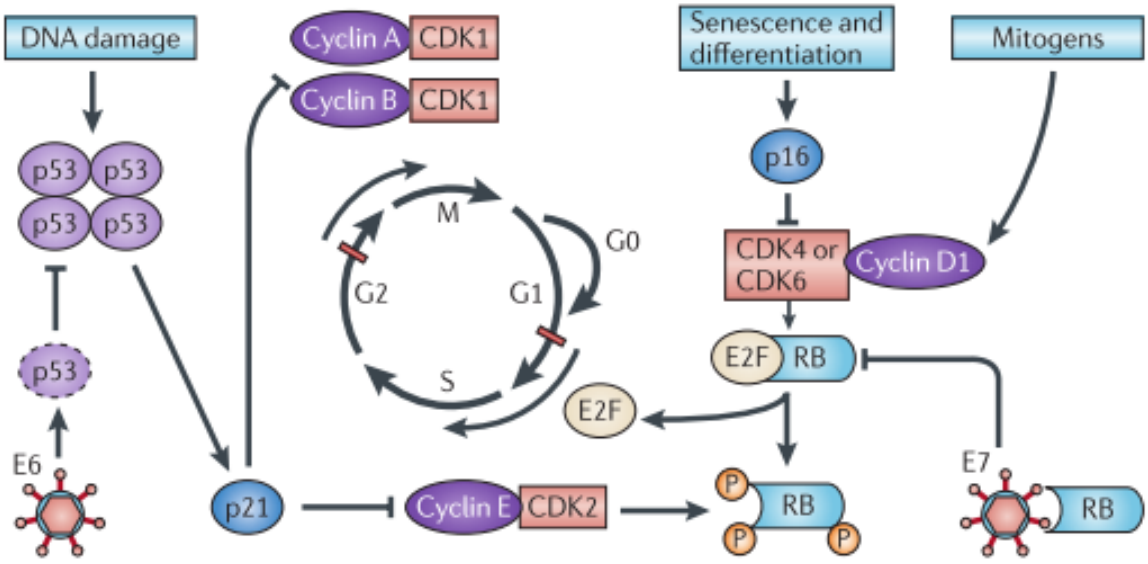

Figure 4: Cell cycle deregulation by human papilloma virus. The cell cycle is regulated by complexes of cyclins and cyclin-dependent kinases (CDKs). In this figure, various inhibitors of these cyclin–CDK complexes are shown. In order to keep the cell cycle running, cells have to pass the G1 restriction point (red bar) that is controlled by the retinoblastoma pocket proteins (Rb). In response to a mitogenic signal, the cyclin D1+CDK4 and cyclin D1+CDK6 complexes are activated. They phosphorylate the Rb pocket proteins, causing release and

activation of E2Fs. E2F inducts cyclin E which forms the cyclin E+CDK2 complex and phosphorylates of RB, initiating S phase. The inhibitor for the cyclin D1+CDK4 and cyclin D1+CDK6 complexes is p16, which is coded by gene CDKN2A. Its expression mediates senescence and differentiation.
A second important control point takes place during G2 phase and its role is to check for any DNA damage. The key protein involved in the response to replication errors and other DNA damage is p53. The presence of DNA-damage leads to increased activity of p53, forming p53 tetramers which act as a stress-induced transcription factor and induce the expression of p21 (also known as CDKN1A). p21 inhibits several cyclin–CDK complexes and holds the cell cycle. The human papillomavirus (HPV) genome encodes two viral oncoproteins: E6 and E7. The E6 protein binds p53 and targets the protein for degradation, whereas the E7 protein 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. (Adopted from Leemans CR et al, Nature reviews. Cancer, 2011)

5.2 p53 v HPV$^-$

In patients with HPV$^-$ carcinomas, the situation with p53 is different. The mutation of an ubiquitous tumor suppressor p53 occurs in 84% of HPV$^-$ tumours[5] and may result in loss-of-function or gain-of-function, and could have a dominant-negative effect. Most of the time, mutations of the *TP53* gene are missense mutations and occur mainly in the DNA-binding domain.[36] The mutation restricts the DNA-binding either by the mutation occurring directly in the amino acids that bind to the p53-responsive element in DNA, or by changing the conformation of p53 protein which leads to the loss of DNA-binding ability. Not only do both mutant variants lose the transcriptional function of wild-type p53, but negative effect where the mutated allele binds and inhibits the remaining functional wild-type p53's ability to suppress cellular transformation.[36]

As mentioned previously, the mutation in the *TP53* gene can also lead to the gain-of-function. For example, it was observed that p53 in HNSCC cells can promote invasive growth[36], resistance to cisplatin therapy[37], and resistance to the programmed cell death initiated by the absence of cell–matrix interactions, i.e., anoikis.[38] Furthermore, gain-of-function p53 mutant forms can mediate genomic instability, drive EMT, regulate angiogenesis, and initiate cellular programming in cancer stem cells. Considering the gain-of-function mutation of p53, the mutated p53 does not act as just one protein but as a group of proteins participating in a large network of tumor-promoting processes in cancer cells. Consequently, finding the drug-based therapies to target mutant p53 in HNSCC is highly challenging.[36]

