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Radioresistance in Head and Neck Squamous Cell Carcinoma — Possible Molecular Markers for Local Recurrence and New Putative Therapeutic Strategies

Federica Ganci, Andrea Sacconi,
Valentina Manciooco, Giuseppe Spriano,
Giulia Fontemaggi, Paolo Carlini and
Giovanni Blandino

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1. Introduction

Head and neck squamous cell carcinoma (HNSCC) comprises 5.5% of all incidence cancers and is the sixth leading cancer worldwide with approximately 600,000 cases reported annually [1, 2]. The vast majority of them are squamous cell carcinomas that originate in the epithelium of the oral cavity, pharynx and larynx. There is a higher incidence rate in males compared to females and the median age of patients with HNSCC is about 60 years [3]. The main risk factors for HNSCC are tobacco smoking and heavy use of alcohol. In particular, alcohol consumption and tobacco smoking have a synergic effect [4]. The contribution of tobacco exposure to HNSCC carcinogenesis is strongly correlated with the time and rate of the person who smokes and has showed to have site-specific differences according to the anatomical regions, with an increase in sensitivity from the oral cavity down to the larynx [5]. In addition, high-risk infection types of human papillomavirus (especially HPV-16 and 18) is emerging as a major cause of a subgroup of HNSCC, particularly those of the oropharynx and oral cavity [2, 6]. The traditional risk factors, tobacco and alcohol use, do not appear to play a contributing role in HPV-positive cancers [7]. However, it is known that HPV-positive and negative tumors have different clinical, pathological and molecular characteristics and that HPV-positive tumors are associated with a more favorable outcome [2, 6] and better response to standard therapy.

Many molecular studies show that these HNSCC may not be as homogeneous as previously supposed. This indicates the need to obtain a more detailed molecular characterization in order to stratify patients better. This ultimately is likely to provide a more rational therapeutic approach, potentially relevant to diagnosis and prognosis of this poorly defined subset of HNSCC cancer.

2. Therapy strategies and molecular mechanisms of radioresistance

HNSCC is typically characterized by locoregional diffusion and low propensity to develop distant metastasis. Due to the lack of symptoms in the early stage of the disease, about two thirds of patients are diagnosed in advanced stage with lymph node metastases. Local recurrence affects about 50-60% of patients and metastases develop in 15-20% of cases [8], with the five-year overall survival rate less than 50% [8, 9]. Locoregional failure is the most common cause of death in patients affected by HNSCC [10]. Recurrence may arise from residual neoplastic cells that survive to the treatment or from underlying field cancerization. Indeed, one key feature of HNSCC is the insurgence of recurrences after seemingly complete surgical resection, probably due to the existence of preneoplastic processes at multiple sites in the mucosa ("field cancerization" hypothesis). These preneoplastic tissues are apparently tumor-free when analyzed at histological level but present several genetic alterations when analyzed at a molecular level [11, 12].

Typically, HNSCC treatment consists of surgical resection followed by ionizing radiation or chemoradiation, or chemoradiation alone. Therapeutic strategy choice depends on disease stage: tumors at early stage are treated with surgery or radiotherapy. Surgery can be performed if complete tumor excision is possible and radiation can be used postoperatively when surgical margins are positive for the presence of tumor cells and/or if lymphovascular invasion by tumor is found. Platinum-based agents, in particular cisplatin (CDDP), are the conventional chemotherapeutic drugs for HNSCC treatment. More advanced cancers require multimodality therapy combining surgery, radiation and chemotherapy. Concurrent chemoradiation is the preferred treatment for advanced inoperable HNSCC [13-15]. These standard therapies have some limitations; they have several side effects and generally more than 50% of HNSCC patients relapse. The toxicities are mainly due to non-selective nature of treatment. However, resistance to chemoradiotherapy frequently occurs and is associated with poor outcome. This is the major clinical problem in HNSCC patients and relies on the fact that recurrence is often related to an intrinsic tumor radioresistance [14].

Molecular mechanisms underlying the resistance to radiotherapy or combined treatments mainly involve intracellular pathways related to cell proliferation, apoptosis, DNA repair and angiogenesis [13, 14, 16, 17]. To date, the main molecular mechanisms for radioresistance are:

- The hypoxia phenomenon
- Alterations in the Epidermal Growth Factor Receptor (EGFR)- PI3K/Akt pathway
- Epithelial Mesenchymal Transition (EMT) process

- The deregulation in p53 signaling cascades
- Alterations in the expression of angiogenic factors
- The presence of cancer stem cells (CSCs) subpopulation in tumor tissue

2.1. Hypoxia

Hypoxia is a common phenomenon present in many tumors and is associated with poor prognosis, malignant transformation and therapy resistance [18, 19]. In solid tumors, including HNSCC, oxygen is frequently reduced as the result of intermittent blood flow arising from the abnormal tumor microvasculature. Under oxygen deficiency, hypoxic tumor cells can activate the expression of hypoxia-inducible genes, functionally related to pro-survival, anti-apoptosis, angiogenesis, DNA-repair and metabolism signaling pathways [18, 20]. In particular, tumor cells switch their glucose metabolism from the oxygen-dependent tricarboxylic acid (TCA) cycle to oxygen-independent glycolysis metabolic pathway; as a consequence, hypoxic cells use glycolysis as main mechanism to produce ATP.

A key transcription factor having a central role in hypoxia-related gene expression changes is hypoxia-inducible transcription factor 1 (HIF-1). In normoxia, HIF-1 α undergoes rapid hydroxylation and degradation. In hypoxia, hydroxylation is prevented, stabilized HIF-1 α binds to HIF-1 β and the heterodimer binds to hypoxia response elements in target genes, such as glycolytic enzymes, angiogenic molecules (among which VEGFA), survival and growth factors (among which EGF, PDGF and TGF- β), chaperons and other apoptosis resistance-related proteins [13, 18, 21].

DNA double-stranded breaks (DSB) are the main DNA lesions leading to cell killing after radiotherapy. Oxygen is known to be a potent radiosensitizer and, through interaction with the radicals formed following radiation, it is essential for the promotion of radiation-induced DNA damage. Oxygen deficiency causes a reduction in reactive oxygen species (ROS) production and a deficit in radiation-induced DNA damage [20]. In agreement with these evidences, cells irradiated in the presence of air are about three times more sensitive than cells irradiated under conditions of severe hypoxia [22].