5.3 Drug resistance related to p53

As previously mentioned, alterations in the *TP53* gene are associated with tumorigenesis by modulating DNA damage repair and apoptotic response.[39] The *TP53* mutations are found in more than 50% of HNSCC cancers and the p53 status was shown to correlate with resistance to cisplatin in HNSCCs.[20] Also, the absence of functional p53 has been shown to play a role in regulating response of the cell to several targeted agents, including EGFR inhibitors, for example cetuximab. The dysregulation or mutation in p53 leads to cell growth which is independent of EGFR. Loss of p53 was found in all of the examined HNSCC cell lines which were resistant to the EGFR inhibitors.[40] Silencing of p53 in a sensitive cell line leads to decreased sensitivity to cetuximab. On the other hand, restoration of functional p53 was sufficient tore-establish the sensitivity to EGFR-inhibitors and radiation.[40] Importantly, p53 also plays a role in resistance to commonly used chemotherapeutic drugs, such as cisplatin. According to the p53 status, HNSCC cell lines differ in their sensitivity to cisplatin. They can be divided into two groups, with nuclear p53 signal and without. The p53 nuclear signal is present if the intact COOH-terminal nuclear localization signal is detected. It shows that the p53 was translocated to the nucleus to affect the transcription. The ones with total loss of nuclear p53 signal are resistant against cisplatin chemotherapy. The loss of nuclear p53 signal is most probably due to a mutation resuting in loss of the COOH-terminally located nuclear localization signal in p53.[39]

5.4 Therapeutic targeting of p53

There are several approaches trying to overcome this resistance by reactivation of p53 or its replacement with wild-type protein. First to mention is adenoviral p53 gene therapy using adenovirus with the affinity to the upper aero-digestive tract. The modified version of adenovirus-5, Ad-p53, has the viral E1 protein region replaced with a human wild-type p53 expression cassette.[41] Adenoviral therapy results in strong wild-type p53 protein expression. Applying combination of Ad-p53 with chemotherapy on patients with stage III increased their survival rate.[41] Thanks to results of gene therapy, Ad-p53 was proved as a promising therapeutic strategy for the treatment of HNSCCs.[41] Other, slightly different way, was to make a virus attacking cell with the lack of functional p53. An adenovirus called Onyx-15, which lacks the E1B gene, was made.[42] In phase I and II limitations occurred therefore phase III was stopped and the technology was licensed to a Chinese company which modified it.[43]

As a second possible way, a small-molecular-weight compound called PRIMA ("p53 reactivation and induction of massive apoptosis") was constructed. It has a capability to change the conformation of mutant p53 to wild-type and to restore p53-dependent transcription.[44,45] Using PRIMA-1 as a single-agent and in combination with regular chemotherapeutics, PRIMA-1 inhibited the proliferation of several cell lines, including Fadu. As expected, G2-arrest and increased nuclear p53 localization was observed. Moreover, PRIMA-1 was not as active in a wild-type p53 line (JHU-O28). Additionally, PRIMA-1 increased the expression of p53-regulated genes and the efficacy of cisplatin in HNSCC cell lines. Similar outcomes were observed with another molecule called CP-31398.[46] Thus, restoration of functional p53 is feasible and sufficient to re-establish sensitivity of HNSCC cells to cisplatin.

Nutlins are *cis*-imidazoline analogs which represent the third way of fighting p53-related resistance. These small molecules interact with MDM2, an endogenous p53 inhibitor, blocking the binding of MDM2 to p53 protein, thereby stabilizing the p53 protein.[47] The function of nutlins explains the outcomes from Roh et al. study that Nutlin-3 is more effective in wild-type line.[46] RITA ("reactivation of p53 and induction of tumor cell apoptosis") is also known to block the interaction between p53 and MDM2.[48] Two different studies have reported that RITA is also more effective in wild-type lines than in the p53-mutant ones.[46,49] In both cases, Nutlin-3 and RITA, the treatment was more successful in combination with cisplatin than as a single-agent alone.[46]

6 Notch signaling pathway

While sequencing the genome of HNSCC cancer cells high frequency of mutations was found in *Notch1* gene (up to 15%); after *TP53* it was the second most frequently mutated gene.[50,51] Notch1 is a mammalian single-pass receptor through which a cell can interact with another cell which expresses certain ligands of *Delta* and *Jagged* families. When the interaction occurs, Notch receptor undergoes enzymatic cleavage. At first, it is cleaved by the ADAM-family metalloproteases. The second cleavage is mediated by γ-secretase, forming a soluble fragment called NICD (Notch intracellular domain). Then the NICD is translocated to the cell's nucleus and associates with other regulatory proteins. Together they form a ternary complex that activates transcription of Notch-target genes, including proteins of the hairy

and enhancer of split (Hes) family, Myc, p21. This pathway is known as the canonical Notch signaling pathway (Figure 5).[52] Notch can act as both an oncogene or a tumor suppressor depending on its precise cellular context. The Notch target genes *c-Myc* and cyclinD1 are oncogenes involved in many types of cancer. Another target gene is p21 which mediates cell cycle arrest and therefore possesses tumor suppressive features.[52]

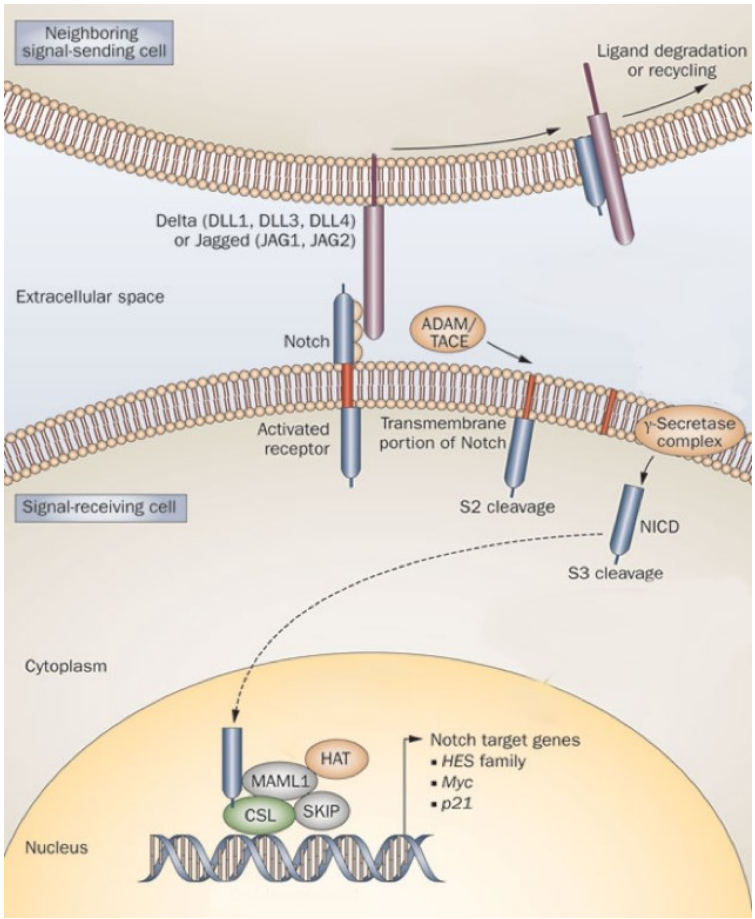

Figure 5: Notch signaling pathway. Activation of the Notch receptor occurs when Delta or Jagged ligands bind to it during cell-to-cell contact. Then the proteolysis of the heterodimer Notch receptor by ADAM and γ-secretase complex follows creating a soluble fragment, NICD. The NICD translocates through cytoplasm to the nucleus where it functions as a transcriptional activator of Notch target genes, including HES, *Myc* and p21. (Adopted from Takebe N et al, Nature reviews. Clinical oncology, 2015)

6.1 Notch in keratinocytes

Several cancers particularly squamous cell carcinomas (SCC) are associated with loss-of-function mutations in *Notch* gene. One of many purposes of Notch signalization is promoting cell differentiation in keratinocytes. The activation of Notch1 leads through the canonical pathway to p21 expression and in consequence of it, the keratinocyte leaves the proliferation cycle and terminally differentiates. In mice, the inhibition of CSL-dependent* Notch signaling leads to development of actinic keratosis which constitutes precursor lesions of squamous cell carcinoma. The mice also expressed more nuclear β-catenin and cyclin D1 than controls; similar expression pattern was also observed in human SCC. Overall, high levels of nuclear β-catenin and cyclin D1 occurred after inhibition of Notch1 in epidermis suggesting that Notch1 may be important for sustained tumor growth.[52] Notch1 can be down-regulated by suppression of p53 activity; therefore, the differentiation

*CSL is a transcriptional factor which is activated by NICD.

is disabled and the keratinocytes can follow the way of dysregulated proliferation. The p53 transcription can be inhibited by EGFR signaling through a mechanism that also involves activation of c-Jun, a direct negative regulator of p53 gene expression. It binds to the p53 promoter in SCC-derived keratinocytes so the knockdown of c-Jun enhances expression of the p53 gene in these cells. The *Notch1* gene expression is also induced in the same way. Taken together, EGFR signaling negatively controls the *Notch1* gene expression by transcriptional downregulation of the p53 gene. Therefore, in the keratinocytes, the EGFR signaling is thought to maintain self-renewal and suppress their differentiation.[53]

6.2 Notch and EMT

Notch signaling pathway has been reported to be involved with the EMT in drug resistant cancer cells.[54] In terms of cancer-stem-cell maintenance, Notch pathway participates in EMT and increases chemoresistance.[55] It was found that in HNSCC, the EMT is related with *c-Myc*, a target gene in Notch signaling, because *c-Myc* down-regulation caused by inhibition of Notch1 was shown to reduce EMT and invasion of the tumor cells.[54] HNSCC cell lines resistant to gefitinib had high expression of the mesenchymal marker vimentin; most of them showed overexpression of fibronectin and they also expressed the E-cadherin repressor ZEB1 which is another hallmark of EMT.[19] Down-regulation of Notch signaling by siRNA leads to partial reversal of the EMT phenotype through a process called MET, mesenchymal-epithelial cell transition.[54]

6.3 Notch and CSCs

According to several studies, the process of EMT generates cells with stem-like properties (cancer stem cells, CSCs) through activated Notch1. CSCs represent a small subset of cancer cells that have the ability to self-renew suppling the tumor mass. Recently, it is believed that CSCs play an important role also in drug resistance and cancer metastasis. CSCs use mechanisms like increased expression of drug transporters and DNA repair systems to resist the cytotoxic effects of drugs. A long-term exposure to the drug induces drug resistance phenotype which could be even passed on daughter cancer cells. CSCs have been found also in the head and neck tumours.[55] Interestingly, levels of all common CSC self-renewal-related markers (CD44, SOX2, ALDH1, Slug) were increased in CSCs, as well as the protein levels of

Notch1 and HES1 were strongly expressed, which indicates that Notch signaling is closely correlated with CSC self-renewal capacity and might play a role in CSC regulation. Some results even support the idea that Notch1 inhibition reduces CSC-related markers and transcription factors involved in self-renewal, which further decreases CSC frequency. Because elimination of CSCs is an important goal in the effort to cure cancer, the Notch pathway is a potential target for treatment of HNSCC.[55]