One of the evidences linking hypoxia to radiation response is a correlation between tumor control and hemoglobin levels [23], which is also related to oxygenation of solid tumors. Indeed, high hemoglobin (Hb) level, prior to and during treatment, has been associated with good prognosis in HNSCC patients treated with radiotherapy [23].

Hypoxia problem is particularly relevant in smoker patients. Indeed, in these HNSCC patients, the low oxygen level is also influenced by the formation of carboxyhemoglobin (COHb) and nicotine vase constrictive effect. As a consequence, the response to treatment and survival of smoker patients is significantly reduced compared to nonsmokers [23].

Given the influence of hemoglobin on tumor oxygenation and radiotherapy response, many researches tried to find methods able to increase Hb level in HNSCC patients having low Hb level, prior to and during radiation treatment; transfusion, or erythropoietin stimulating agents, are some of them, but unfortunately did not result in improved outcome or response

to therapy [23]. To date, the main radiosensitizing and cytotoxic agents used in the clinical practice for hypoxic cells targeting are nitroimidazoles, which have also been shown to improve locoregional control, when applied in conjunction with radiation [20].

There is also interest in the use of nitroimidazoles as noninvasive hypoxia markers [24, 25]. Indeed, it remains difficult to identify hypoxic tumors and those patients most likely to benefit from hypoxia modification therapy. Under hypoxic conditions, nitroimidazoles are converted into reactive intermediates, which then become covalently bound to macromolecules within the cell. Nitroimidazoles labeling with an appropriate isotope or immunologically recognizable marker allows the bio-reduced compound to be detected, indicating the presence of hypoxia.

Additional indirect non-invasive techniques being explored to identify hypoxic tumors include measuring the immunohistochemical expression of hypoxia-regulated proteins, such as carbonic anhydrase 9 (CA9) and HIF-1 α [26, 27]. This represents an attractive approach for routine clinical use, but is limited by the variability of expression of these markers within a tumor and by the lack of hypoxia specificity of individual proteins. An attempt to overcome these problems has been carried out by searching for tumor hypoxia gene signatures by meta-analysis of transcriptome datasets [28–30]. Winter and colleagues defined an *in vivo* hypoxia metagene by clustering around the RNA expression of a set of known *in vitro* hypoxia-regulated genes; this signature was also validated as a prognostic factor for recurrence-free survival in an independent data set [30].

2.2. Alterations in the Epidermal Growth Factor Receptor (EGFR)-PI3K/Akt pathway

Epidermal growth factor receptor (EGFR) is a transmembrane protein with tyrosine kinase activity that is overexpressed in about 90% of HNSCC, even if its expression is highly variable according to different subgroups of head and neck tumors as well as within the same tumor type [2, 14]. Stimulation by extracellular soluble ligands as epidermal growth factor (EGF) and transforming growth factors (TGFs) induces a conformational change leading to receptor heterodimerization with one of its family members (ErbB2, ErbB3, ErbB4); this causes auto-phosphorylation of the receptor intracellular domain and subsequent internalization followed by the activation of multiple signaling pathways, such as Ras-MAPKs (mitogen-activated protein kinases), extracellular signal-regulated kinases (ERKs), phosphatidylinositol-3-kinase-AKT (PI3-K/AKT), signal transducers and activators of transcription (STAT) and phospholipase C gamma (PLC-g) pathways [20].

High EGFR expression correlates with poor prognosis and resistance to conventional radiotherapy. EGFR expression can also be activated by the ionizing radiation itself, leading to increased radioresistance [20]. EGFR activation is also involved in increased proliferation rate and consequent repopulation, rendering radiotherapy ineffective [14].

Key proteins activated by EGFR are AKT and Ras; the first one is a kinase which phosphorylates multiple downstream effectors, stimulating cell survival and inhibiting apoptosis; Ras is a cell membrane protein able to stimulate a tyrosine kinase cascade, including B-RAF, MEK, MAPK proteins, by which Myc, FOS and Jun translocate in the nucleus finally promoting cell

proliferation. This cascade is also able to stimulate the production of EGFR monomers, TGFs and amphiregulin (AREG), contributing to paracrine EGFR activation [14]. Other proteins activated by EGFR are cyclin D1 and Pim-1, involved in cell cycle progression and inhibition of apoptosis; for the activation of both, the signal is mediated by STATs proteins [31, 32]. In addition, the interaction between EGFR-PI3-K/AKT and HIF pathways was also observed under hypoxic conditions, providing evidences on the correlation between EGFR signaling and the induction of angiogenic proteins, such as VEGFA, which is a downstream target of HIF-1 [20]

A subgroup of HNSCC (40%) expresses a truncated splicing variant of the EGFR, called EGFRvIII, in which the ligand-binding domain is altered, due to the deletion of amino acids 6-273. This alteration causes a permanent phosphorylation and activation of the receptor, also in the absence of EGF and TGFs ligands binding. As wild-type EGFR, EGFRvIII is implicated in increased cell proliferation, cell survival, motility and invasion. This variant is absent in normal tissues [17].

Besides EGFR overexpression, other mechanisms are involved in PI3K/Akt signaling hyperactivation, such as Ras activation, PI3-K gene mutation, Akt gene amplification and loss of tumor suppressor protein PTEN [14].

2.3. Epithelial Mesenchymal Transition (EMT) process

Another important mechanism by which radiotherapy can fail in HNSCC is epithelial to mesenchymal transition (EMT) process. When EMT occurs, epithelial cells change in mesenchymal phenotype which is characterized by reduction of the matrix contact, cell-cell adhesion followed by an increase in cell migration and motility. A crucial step of EMT is the loss of E-cadherin, a strong epithelial marker involved in adherens junction that anchors epithelial cells to each other [33]. A reduction of E-cadherin level was observed in HNSCC, especially in poorly differentiated tumors. In addition, many studies have demonstrated that aberrant E-cadherin expression is associated with poor outcome and local recurrence in HNSCC [34]. Loss or decrease of E-cadherin expression causes the translocation of β -catenin protein from the cell membrane to the nucleus to induce transcription of EMT-related genes, such as TWIST and SNAIL1 [33]. Another important protein involved in EMT is vimentin, which is an intermediate filament protein used as a marker for mesenchymal cells and is associated with the migratory phenotype, local recurrence and survival in HNSCC [34, 35]. Also fibronectin, a glycoprotein which mediates cellular interaction with extracellular matrix, plays an important role in migration, growth and adhesion of cells; its expression can be promoted by SNAIL and TWIST transcription factors [33]. Fibronectin is expressed at high level in tumors and blood plasma of HNSCC patients and has been proposed as biomarker of poor response to radiotherapy [36].