6.4 Drug resistance related to the Notch pathway

Notch might play an important role in chemoresistance to many types of drugs including the one that are frequently used in the treatment of HNSCC, for example cisplatin, docetaxel, 5-fluorouracil.[55] Many studies have suggested that Notch may play a role in the mechanism of cisplatin resistance. In HNSCC, *Notch1* was highly expressed in cisplatin-resistant tumors in HNSCC patients. To prove this correlation, inhibition of Notch signaling was found to increase the cisplatin sensitivity in cisplatin-resistant HNSCC. These results lead to an idea that inactivation of Notch pathway could be a promising treatment strategy for patients.[56] Similarly, the clinical effect of docetaxel can be also disappointing due to the drug resistance. Recently, it was found that down-regulation of Notch-1 signaling increases chemosensitivity to docetaxel in HNSCC, also indicating the promising results of Notch signaling inhibition by DAPT which inhibits the γ-secretase. The same conclusion was made when combining 5-fluoroacil chemotherapy with DAPT.[55] The activation of Notch signaling is linked with chemoresistance of the tumors; therefore, the inactivation of the pathway could be a potential therapeutic target on the way to overcome chemoresistance.[54]

7 miRNAs

MicroRNAs (miRNAs) are small, non-coding RNAs. Their discovery is one of the most important discoveries in the modern biology; there is evidence for the role of miRNAs in tumorigenesis, process of metastasis, and drug resistance.[57] It is well known that the miRNAs exert their regulatory effects in post-transcriptional regulation of genes by binding to the 3' untranslated region (3'UTR) of target messenger RNA (mRNA). The pairing of RNAs results in degradation of the mRNA.[58]

Some miRNAs function either as tumor suppressors or have a oncogenic activity.[58] For example, miR-10b is down-regulated in HNSCC cell lines which induces cancer cell proliferation, migration, and invasion.[59] In some cases, the oncogenic miRNA is up-regulated. For example, the up-regulation of miR-155 in oral squamous cell carcinoma (OSCC) is associated with poor prognosis due to induction of metastasis.[60] One of the tumor suppressor miRNAs in HNSCC is let-7d which most probably targets RAS. Let-7d was found to be significantly decreased in regional metastatic lymph nodes of patients with OSCC.[61] Similarly, the low expression of miR-153 is related to metastasis. miR-153 prevents EMT and returns the mesenchymal phenotype of cells to epithelial-like cells.[62] Many miRNAs operate as a tumor suppressors in HNSCC being involved in several processes of tumorigenesis.

7.1 miR-34

The miR-34 family is composed of three processed miRNAs of which the miR-34a is the most dominant. The expression of miR-34a has been found to be lower in HNSCC, suggesting that miR-34a could act as a tumor suppressor. Recently, Wu et al. reported that the expression of miR-34a is down-regulated in nasopharyngeal carcinoma. Since miR-34a inhibits Notch1 expression at the transcriptional level by binding to the Notch1 mRNA, overexpression of miR-34a can lead to decreased expression levels of Notch1, and therefore decrease in cell proliferation.[63] In HNSCC, up-regulation of miR-34b and miR-34c has been reported. MiR-34b role appears to be a tumor-specific; it is consistently reported in HNSCC, playing an important role in tumorigenesis-related molecular mechanisms including EMT.[64]

7.2 miR-1 and miR-206

Other miRNAs, miR-1 and miR-206, were significantly down-regulated in HNSCC suggesting their tumor-suppressive character. Genes which are directly down-regulated by these miRNAs, are coding for EGFR and hepatocyte growth factor receptor (c-MET). Moreover, ectopic expression of miR-1 and miR-206 reduces the levels of p-ERK1/2 and p-ATK which happen to be downstream signaling molecules of c-MET and EGFR. By increasing the level of miR-1 and miR-206, inhibition of cell proliferation, migration, and invasion, was demonstrated (in comparison with the mock or miR-control-transfected cells). This suggests that these miRNAs act as tumor suppressors. Since both c-MET and EGFR mediate signals contributing to cancer aggressiveness and drug resistance, and are overexpressed in HNSCC

clinical specimens, miR-1 and miR-206 might provide a new understanding of HNSCC molecular mechanisms of progression and metastasis. MiRNAs like miR-1 and miR-206 might even bring us to new strategies for treatment. In addition, it was discovered that in HNSCC, the miR-1 also down-regulates the Transgelin-2 protein gene *TAGLN2* which's expression in colorectal cancer was correlated with lymph node and distant metastasis. The data also show a role of Transgelin-2 in cell migration and invasion, proposing its oncogenic function.[58]

Altogether, the miRNAs have a promising future in cancer treatment. The miRNA expression profile can be used to distinguish the cancers from benign tumors and normal tissues, as well as to predict the origin of un-known primary tumors in the head and neck area.[65] For example, miR-125a and miR-200a are under-expressed in saliva of OSCC patients compared to healthy controls.[66] Decreased expression levels of miR-205 and let-7d are significantly associated with higher frequency of locoregional recurrence and shorter survival in patients with HNSCC.[65]

8 Conclusion

The head and neck squamous cell carcinoma is a heterogeneous disease at both the anatomical and molecular levels; therefore, common treatment indeed fails. Despite multi-modal approach, 40% to 60% of the patients with locally advanced HNSCC relapse. It is important to recognize HPV-negative and HPV-positive cancers as biologically and clinically different, with HPV-positive tumors having more favorable survival outcomes. Currently, surgery and radiation are the cornerstones of therapy, with increasing roles for chemotherapy and targeted therapy, especially because of organ preservation and increasing effectiveness. New molecular methods may help to diagnose tumors earlier and may provide more accurate staging systems with easier way to find metastases. In some cases, the mechanisms of resistance are already known and others are still a subject of research. Therefore, overcoming the resistance seems to be difficult, even though there have been successful clinical trials. In most of the resistant HNSCC cell lines, many important genes involved in processes of tumorigenesis are mutated or altered, supporting the resistance. One of these processes is EGFR-related signaling through which tumor cells gain the resistance to common drugs, for example methotrexate or doxorubicin, and to the

targeted therapeutic agents. Also, the down-stream PI3K/Akt/mTOR signaling pathway is affected and plays an important role in resistance to EGFR inhibitors. The difference between HPV^+ and HPV^- tumors is crucial when it comes to the p53-related pathways. The p53 is dysregulated in most of the HNSCC tumors, however, the alterations differ according to the HPV status. Alterations in p53 are involved in the chemoresistance frequently used drugs, such as to cisplatin, and lower the sensitivity to targeted therapy. Up-regulation of Notch supports EMT, appearance of CSCs, and resistance to many chemotherapeutic drugs. Current data show that an important role in tumorigenesis and drug resistance have miRNAs. MiRNAs play different roles in development, progression, and metastasis of HNSCC. They can act as oncogenes or as tumor suppressors; because of that they seem to have great potential to become novel molecular biomarkers for the diagnosis of cancer, metastatic site, cancer stage, and its progression.