2.4. TP53 gene deregulation

TP53 is a tumor suppressor gene, which functions in carcinogenesis by initiating G1 arrest in response to certain DNA damages and apoptosis. About 40-70% of HNSCC has mutation in TP53 gene, leading to inactivation of its product [37]. Indeed, mutant p53 proteins are unable

to transcriptionally regulate wt-p53 target genes and to exert antitumor effects such as apoptosis, growth arrest, differentiation and senescence. On the other hand, countless evidence has demonstrated that at least certain mutant forms of the p53 protein may possess gain of function activity, thereby positively contributing to the development, maintenance and spreading of many types of tumor, including HNSCC [38, 39]. The prognostic role of p53 alteration in HNSCC is controversial. However, generally, deregulation of p53 protein predicts shorter overall survival, local recurrence and cancer treatment failure [40-44].

In particular, p53 alteration leads to an impaired capability to arrest cell cycle and to inhibit apoptosis. In addition, in this condition also DNA damage repair results compromised. As a consequence, tumor cells carrying *TP53* mutation are less sensitive to radiation-induced cell death and are unable to restore DNA integrity, thus accumulating several genetic mutations which lead to increased tumor heterogeneity and finally to resistance to conventional therapy [14].

In addition, several evidences show that its prognostic value depends on the *TP53* protein domain affected by mutation [43-46]. One of the main classifications of *TP53* mutation used in HNSCC is “disruptive” versus “not-disruptive”; any mutation in L2 or L3 loop of the DNA-binding domain resulting in a polarity change of the protein or any stop codon was classified as disruptive [44]. Disruptive *TP53* mutations were associated with poor outcome and increased radioresistance [44, 46]. Other studies have proposed an alternative classification by which mutations in DNA-binding regions, especially in L2 and L3+LSH motifs, were associated with poorer prognosis and clinical response to radiotherapy [45].

Of note, emerging evidences show that senescence may play a role in the radiation response by wild-type p53 [47]. Senescence is a form of cell cycle arrest in which cells lack replicative potential while remaining metabolically active, and was found to correlate with radiosensitivity in HNSCC [46]. In the proposed model, in the presence of *TP53* wild type or nondisruptive mutation, radiation promotes the induction of ROS production and p21 protein expression, which are critical mediators of cellular senescence. *TP53* disruptive mutations cause cellular senescence inhibition by reduction of radiation-induced ROS, thus driving resistance to radiotherapy [46].

2.5. Alterations in the expression of angiogenic factors

Angiogenesis is a process by which new blood vessels grow up from preexisting capillaries. Because expanding tumors have a continuous need for oxygen and nutrients, tumor cells induce angiogenesis. In particular, by secreting a variety of growth factors they activate the endothelial cells, constituting the inner lining of blood vessels, which produce proteases that degrade the basal membrane and extracellular matrix components. As a consequence, the endothelial cells can proliferate and migrate forming new capillary beds. Because in tumors new blood vessels are irregular and disorganized, the oxygen supply inside the tissue is not homogenous, resulting in continuous angiogenesis stimulation [48].

The main actors of this process are vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and matrix metalloprotease (MPP) family proteins.

VEGF family consists of seven ligands, which play a central role in the formation of new blood vessels; VEGFA is the best known agent that induces angiogenesis by binding two receptor tyrosine kinases, VEGFR-1 and VEGFR-2. VEGFA is able to promote development of the vascular system, cell migration, survival and induction of MMPs [13]; it also activates PI3K/AKT and Ras/MAPK signaling pathways [49]. There are increasing evidences that angiogenic response of irradiated tumor cells is related with decreasing radiation sensitivity and head and neck cancer progression. In a meta-analysis of 12 studies including 1002 patients affected by cancer of oral cavity, pharynx and larynx, VEGF expression positivity was associated with a two folds higher risk of death at 2 years of follow-up [50].

Release of VEGF and bEGF by epithelial tumor cells after irradiation is a common response mechanism by which cancer cells may survive and become protected from radiation-induced cell death [51]. Therefore, the level of VEGF and bEGF prior to and during treatment may be relevant for successful therapy.

2.6. Cancer Stem Cells (CSCs)

Cancer stem cells (CSCs) have been defined by Clarke et al., as a small tumor subpopulation possessing the capability to self-renewal and causing the heterogeneous lineage of cancer cells inside the tumor [52]. They are functionally defined as a subset of tumor cells with ability of self-renewal and multipotency, serving as progenitors of cancer cells. The characteristics by which CSCs can be distinguished to other cancer cells are the following [53]:

1. Promotion of tumorigenesis when they are transplanted in immunosuppressed mice.
2. Expression of specific cell surface markers (such as CD44, CD133, ALDH1, CD24) and formation of tumor spheres.
3. Tumors arising from CSCs have a heterogeneous cells population composed by tumorigenic and non-tumorigenic cells.
4. Capacity of self-renewal in serialized transplants over several generations.

The presence of this subpopulation has been identified in several tumors, including HNSCC where its ability to maintain tumor population, metastasize and to be resistant to radiochemotherapy has been shown [53-55].

The origin of CSCs has not been clearly defined; in HNSCC, it has been proposed that a chronic inflammation caused by permanent tobacco, alcohol use, mechanic irritation or viral infection, in association with genetic predisposition, lead to the accumulation of various genetic alterations and finally to the manifestation of a malignant phenotype [53].

In addition, during tumor progression, some CSCs, through an EMT process, can acquire the ability to infiltrate and metastasize. On the other hand, EMT is involved in the acquisition of cancer stem cells properties; at the molecular level, the transcription factor Twist induces downregulation of E-cadherin while promoting expression of Bim1, which has an essential role in self-renewal of CSCs. In agreement with these data, high expression of Bim1 and Twist are associated with a poor prognosis in HNSCC [53].

In HNSCC patients, high percentage of CD44 positive cells was associated with higher rate of treatment failure in general, while cells expressing CD44, CD24, Oct4 and integrin β 1 were associated with poor outcome after radiotherapy [56, 57]. From a clinical point of view, these evidences suggest that the patients can be cured if CSCs are completely eliminated.

3. Potential molecular markers for local recurrence and radioresistance

One of the current major research questions in the management of HNSCC disease addresses the prediction and treatment of local recurrence. As described before, mortality of patients with HNSCC is primarily driven by tumor cell radioresistance leading to local recurrence. Due to the heterogeneous nature of tumors, the identification of markers with prognostic or predictive value to be used as a complement to conventional diagnostic methods is a complex challenge. Indeed, although advance in expression technologies, current studies have provided ambiguous results.