Over the past several years immuno-oncology has evolved and become a novel promising strategy for cancer therapy. A promising role in the future treatment strategy seems to play inhibition of the immune check-points. These inhibitors, such as pembrolizumab or nivolumab, target the interaction between programmed death receptor 1/ programmed death ligand 1 (PD-1/PDL-1) or PDL- 2. The inhibitor of PD-1 is a humanized IgG4 mAb called nivolumab and it is currently being evaluated for the treatment of HNSCCs. The second, anti-PD-1 mAb is pembrolizumab which is in phase II clinical trial in patients with metastatic HNSCC applied after standard platinum-based therapy. Multiple clinical trials are ongoing and impressive clinical data have revealed the potential of immunotherapy for HNSCC patients; better patient survival and reduced morbidity were observed.

9 Abbreviations

Akt	Protein Kinase B, PKB
ADAM	A Disintegrin and Metalloproteinase
ATP	Adenosintrifosfát
c-MET	Hepatocyte Growth Factor Receptor
CDK	Cyclin-Dependent Kinase
CDKN2A	Cyclin-Dependent Kinase Inhibitor 2A
CK2	Casein Kinase 2
CSCs	Cancer Stem Cells
CSL	Cbf1/Su(H)/Lag-1
DLL	Delta-Like Ligand
DNA	Deoxyribonucleic Acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial–Mesenchymal Transition
ERK1/2	Extracellular Regulated Kinase
FDA	Food and Drug Administration
HA	Hyaluronic Acid
HAT	Histone Acetyltransferase
HES	Hairy and Enhancer of Split
HNSCC	Head and Neck Squamous Cell Carcinoma
HPV	Human Papillomavirus
mAbs	Monoclonal Antibodies
MAML1	Mastermind-Like Protein 1
MAML1	Mastermind-Like 1
MAPK	Mitogen-Activated Protein Kinase
MDM2	Mouse Double Minute 2 Homolog
MET	Mesenchymal-Epithelial Transition
miRNA	Micro Ribonucleic Acid
mRNA	Messenger Ribonucleic Acid
mTOR	Mechanistic Target of Rapamycin
mTORC	Mechanistic Target of Rapamycin Complex
NICD	Notch Intracellular Domain
OSCC	Oral Squamous Cell Carcinoma
PD-1	Programmed Death Receptor 1
PDK1	Phosphoinositide-Dependent Kinase 1
PDL-1/2	Programmed Death Ligand 1/2
PI3K	Fosfatidylinositol-3-Kináza
PIK3CA	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha
PIP2	Phosphatidylinositol 4,5-Bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-Trisphosphate
PKB	Protein Kinase B
PRIMA	P53 Reactivation and Induction of Massive Apoptosis
PTEN	Phosphatase and Tensin Homolog
RB	Retinoblastoma
RITA	Reactivation of P53 And Induction of Tumor Cell Apoptosis

RNA	Ribonucleic Acid
RTK	Receptor Tyrosine Kinases
SCC	Squamous Cell Carcinoma
SKIP	Ski-Interacting Protein
STAT	Signal Transducer and Activator of Transcription
TACE	TNF-A-Converting Enzyme
TGF-α	Transforming Growth Factor Alpha
TK	Tyrosine Kinase
TKI	Tyrosine Kinase Inhibitor
TSC	Tuberous Sclerosis Proteins
ZEB1	Zinc finger E-box-binding homeobox 1