Among the prognostic markers proposed in HNSCC, as described in the previous paragraph, the presence of mutation in *TP53* gene predicts the development of locoregional recurrence by increasing the radioresistance in tumor cells (Table 1).

Additional molecular markers predicting high local recurrence development and response to radiotherapy are summarized in Table 1.

Gene	Function	References
<i>TP53</i>	A tumor-suppressor regulating cell cycle progression, apoptosis and cell survival.	[2, 42, 44, 46, 58]
HIF-1 α	A transcription factor induced under hypoxic condition and promoting EMT, angiogenesis, cell migration and metastasis.	[16]
PTEN	A tumor suppressor gene regulating signaling pathways controlling cell proliferation and apoptosis.	[59-62]
Fibronectin	It is a glycoprotein of the extracellular matrix, which plays a major role in cell adhesion, growth, migration, and differentiation.	[36]
EGFR	Transmembrane TK acting as a central transducer in multiple pathways that mediate cell cycle progression, angiogenesis, inhibition of apoptosis, tumor invasion and metastasis.	[14, 20]
VEGFs	Ligands of transmembrane TK promoting cell proliferation, migration and survival of endothelial cells during tumor growth.	[13, 14, 20, 51]
Cox2	Catalytic enzyme decreasing apoptosis, increasing inflammation and important for tumor progression.	[63]

Gene	Function	References
p-AKT (Ser473)	It is a serine/threonine-specific protein kinase that plays a key role in multiple cellular processes such as glucose metabolism, apoptosis, cell proliferation, transcription and cell migration.	[64]
Cyclin B1	It is a regulatory protein involved in mitosis. It begins to increase during G2, peaks in mitosis, and is rapidly degraded before the cell cycle is completed. By the interaction with cdk1, cyclin B1 promotes cell progression.	[65, 66]
E-cadherin/ Vimentin	They are protein markers of EMT; E-cadherin is a marker of epithelial cells, while vimentin is a marker of mesenchymal cells.	[34, 35]
Yap/BCL-2/ c-met/VEGF/ Clauding	They are genes involved in cell proliferation, migration, inhibition of apoptosis and angiogenesis.	[67]
Rac1	It is a member of Rac family of Rho GTPase involved in intracellular adherens junction, epithelial differentiation and regulation of motility.	[68, 69]
Pim-1	It is an oncogene with serine/threonine kinase activity mainly involved in cell cycle progression, apoptosis and transcriptional activation.	[32]
CD10	Cell surface antigen associated with CSCs.	[70]
FOXM1	It is a gene involved in cell cycle regulation, which is associated with radioresistance only in quiescent cells.	[71]
15-gene hypoxia classifier composed by ADM, ALDOA, ANKRD37, BNIP3, BNIP3L, C3orf28, EGLN3, KCTD11, LOX, NDRG1, P4HA1, P4HA2, PDK1, PFKFB3, SLC2A1	These genes are able to classify more or less hypoxic tumors. Tumors classified as high hypoxic and treated with radiotherapy show a poor outcome respect to low hypoxic ones. Accordingly, more hypoxic tumors have a better response to radiosensitiser nimorazole respect to those classified as low hypoxic.	[29]
Gene expression model of intrinsic tumor radiosensitivity based on the expression of 10 genes composed by Androgen Receptor (AR), c-Jun, STAT1, PKC, RelA, c-Abl, SUMO-1, CDK1 (p34), HDAC1, IRF1	The authors developed a radiosensitive predictive model using 10 genes comprised in central pathways involved in radioresistance. This linear regression algorithm generates a radiosensitive index RSI having a prognostic value in HNSCC datasets.	[72]

Table 1. Biomarkers predicting local recurrence and radioresistance in head and neck cancers.

3.1. MicroRNAs as new potential biomarkers predicting radiotherapy response

A class of small non-coding RNAs termed microRNAs (miRNAs) has recently been indicated as biomarker of some type of cancers [73]. miRNAs are endogenous, small, non-coding RNAs of 17-25 nucleotides that are thought to regulate approximately 30% of human genes at posttranscriptional level, primarily through their partial complementarity with the coding region or 3' untranslated region (UTR) of target mRNAs. This leads to translational repression and/or degradation of target mRNA, therefore regulating gene expression [74]. They are involved in essential biological activities such as cellular differentiation, proliferation, development, apoptosis and cell cycle regulation. The roles of miRNAs in cancer have been extensively investigated in the past few years. The relevance of miRNAs in cancer was suggested by the observed changes in expression patterns and recurrent amplification as well as deletion of miRNA genes in cancer [75]. It has been shown that there are two types of cancer-related miRNAs: oncogenic or tumor suppressor miRNAs [74].

Several investigators have empathized the role of miRs as biomarkers for HNSCC [42] and the usefulness of miRs as prognostic factors has only begun to be explored. Moreover, miRNA expression may predict the efficacy of therapies, including radiotherapy [76]. Data from the study of miR-205 and let-7d expression showed their association with locoregional occurrence and shorter survival [77]. In addition, high expression of miR-205 can be used to detect positive lymph nodes, suggesting that this miR can be considered as a marker for metastatic HNSCC [78]. A similar study has shown that lower expression levels of miR-451 in HNSCC tumors are associated with recurrence [79]. Another recent work reported that downregulated miR-125b expression was associated with proliferation and radioresistance mechanisms, probably through ICAM2 signaling [80]. In addition, miR-17-5p expression has been shown to be induced in irradiated oral cancer cells and it downregulates p21 protein expression, contributing to radioresistance [81].

Furthermore, we also identified microRNAs signatures (miR-17-3p, miR-18b-5p, miR-324-5p, miR-19a-3p, miR-200a-3p, miR-331-3p, miR-21-3p, miR-21-5p, miR-205-5p, miR-151a-3p, miR-96-5p and miR-429) that are able to predict the risk of local recurrence and poor outcome in HNSCC tumors, and that are more powerful as biomarkers when compared to traditional prognostic indicators [42]. Finally, some evidences support the possibility to use miRNA detected in plasma as radio-responsive biomarkers for different types of cancer, including HNSCC. Accordingly, in HNSCC patients, the authors have detected changes in the abundance of circulating miRNAs (miR-425-5p and miR-93-5p) during radiochemotherapy. In addition, the researchers have demonstrated that the altered plasma miRNA changes after the therapy are the results of miRNAs release from damaged tumor cells [82].