10 Literature

1. NIH. National Cancer Institute. Head and Neck Cancers. https://www.cancer.gov/types/head-and-neck/head-neck-fact-sheet. Published 2017.
2. International Agency for Research on Cancer. Globocan. Estimated incidence, mortality and 5-year prevalence: both sexes. http://globocan.iarc.fr/Pages/fact_sheets_population.aspx. Published 2012.
3. Marur S, Forastiere AA. Head and Neck Squamous Cell Carcinoma: Update on Epidemiology, Diagnosis, and Treatment. *Mayo Clin Proc*. 2016;91(3):386-396. doi:10.1016/j.mayocp.2015.12.017.
4. Chaturvedi AK, Anderson WF, Lortet-Tieulent J, et al. Worldwide trends in incidence rates for oral cavity and oropharyngeal cancers. *J Clin Oncol*. 2013;31(36):4550-4559. doi:10.1200/JCO.2013.50.3870.
5. Cancer Genome Atlas Network. Comprehensive genomic characterization of head and neck squamous cell carcinomas. *Nature*. 2015;517:578-582. doi:10.1038/nature14129.
6. Česká lékařská společnost Jana Evangelisty Purkyně. Czech Society for Oncology. Chemotherapy; treatment with cytostatics. http://www.linkos.cz/databaze-onkologickych-leciv/chemoterapie-neboli-lecba-cytostatiky/. Published 2017.
7. Pignon JP, Bourhis J, Domenge C, Designé L. Chemotherapy added to locoregional treatment for head and neck squamous-cell carcinoma: three meta-analyses of updated individual data. MACH-NC Collaborative Group. Meta-Analysis of Chemotherapy on Head and Neck Cancer. *Lancet*. 2000;355(9208):949-955. doi:10.1016/S1278-3218(00)00073-1.
8. Wang SJ, Bourguignon LYW. Hyaluronan and the interaction between CD44 and epidermal growth factor receptor in oncogenic signaling and chemotherapy resistance in head and neck cancer. *Arch Otolaryngol - Head Neck Surg*. 2006;132(7):771-778. doi:10.1001/archotol.132.7.771.
9. Yamano Y, Uzawa K, Saito K, et al. Identification of cisplatin-resistance related genes in head and neck squamous cell carcinoma. *Int J Cancer*. 2010;126:437–449. doi:10.1002/ijc.24704.
10. Amato VD, Rosa R, Amato CD, et al. The dual PI3K / mTOR inhibitor PKI-587 enhances sensitivity to cetuximab in EGFR-resistant human head and neck cancer models. *Br J Cancer*. 2014;110:2887-2895. doi:10.1038/bjc.2014.241.
11. Bonner J a, Harari PM, Giralt J, et al. Radiotherapy plus cetuximab for squamous-cell carcinoma of the head and neck. *N Engl J Med*. 2006;354(6):567-578. doi:10.1056/NEJMoa053422.
12. Ang KK, Zhang Q, Rosenthal DI, et al. Randomized phase III trial of concurrent accelerated radiation plus cisplatin with or without cetuximab for stage III to IV head

and neck carcinoma: RTOG 0522. *J Clin Oncol*. 2014;32(27):2940-2950. doi:10.1200/JCO.2013.53.5633.

13. Giralt J, Trigo J, Nuyts S, et al. Chemoradiotherapy with or without panitumumab in patients with unresected, locally advanced squamous-cell carcinoma of the head and neck (CONCERT-1): A randomised, controlled, open-label phase 2 trial. *Lancet Oncol*. 2015;16(2):221-232. doi:10.1016/S1470-2045(14)71198-2.

14. Sok JC, Coppelli FM, Thomas SM, et al. Human Cancer Biology Mutant Epidermal Growth Factor Receptor (EGFRvIII) Contributes to Head and Neck Cancer Growth and Resistance to EGFR Targeting. *Clin Cancer Res*. 2006;12(17):5064-5073. doi:10.1158/1078-0432.CCR-06-0913.

15. Nagalakshmi K, Jamil K, Pingali U, Reddy MV, Attili SS V. Epidermal growth factor receptor (EGFR) mutations as biomarker for head and neck squamous cell carcinomas (HNSCC). *Biomarkes*. 2014;19(3):198-206. doi:10.3109/1354750X.2014.895852.

16. Amador ML, Oppenheimer D, Perea S, et al. An Epidermal Growth Factor Receptor Intron 1 Polymorphism Mediates Response to Epidermal Growth Factor Receptor Inhibitors. *Cancer Res*. 2004;64(24):9139-9143.

17. Szabó B, Nelhubel GA, Kárpáti A, et al. Clinical significance of genetic alterations and expression of epidermal growth factor receptor (EGFR) in head and neck squamous cell carcinomas. *Oral Oncol*. 2011;47:487-496. doi:10.1016/j.oraloncology.2011.03.020.

18. Chen LF, Cohen EE, Grandis JR. New strategies in head and neck cancer: understanding resistance to epidermal growth factor receptor inhibitors. *Clin Cancer Res*. 2010;16(9):2489-2495. doi:10.1158/1078-0432.ccr-09-2318.

19. Frederick BA, Helfrich BA, Coldren CD, et al. Epithelial to mesenchymal transition predicts gefitinib resistance in cell lines of head and neck squamous cell carcinoma and non – small cell lung carcinoma. *Mol Cancer Ther*. 2007;6(6):1683-1691. doi:10.1158/1535-7163.MCT-07-0138.

20. Wang SJ, Bourguignon LYW. Hyaluronan-CD44 promotes phospholipase C-mediated Ca2+ signaling and cisplatin resistance in head and neck cancer. *Arch Otolaryngol - Head Neck Surg*. 2006;132(1):19-24. doi:10.1001/archotol.132.1.19.

21. Freudlsperger C, Burnett JR, Friedman JA, et al. EGFR – PI3K – AKT – mTOR signaling in head and neck squamous cell carcinomas : attractive targets for molecular-oriented therapy. *Expert Opin Ther Targets*. 2011;15(1):63-74. doi:10.1517/14728222.2011.541440.

22. Stambolic V, Suzuki A, Pompa L De, et al. Negative Regulation of PKB / Akt-Dependent Cell Survival by the Tumor Suppressor PTEN. *Cell*. 1998;95(1):29-39.

23. Feng Z, Zhang H, Levine AJ, Jin S. The coordinate regulation of the p53 and mTOR

pathways in cells. *PNAS*. 2005;102(23):8204–8209.

24. Qiu W, Sch F, Li X, et al. PIK3CA Mutations in Head and Neck Squamous Cell Carcinoma. *Clin Cancer Res*. 2006;12(5):1441-1446. doi:10.1158/1078-0432.CCR-05-2173.

25. Network TCGA. Comprehensive genomic characterization of head and neck squamous cell carcinomas. *Nature*. 2015;517(7536):576-582. http://dx.doi.org/10.1038/nature14129.

26. Bauman JE, Arias-Pulido H, Lee SJ, et al. A phase II study of temsirolimus and erlotinib in patients with recurrent and/or metastatic, platinum-refractory head and neck squamous cell carcinoma. *Oral Oncol*. 2013;49(5):461-467. doi:10.1016/j.oraloncology.2012.12.016.