4. Molecular strategies and future application in the treatment of HNSCC

Conventional HNSCC treatment consists of surgical resection followed by ionizing radiation or chemoradiation. In case of local advance/inoperable HNSCC, the typical treatment is concomitant platinum-based chemoradiotherapy. These standard therapies have some

limitations; the surgery can result in disfigurement and functional impairment, while the radiochemotherapy, although it is an organ-preserving treatment, can cause several side effects including mucositis, oral candidiasis, loss of taste, xerostomia and osteoradionecrosis [83, 84]. In addition, overall five-year survival rate is lower than 50% in HNSCC patients. Therefore, resistance to chemoradiotherapy often occurs and is associated with recurrences and poor outcomes; this represents a major clinical problem for HNSCC patients [14].

The understanding of the molecular perturbations in the cells of carcinomas recurring after irradiation could help to identify more specific target proteins and design novel therapeutic agents that will help improving therapy outcome in patients with HNSCC recurrences.

Tumor cells repopulation is a common effect observed in radiotherapy failure. A method to decrease this phenomenon, called Accelerated Radiotherapy (AR), is the reduction of overall radiation treatment time maintaining the total dose constant [14]. This therapeutic approach has produced excellent results in patients with advanced HNSCC [85]. In addition, several studies have shown that patients overexpressing EGFR protein, result to be more sensitive and consequently to have a better response to AR [14].

Besides the modification of radiotherapy modalities, there are several therapeutic strategies, such as, for example, immunotherapy, that can be combined with radiation, and are subjected to clinical development [86] (Table 2). Currently, two of the main intriguing targets for new targeted therapy are EGFR and VEGFR [86]. Both targeted therapies can be subdivided in monoclonal antibodies and tyrosine kinase inhibitors.

4.1. EGFR targeted therapy

The role of EGFR signaling in radioresistance was widely discussed in the paragraph 2.2. Many evidences suggest that the use of EGFR inhibitors in combination with radiotherapy improves the outcomes of HNSCC patients respect to those treated with radiotherapy alone [14].

4.1.1. EGFR monoclonal antibodies

One of the main antibodies targeting EGFR is called cetuximab. Other anti-EGFR antibodies under active investigations in combination with chemoradiotherapy in HNSCC are panitumumab, zalutumumab and nimotuzumab (Table 2).

Cetuximab: It is a chimeric IgG1 mAb, which by the recognition of determinants expressed on the extracellular domain of EGFR, antagonize normal receptor interaction, preventing the activation of the downstream signaling pathways [17]. Based on the results obtained from the clinical trials, since 2006 it has been approved by the Food and Drug Administration (FDA) in association with radiotherapy [14, 86]. However, a meta-analysis studying 15 trials and focusing on the comparison of the two currently combined modality therapies show that chemoradiotherapy respect to radiotherapy plus cetuximab is associated with a better overall survival and locoregional recurrence in advanced HNSCC [87]. In addition, some HNSCC patients develop a resistance to anti-EGFR therapy mainly due to k-Ras deregulation in absence of its mutation [14, 17, 88] and the presence of the variant EGFRvIII in tumor cells [17].

In this last case, the deletion presents in this variant cause a reduction in the binding affinity of monoclonal antibodies raised with wild type EGFR [17].

Panitumumab: Preclinical evidences show that it increases radiosensitivity by the radiation-induced DNA damage and preventing the translocation of EGFR in the nucleus. Currently, therapy combining radiation in combination with panitumumab is undergoing phase III clinical trials [86]. In addition, a phase III trial performed in advanced HNSCC patients to compare 5-FU and cisplatin treatment in presence and not of panitumumab have not shown an important improvement of the clinical outcome [89].

Zalutumumab: Several studies on phase I/II trial were performed using this drug at different doses in combination to radiation and/or chemotherapy; the results are encouraging and a phase III is ongoing [86].

Nimotuzumab: Preclinical studies show that it has antiproliferative, antiangiogenic and proapoptotic effects and it is well tolerated in HNSCC patients treated with radiation [86]. However, it has been demonstrated that cetuximab is more effective in comparison to nimotuzumab in enhancing radiosensitivity in high-EGFR expressing cells [90].

In conclusion, antibody anti-EGFR in combination with radiation therapy was well tolerated in HNSCC patients; currently, the best-studied mAb are cetuximab and panitumumab. Both improve radiosensitivity and overall survival in advanced HNSCC treated with radiation. However, the addition of cetuximab to conventional chemoradiotherapy has not shown a significant improvement in clinical outcome and the results obtained from the treatment of a large number of patients in multi-centered trials has shown that the treatment is effective in about 20% of cases [17].

To date, the use of cetuximab in combination with radiation represents a standard clinical approach, particularly in HNSCC patients who cannot tolerate chemotherapy [86].

4.1.2. EGFR tyrosine kinase inhibitors

Another group of agents targeting EGFR are small molecule tyrosine kinase inhibitors (TKIs). They act preventing EGFR autophosphorylation and consequently its activation by the occupation of the EGFR intracellular ATP-binding domain [17]. The two best studied TKIs are gefitinib (Iressa) and erlotinib (Tarceva). Others are called lapatinib and afanitib (Table 2).

Gefitinib: Preclinical studies show that gefitinib treatment on HNSCC cells can inhibit cell proliferation, decrease cell survival and enhance tumor cell radiosensitivity [91]. In addition, encouraging results were obtained in the clinical studies when gefitinib was combined with VEGFR inhibitors or other targets, suggesting the possibility to use it as possible neoadjuvant agent. Besides that, clinical trials combining gefitinib with chemoradiotherapy have not yet demonstrated a significant improvement respect to conventional therapy [86].

Erlotinib: Encouraging results were obtained from preclinical studies showing that the combination of erlotinib with radiation and/or VEGFR inhibitors improve treatment efficacy by the inhibition of tumor growth, proliferation and vessel density [92, 93]. However, to date,

there is no convincing clinical evidence that the addition of erlotinib to conventional therapy is universally beneficial [86]

Lapatinib and Afatinib: They are orally active EGFR and HER2 inhibitors, which seem to be well tolerated from HNSCC patients. Interestingly, in p16-negative HNSCC patients, a large difference in clinical outcome was observed in patients treated with lapatinib versus placebo. Phase III trials are ongoing in HNSCC for both molecules [86].

4.2. VEGF targeted therapy

As explained in the paragraph 2.5, VEGF is one of the most important regulators of angiogenesis; its upregulation is a common event in HNSCC and it is associated with radioresistance and poor prognosis.