27. Mandal M, Younes M, Swan EA, et al. The Akt inhibitor KP372-1 inhibits proliferation and induces apoptosis and anoikis in squamous cell carcinoma of the head and neck. *NIH Public Access*. 2006;42(4):430-439.

28. Rebucci M, Peixoto P, Dewitte A, et al. Mechanisms underlying resistance to cetuximab in the HNSCC cell line: Role of AKT inhibition in bypassing this resistance. *Int J Oncol*. 2011;38:189-200. doi:10.3892/ijo.

29. Seung HO, Jin Q, Kim ES, Khuri FR, Lee HY. Insulin-like growth factor-I receptor signaling pathway induces resistance to the apoptotic activities of SCH66336 (Lonafarnib) through Akt/mammalian target of rapamycin-mediated increases in survivin expression. *Clin Cancer Res*. 2008;14(5):1581-1589. doi:10.1158/1078-0432.CCR-07-0952.

30. Kuo SZ, Blair KJ, Rahimy E, et al. Salinomycin induces cell death and differentiation in head and neck squamous cell carcinoma stem cells despite activation of epithelial-mesenchymal transition and Akt. *BMC Cancer*. 2012;12(1):556. doi:10.1186/1471-2407-12-556.

31. Argiris A, Cohen E, Karrison T, et al. A Phase II Trial of Perifosine, An Oral Alkylphospholipid, In Recurrent or Metastatic Head and Neck Cancer. *Cancer Biol Ther*. 2006;5(7):766-770. doi:10.4161/cbt.5.7.2874.

32. Zhou G, Liu Z, Myers JN. TP53 Mutations in Head and Neck Squamous Cell Carcinoma and Their Impact on Disease Progression and Treatment Response. *J Cell Biochem*. 2016;117(12):2682-2692. doi:10.1002/jcb.25592.

33. Slebos RJC, Yi Y, Ely K, et al. Gene expression differences associated with human papillomavirus status in head and neck squamous cell carcinoma. *Clin Cancer Res*. 2006;12(3 I):701-709. doi:10.1158/1078-0432.CCR-05-2017.

34. Gillison ML, Koch WM, Capone RB, et al. Evidence for a Causal Association Between Human Papillomavirus and a Subset of Head and Neck Cancers. *J Natl Cancer Inst*.

2000;92(9):709-720.

35. Smith EM, Rubenstein LM, Hoffman H, Haugen TH, Turek LP. Human papillomavirus, p16 and p53 expression associated with survival of head and neck cancer. *Infect Agent Cancer*. 2010;5:4. doi:10.1186/1750-9378-5-4.

36. Neskey DM, Osman AA, Ow TJ, et al. Evolutionary action score of TP53 identifies high-risk mutations associated with decreased survival and increased distant metastases in head and neck cancer. *Cancer Res*. 2015;75(7):1527-1536. doi:10.1158/0008-5472.CAN-14-2735.

37. Osman AA, Neskey DM, Katsonis P, et al. Evolutionary action score of TP53 coding variants is predictive of platinum response in head and neck cancer patients. *Cancer Res*. 2015;75(7):1205-1215. doi:10.1158/0008-5472.CAN-14-2729.

38. Xie T-X, Zhou G, Zhao M, et al. Serine Substitution of Proline at Codon 151 of TP53 Confers Gain of Function Activity Leading to Anoikis Resistance and Tumor Progression of Head and Neck Cancer Cells. *Laryngoscope*. 2013;123(5):1416-1423. doi:10.1002/lary.23846.

39. Mandic R, Schamberger CJ, Müller JF, et al. Reduced cisplatin sensitivity of head and neck squamous cell carcinoma cell lines correlates with mutations affecting the COOH-terminal nuclear localization signal of p53. *Clin Cancer Res*. 2005;11(19):6845-6852. doi:10.1158/1078-0432.CCR-05-0378.

40. Huang S, Benavente S, Armstrong EA, Li C, Wheeler DL, Harari PM. P53 modulates acquired resistance to EGFR inhibitors and radiation. *Cancer Res*. 2011;71(22):7071-7079. doi:10.1158/0008-5472.CAN-11-0128.

41. Li Y, Li L, Wang L, et al. Selective intra-arterial infusion of rAd-p53 with chemotherapy for advanced oral cancer : a randomized clinical trial. 2014.

42. Nemunaitis BJ, Khuri F, Ganly I, et al. Phase II Trial of Intratumoral Administration of ONYX- 015 , a Replication-Selective Adenovirus , in Patients With Refractory Head and Neck Cancer. *J Clin Oncol*. 2001;19(2):289-298. doi:10.1200/JCO.2001.19.2.289.

43. Elser C, Siu LL, Winquist E, et al. Phase II Trial of Sorafenib in Patients With Recurrent or Metastatic Squamous Cell Carcinoma of the Head and Neck or Nasopharyngeal Carcinoma. *J Clin Oncol*. 2007;25(24):3766-3773. doi:10.1200/JCO.2006.10.2871.

44. Bykov V, Issaeva N, Shilov A, et al. Restoration of the tumor suppressor function to mutant p53 by a low-molecular-weight compound. *Nat Med*. 2002;8(3):282-288.

45. Li Y, Mao Y, Brandt-rauf PW, Williams AC, Fine RL. Selective induction of apoptosis in mutant p53 premalignant and malignant cancer cells by PRIMA-1 through the c-Jun-NH 2 -kinase pathway. *Mol Cancer Ther*. 2005;4(6):901-909. doi:10.1158/1535-7163.MCT-04-0206.