4.2.1. VEGF monoclonal antibodies

Bevacizumab (Avastin) (Table 2) is the main recombinant anti-VEGFA monoclonal antibody under active investigation for HNSCC therapy. Preclinical evidences show that bevacizumab is able to act as radiation sensitizer in HNSCC cells, to reduce angiogenesis and tumor growth [86]. Phase I/II clinical trials performed using bevacizumab in combination with conventional chemoradiotherapy in HNSCC have shown that although this combined modality therapy is possible, to date there is no strong evidence that the addition of bevacizumab to chemoradiotherapy causes an improvement of the overall survival in HNSCC patients [13]. Future investigations are necessary to define the effectiveness of this molecule in the treatment of HNSCC.

4.2.2. VEGFR tyrosine kinase inhibitors

To date, the known VEGF tyrosine kinase inhibitors are: vandetanib (ZD6474), sunitinib, sorafenib and linifanib (ABT-869) (Table 2).

Vandetanib: It is an orally multi-kinase inhibitor targeting EGFR, VEGFR-2 and RET tyrosine kinases. Preclinical evidences show that the administration of vandetanib enhances the antitumor effects of radiation therapy by inhibition of both EGFR and VEGFR signaling in HNSCC human tumor xenografts; in particular, the authors demonstrate that radiation plus vandetanib treatment is effective in both overexpressing EGFR tumor cells and EGFR- null cells [94]. In addition, vandetanib restores HNSCC cells sensitivity to cisplatin and radiation *in vivo* and *in vitro* by promoting an increase of apoptosis and a decrease of microvessel density [95]. A randomized phase II clinical trial using a combination of cisplatin and radiation with or without vandetanib in advanced HNSCC is under consideration [13].

Sunitinib: It is an orally multi-kinase inhibitor targeting VEGFR, PDGFR and c-Kit tyrosine kinases. Preclinical and clinical studies show that sunitinib has low activity as monotherapy, but in combination with cetuximab and radiation, it causes a strong tumor inhibition effect by a complete abolition of tumor growth. Specifically, the combination of cetuximab and sunitinib causes a decrease of cell proliferation and enhances cell differentiation, while a decrease in

tumor vessels number was observed when the radiation treatment was added [96]. These results encourage future clinical investigations regarding the sunitinib and chemoradiotherapy treatment combination.

Sorafenib: It is an oral inhibitor of serine/threonine protein kinase b-Raf, C-Raf, VEGFR and PDGFR. Preclinical evidences show that sorafenib in combination with chemoradiation is able to enhance a more effective antitumor effect by the inhibition of cell growth, clone formation, cell migration and invasion compared to chemoradiation or radiation alone. This therapy combination is also able to inhibit tumor angiogenesis [97]. In addition, sunitinib can increase the antiproliferative effect of chemoradiotherapy by inhibiting the Raf/MEK/ERK signaling pathway and consequently downregulating the expression of the DNA repair proteins ERCC-1 and XRCC-1 [13]. Although these results suggest that sorafenib could enhance the effectiveness of chemoradiotherapy, ongoing phase I/II clinical trials will determine the real efficacy of sorafenib in HNSCC patients.

Linifanib: It is a novel ATP-competitive tyrosine kinase inhibitor of the VEGF and PDGF receptor family members. Preliminary data on HNSCC cells show that linifanib can act as radiation sensitizer since its combination with radiation is more effective compared to radiation or chemoradiation alone [13].

4.3. Other targeted therapies

As explained in the paragraph 2 relative to molecular mechanisms of radioresistance, there are many actors playing a key role in the failure of radiotherapy in HNSCC. As a consequence, targeted therapies against other molecules besides EGFR and VEGF family proteins were developed and their characterization is still ongoing. Among them, there are Src-family kinase inhibitors such as dasatinib; proteasome inhibitors as bortezomib, cyclooxygenase(Cox)-2 inhibitor (celecoxib); PI3K/Akt/mTOR inhibitors as wortmannin, perifostine and temsirolimus; and therapies targeting c-Met signaling pathway [14, 86] (Table 2).

Briefly, Src-kinase inhibitor dasatinib promotes radiosensitization by decreasing EGFR phosphorylation, its translocation in the nucleus and consequently, its association with DNA-protein kinases, blocking DNA repair pathways [98, 99]. Evidences on proteasome inhibitor bortezomib show its capability to act as radiosensitizer; specifically, it promotes the upregulation of PTEN activity and downregulation of p-Akt, leading to an increase of apoptosis of tumor cells [100-102]. Cox inhibitor celecoxib leads to a decrease of VEGFR expression and angiogenesis [103]. Next, mTOR inhibitors cause a reduction of angiogenesis and an induction of cell death by autophagy [86, 104]. Finally, given the important role discussed in the paragraph 2 on the significance of CSCs subpopulation in radioresistance, an emerging concept is the combined use of standard chemoradiotherapy with cancer stem cells targeted therapy. Preclinical study on CD44 expressing HNSCC cells combine radiation with anti-CD44 antibodies; the results show an increase in local tumor control in patients treated with radiation plus anti-CD44 antibodies compared to those treated with radiation alone in vivo [105].

Anticancer Agent	Type of agent	Target of Agent	Phase of development in HNSCC
Cetuximab	mAb IgG1	EGFR	Approved by FDA, phase III/IV
Panitumumab	mAb IgG2	EGFR	Phase III
Zalutumumab	mAb IgG1	EGFR	Phase III
Nimotuzumab	mAb	EGFR	Phase III
Gefitinib	TKI	EGFR	Phase I/II
Erlotinib	TKI	EGFR	Phase I/II
Afatinib	TKI	EGFR/HER2	Phase III
Lapatinib	TKI	EGFR/HER2	Phase III
Bevacizumab	mAb	VEGFA	Phase III
Vandetanib	TKI	VEGFR/EGFR	Phase I
Sunitinib	TKI	VEGFR/PDGFR/kit	Phase I
Sorafenib	TKI	VEGFR/PDGFR/Raf	Phase I
MM-121	mAb IgG2	HER-2	Preclinical phase
Pertuzumab	mAb IgG1	HER-3	Preclinical phase
AV-203	mAb IgG1	HER-3	Phase I
RO5479599	mAb	HER-3	Preclinical phase
Motesanib	TKI	VEGFR/PDGFR/kit	Preclinical phase
Dasatinib	TKI	Src family kinase	Phase I/II
Bortezomib	Proteasome inhibitor	26S proteasome	Phase I
Celecoxib	Nonsteroidal anti-inflammatory inhibitor	Cox-2	Phase I
Everolimus	Inhibitor derived from rapamycin	mTor	Phase I
Temsirolimus	Inhibitor derived from rapamycin	mTor	Phase I
Onartuzumab	mAb	c-Met	Preclinical phase
Cixutumumab	mAb IgG1	IGFR	Phase 0/II
Ficlatuzumab	mAb IgG1	HGF	Phase I
AMG 102	mAb IgG2	HGF	Preclinical phase
Fresolimumab	mAb IgG4	TGF- β	Preclinical phase

Table 2. List of molecular targeted therapies combined with radiotherapy under consideration for treatment of HNSCC patients (clinicaltrials.gov) [13, 86, 106].