46. Roh J, Kang S, Minn I, Califano J, Sidransky D, Koch W. p53-Reactivating small molecules induce apoptosis and enhance chemotherapeutic cytotoxicity in head and neck squamous cell carcinoma. *Oral Oncol*. 2011;47(1):8-15. doi:10.1016/j.oraloncology.2010.10.011.

47. Vassilev LT, Vu BT, Craves B. In Vivo Activation of the p53 Pathway by Small-Molecule Antagonists of MDM2. *Science (80-)*. 2004;303:844-849.

48. Issaeva N, Bozko P, Enge M, et al. Small molecule RITA binds to p53, blocks p53-HDM-2 interaction and activates p53 function in tumors. *Nat Med*. 2004;10(12):1321-1329. doi:10.1038/nm1146.

49. Roh J, Ho J, Jin S, Hwan C, Young J, Koch WM. The p53-reactivating small-molecule RITA enhances cisplatin-induced cytotoxicity and apoptosis in head and neck cancer. *Cancer Lett*. 2012;325(1):35-41. doi:10.1016/j.canlet.2012.05.020.

50. Stransky N, Egloff AM, Tward AD, et al. The mutational landscape of head and neck squamous cell carcinoma. *Science (80-)*. 2012;333(6046):1157-1160. doi:10.1126/science.1208130.The.

51. Agrawal N, Frederick MJ, Pickering CR, et al. Exome Sequencing of Head and Neck Squamous Cell Carcinoma Reveals Inactivating Mutations in NOTCH1. *Science (80-)*. 2011;333(6046):1154-1157. doi:10.1126/science.1206923.Exome.

52. Proweller A, Tu L, Lepore JJ, et al. Impaired notch signaling promotes De novo squamous cell carcinoma formation. *Cancer Res*. 2006;66(15):7438-7444. doi:10.1158/0008-5472.CAN-06-0793.

53. Kolev V, Mandinova A, Guinea-Viniegra J, et al. EGFR signalling as a negative regulator of Notch1 gene transcription and function in proliferating keratinocytes and cancer. *Nat Cell Biol*. 2008;10(8):902-911. doi:10.1038/ncb1750.

54. Inamura N, Kimura T, Wang L, Yanagi H, Tsuda M. Notch1 regulates invasion and metastasis of head and neck squamous cell carcinoma by inducing EMT through c-Myc. *Auris Nasus Larynx*. 2016:1-11. doi:10.1016/j.anl.2016.08.003.

55. Zhao Z, Zhang L, Huang C, Ma S, Bu L, Liu J. NOTCH1 inhibition enhances the efficacy of conventional chemotherapeutic agents by targeting head neck cancer stem cell. *Sci Rep*. 2016;6(24704):1-12. doi:10.1038/srep24704.

56. Gu F, Ma Y, Zhang Z, Zhao J. Expression of Stat3 and Notch1 is associated with cisplatin resistance in head and neck squamous cell carcinoma. *Oncol Rep*. 2010;23:671-676. doi:10.3892/or_00000683.

57. Zhang J, Wang Y, Chen X, Zhou Y, Jiang F, Chen J. MiR-34a suppresses amphiregulin and tumor metastatic potential of head and neck squamous cell carcinoma (HNSCC). *Oncotarget*. 2015;6(10).

58. Nohata N, Sone Y, Hanazawa T, Fuse M. miR-1 as a tumor suppressive microRNA

targeting TAGLN2 in head and neck squamous cell carcinoma. *Oncotarget*. 2011;2(1-2):29-42.

59. Lu YC, Chen YJ, Wang HM, et al. Oncogenic function and early detection potential of miRNA-10b in oral cancer as identified by microRNA profiling. *Cancer Prev Res*. 2012;5(4):665-674. doi:10.1158/1940-6207.CAPR-11-0358.

60. Ni Y-H, Huang X-F, Wang Z-Y, et al. Upregulation of a potential prognostic biomarker, miR-155, enhances cell proliferation in patients with oral squamous cell carcinoma. *Oral Surgery, Oral Med Oral Pathol Oral Radiol*. 2014;117(2):227-233. doi:10.1016/j.oooo.2013.10.017.

61. Chang C, Hsu C, Chang C, et al. Let-7d functions as novel regulator of epithelial-mesenchymal transition and chemoresistant property in oral cancer. *Oncol Rep*. 2011;26:1003-1010. doi:10.3892/or.2011.1360.

62. Xu Q, Sun Q, Zhang J, Yu J, Chen W, Zhang Z. Downregulation of miR-153 contributes to epithelial-mesenchymal transition and tumor metastasis in human epithelial cancer. *Carcinogenesis*. 2013;34(3):539-549. doi:10.1093/carcin/bgs374.

63. Wu R, Qiu E, Lin R, Wang J, Lin H. Regulation of nasopharyngeal carcinoma cell proliferation by targeting Notch1 with miR-34a. *Int J Clin Exp Pathol*. 2016;9(9):8811-8816.

64. Chen D, Cabay RJ, Jin Y, et al. MicroRNA Deregulations in Head and Neck Squamous Cell Carcinomas. *J oral Maxillofac Res*. 2013;4(1):e2. doi:10.5037/jomr.2013.4102.

65. Childs G, Fazzari M, Kung G, et al. Low-level expression of microRNAs let-7d and miR-205 are prognostic markers of head and neck squamous cell carcinoma. *Am J Pathol*. 2009;174(3):736-745. doi:10.2353/ajpath.2009.080731.

66. Park NJ, Zhou H, Elashoff D, et al. Salivary microRNA: Discovery, Characterization, and Clinical Utility for Oral Cancer Detection. *Clin Cancer Res*. 2009;15(17):5473. doi:10.1158/1078-0432.CCR-09-0736.Salivary.