4.4. Therapy by reactivation or elimination of mutant p53 protein

The vast majority of HNSCC show mutations in *TP53* gene; several evidences have shown that mutant p53 protein loses its function as tumor suppressor and acquires new oncogenic functions by which it promotes resistance to cisplatin and radiation treatment. The transfection

of wild-type *TP53* into cell lines induces growth arrest and reduces tumorigenicity in nude mice. This suggested that restoring p53 function in HNSCC could inhibit cell growth [107]. HNSCC has been one of the first tumor localities to benefit from gene transfer therapy. Several strategies have been developed to restore p53 function in HNSCC [14, 108].

Gene therapy: The most used vector for p53 gene therapy in HNSCC is the adenovirus, for its high affinity with the cells of the upper aerodigestive tract. A series of modified p53 adenoviruses (Ad-p53) are able to induce apoptosis and sensitize HNSCC cells to radiotherapy [109, 110]. Therefore, a phase I/II clinical trial based on the injection of Ad-p53 in HNSCC patients was performed and has shown that Ad-p53 is a promising therapeutic strategy [111, 112]. A phase III study based on the comparison of Ad-p53 to methotrexate treatment in advanced HNSCC show that overall, there is no significant difference in clinical outcome between these two subgroups of treated HNSCC, but, interestingly, Ad-p53 treatment was associated with a significant increase of survival in specific subgroup of HNSCC patients, having *TP53* wild type but inactivated by the upregulation of p53 inhibitors Mdm-2 or Mdm-4 [113]. This evidence suggests the possibility to select HNSCC patients who are most likely to benefit from Ad-p53 therapy. Another phase III clinical trial based on the use of recombinant Ad-p53 (gendicine) injection in combination with radiation shows encouraging results [114].

Virus targeting p53 deficient cells: This therapeutic strategy is based on the elimination of mutant p53. The efficient replication of adenovirus requires the neutralization of p53 function through E1B viral protein. ONYX-015 is an engineered adenovirus that does not express E1B protein and consequently is able to induce viral replication and cell death only in tumor cells carrying *TP53* mutations. Phase I/II clinical trials performed in HNSCC patients have shown that intravenous administration of ONYX-15 is feasible and while the treatment with ONYX-15 alone gave only marginal effects, its combination with cisplatin and 5-fluorouracil had a more profound impact on the response of patients [115]. Other clinical trials will be necessary to evaluate its real effectiveness in HNSCC treatment.

Molecules reactivating mutant p53: They are small molecules able to alter the conformation of mutant p53 to wild type, leading to the restoration of its tumor suppression function. Among them, glycerol treatment is able to reactivate p53 wild-type functions in HNSCC cell lines carrying mutant p53 by its ability to refold proteins [116]. Due to its toxicity, glycerol use is not so feasible in HNSCC patients. As a consequence, a series of other similar molecules was developed. Among them, PRIMA and CP-31389 were tested in HNSCC cell lines carrying mutant p53 and have demonstrated to inhibit proliferation and promote apoptosis by the induction of p53-related genes expression, including p21, Bax, Puma and Noxa [117]. Currently, there are no clinical data testing real effectiveness of these molecules in the treatment of HNSCC patients.

Molecules disrupting p53 inhibitors: In tumor cells, the function of p53 protein can be compromised not only by the presence of mutation on its gene, but also by upregulation of its inhibitors. The main p53 natural inhibitor is MDM2, which functions binding p53 protein and promoting its degradation. Nutlins and their derivate RITA are a class of small molecules able to prevent the binding MDM2-p53, restoring p53 tumor suppressor function. Therefore, Nutlins and RITA treatment leads to an increase of nuclear p53 levels, inhibition of prolifera-

tion, increase of cell death and antitumor efficacy of cisplatin [108]. Therapy treatment based on these molecules is more effective in tumor cells carrying p53 wild-type compared with mutant p53-carrying cells.

In addition, in a subset of HPV-related HNSCC, the activity of p53 can be also inhibited by the exogenous viral oncoprotein E6. Specifically, it acts by interacting with E6AP protein to degrade p53 via proteasome pathway and with p300 to prevent p53 acetylation. Treatment of HNSCC cell lines with the small molecule CH1iB, disrupting the binding of E6 HPV16 protein and p300, promotes an increase of the p53 acetylation levels and therefore an increase of p53 transcriptional activity. Additionally, Ch1iB shows an anticancer effect also due to its capability to reduce cancer stem cells population and by sensitizing tumor cells to cisplatin treatment in HPV positive cells [14, 108].

4.5. microRNAs as therapeutic agents

The role of microRNAs as predictors and modifiers of chemoradiotherapy in several kinds of human cancers, including HNSCC, has been shown [118]. For instance, miR-125b transfection on oral cancer cell lines enhances radiosensitivity to X-ray irradiation [80]. In addition, changes in the abundance of circulating miRNAs during radiochemotherapy has been detected and has been shown to reflect the therapy response of primary HNSCC cells after an in vitro treatment [82]. Finally, in our laboratory, we have demonstrated that the expression of signatures of *TP53* mutation-associated miRNAs, composed of 12 and 4 miRNAs, predicts, respectively, the risk of local recurrence insurgence and poor outcome, independently from other relevant prognostic indicators [42]. These evidences suggest the possibility of monitoring changes in miRNAs expression before to and during treatment in order to estimate the effectiveness of certain therapies. At the same time, another possibility for future application of miRNAs in therapy is the modulation of deregulated miRNAs concentration by molecules that replace downregulated miRNAs or using antagonists that binds overexpressed miRNAs [119]. Evidence supporting this possibility has been shown for the treatment of HCV infection; this phase II clinical study is based on the treatment of HCV infected patients with Miravirsen by which miR-122 is sequestered [120]. Miravirsen is the first miR-targeted drug to receive Investigation New Drug (IND) acceptance from FDA [121]. To date, there is only one clinical trial available in cancer patients; in particular, the treatment of liver cancer with MRX34, which is a molecule mimicking miR-34, is ongoing, in order to evaluate its maximum tolerated dose and its pharmacokinetics in patients [119].

4.6. TRAIL and Smac mimetics molecules

Recently, two classes of novel therapeutic agents targeting specific molecules involved in apoptosis pathway have emerged. The first one is the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). It is able to induce cell death by binding to its corresponding cell surface receptor TRAIL-R1/R2 and activating the apoptotic pathways [122-124]. A second class of targeted anticancer agents is composed by Smac mimetics (SMs). They mimic the function of endogenous proapoptotic mitochondrial protein Smac/Diablo [125]. In response to a death stimulus, it is released in the cytoplasm and inhibits the antiapoptotic activity of IAP proteins

[126]. Both TRAIL and SMs have been tested in several cancer models [123, 125, 127]. A study testing the sensitivity to TRAIL and SMs treatment on HNSCC cell lines show that both molecules are highly effective in killing tumor cells. In addition, caspase 8 and TNF- α expression was identified as biomarker for predicting, respectively, TRAIL and SMs sensitivity [128]. These preliminary results encourage future investigations on the possibility to use them as targeted HNSCC treatment.

4.7. Therapeutic activity of molecules derived from plants

Antineoplastic effects of molecules derived from plant extracts have recently gained increasing attention as an additive to traditional therapies of cancer, including HNSCC.

One of the most studied molecules derived from plants for HNSCC treatment is curcumin (diferuloylmethane). It is a polyphenol derived from the *Curcuma longa* plant, commonly known as turmeric. Curcumin, which has been used extensively in Ayurvedic medicine for centuries, is a pleiotropic molecule able to interact with multiple molecular targets and signal transduction pathways, and has a variety of therapeutic properties, including antioxidant, analgesic, anti-inflammatory and antiseptic activity [15]. More recently, curcumin has been found to possess anti-cancer activities, acting on several biological pathways involved in mutagenesis, oncogene expression, cell cycle regulation, apoptosis, tumorigenesis and metastasis [15]. For instance, it is able to inhibit the transcription factor NF- κ B and downstream gene products (including c-myc, bcl-2, COX-2, NOS, cyclin D1, TNF- α , interleukins and MMP-9). Additionally, curcumin affects a variety of growth factor receptors and cell adhesion molecules involved in tumor growth, angiogenesis and metastasis [15]. As a natural product, curcumin is not toxic. It is a potent antitumor agent also in HNSCC and can be used to overcome chemoradiotherapy resistance. Indeed, the treatment of HNSCC cell lines with a molecule derived from curcumin (H-4073) inhibits cell proliferation, angiogenesis and significantly sensitizes the cells to cisplatin treatment. H-4073 mediated its antitumor effects by inhibiting JAK/STAT3, FAK, Akt and VEGF signaling pathways that play important role in cell proliferation, migration, survival and angiogenesis [129]. Another study shows that curcumin sensitizes to radiation HPV-negative HNSCC cells with high levels of Thioredoxin reductase (TrxRs). Indeed, in this work it has been demonstrated that the efficacy of curcumin in sensitizing tumor cells to radiation depends on its ability to inhibit TrxRd1. TrxRs are a family of NADPH-dependent flavoproteins, which are involved in several redox-regulated cellular functions as transcription, DNA repair, proliferation, angiogenesis and apoptosis. Specifically, high levels of TrxRd1 isoform were found in HNSCC and were associated with poor outcome [130]. Finally, data from a very recent study shows that curcumin is more effective, in terms of inhibition of cancer growth, when combined with another non-flavonoid polyphenol called Resveratrol [131].

Another intriguing natural anticancer Chinese medicine is Gamboge. It acts as anti-inflammatory agent, detoxifying and apoptotic inducer in different type of cancer cells. Interestingly, the Gamboge derivative Compound 2 (C2) is able to inhibit growth also in HNSCC stem cells. Indeed, it can inhibit formation of tumor spheres and repress the

expression of multiples genes related to cancer stem cell phenotype by blocking the activation of EGFR pathways [132]. Since one of the main causes of failure in HNSCC treatment is the enrichment of CSCs population, which are resistant to current therapy, the future use of this molecule in combination with chemoradiotherapy could prevent the selective enrichment of CSCs after HNSCC conventional treatment.

5. Conclusions

Radioresistance strongly affects the clinical outcome of HNSCC patients. The key mechanisms by which radioresistance occur have been associated with deregulation of several molecular signaling pathways such as EGFR, VEGFR and p53. Recently, it has been shown that the enrichment of a small population of tumor cells, named cancer stem cells, also plays an important role in the failure of conventional HNSCC treatment. In addition, current treatments are associated with high toxicity and side effects. The basis of treatment decisions are mainly based on TNM staging, but patients with the same staging have different response to therapy. Several molecular targeted therapies are actively under investigation in order to improve the effectiveness of current therapy. Only a few of these strategies have been tested in clinical trials and to date cetuximab is the unique targeted therapy approved from FDA. However, this treatment showed efficacy in about 20% of HNSCC patients. In addition, due to the heterogeneous nature of these tumors, the study of molecular prognostic and predictive factors has been motivated by the necessity to predict radiosensitivity of patients and to define more homogenous groups of patients for treatment selection. Indeed, personalized treatment plans based on biomarkers could improve overall survival and reduce morbidity. Although several evidences have shown that many molecules, as proteins and microRNAs, can potentially predict response to therapy and clinical outcome, to date, the HNSCC treatment decision is uniquely based on TNM staging and HPV infection. One of the reasons of the difficulties to find efficacious biomarkers is the disagreement between these studies; this mainly relies on the variety of tumor sites, sensitivity of the techniques used, quality of the specimens studied and the arbitrary cut-off values set.

Author details

Federica Ganci¹, Andrea Sacconi¹, Valentina Manciocco¹, Giuseppe Spriano¹,
Giulia Fontemaggi¹, Paolo Carlini² and Giovanni Blandino^{1*}

*Address all correspondence to: blandino@ifo.it

1 Translational Oncogenomics Unit, National Cancer Institute “Regina Elena”, Rome, Italy

2 Medical Oncology A, National Cancer Institute “Regina Elena”, Rome, Italy

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