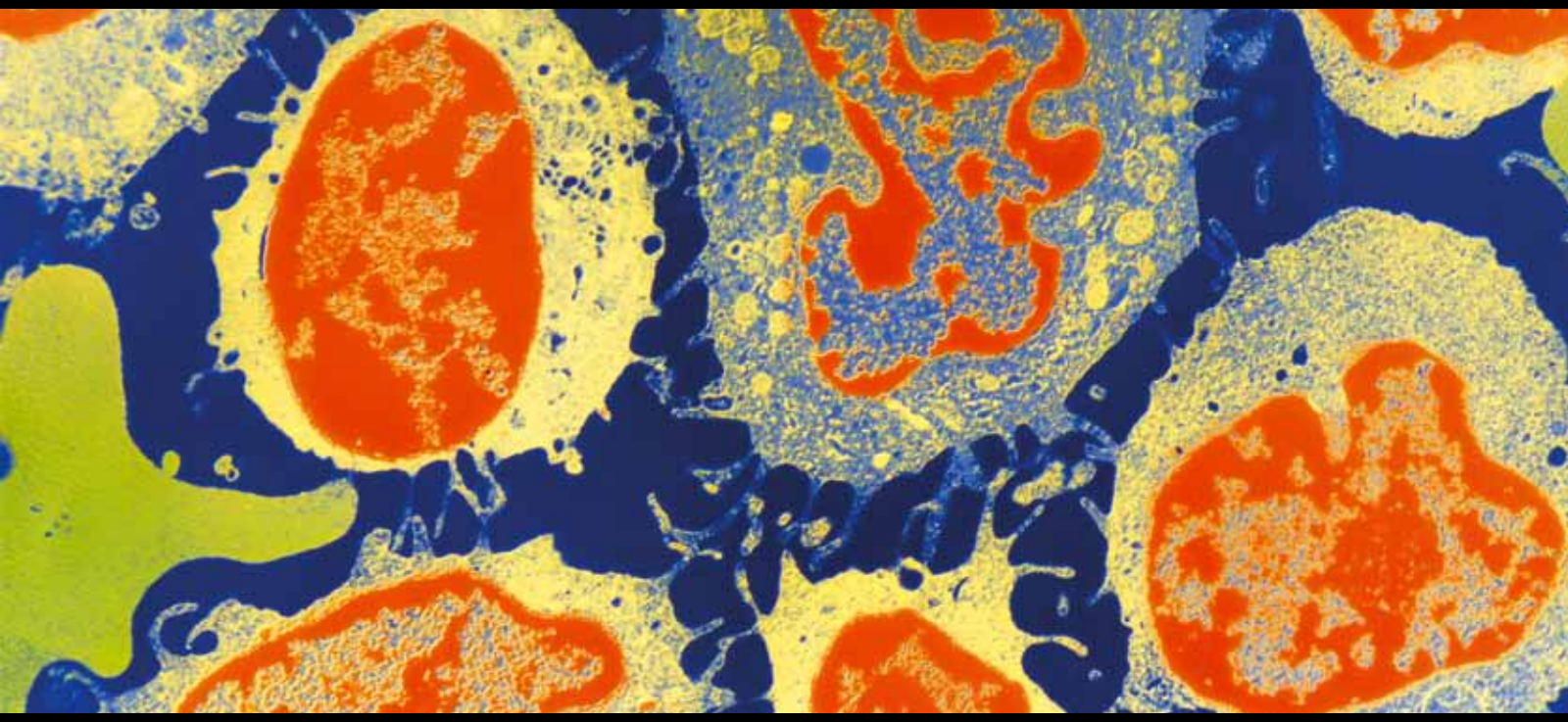
Journal of Oncology

Head and Neck Cancer

Guest Editors: Amanda Psyrri, Barbara Burtness, Paul M. Harari, Jan Vermorken, Lisa Licitra, and Clarence T. Sasaki



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Editorial Board

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Editorial

Head and Neck Cancer

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Head and neck cancer is a major cause of morbidity and mortality worldwide. The most common cancer in the head and neck area is head and neck squamous cell carcinoma (HNSCC) which is the 6th most common cause of cancer worldwide. Thyroid cancer accounts for approximately 37000 new cases per year in the United States and 1,600 deaths. The purpose of this special issue is to provide an update on recent advances in the understanding of head and neck tumorigenesis and their implications in clinical practice.

The most extensively studied pathway for targeted therapy in HNSCC is the Epidermal Growth Factor Receptor (EGFR) pathway. The EGFR-directed monoclonal antibody, cetuximab, is FDA- and EMA-approved for the treatment of HNSCC. Although the vast majority of HNSCCs contain high EGFR levels, clinical responses to EGFR-targeting therapies have been the modest. Molecular predictors for response to EGFR-targeted therapies in HNSCC are needed. The review by Egloff et al. provides a comprehensive and updated overview of candidate predictive markers in response to EGFR-targeted therapies in HNSCC including Src family kinases and describes recent clinical trials combining Src- and EGFR-targeted therapeutics. Fountzilas et al. analyzed retrospectively 37 patients with locally advanced HNSCC treated with concomitant radiotherapy, weekly cisplatin, and cetuximab for a series of biomarkers (tumor EGFR, MET, ERCC1, and p-53 protein and/or gene expression, MMP9 mRNA) and correlated those with treatment response. MMP9 was the only biomarker tested that appears to be of predictive value in cetuximab-treated patients. Validation of this finding in large independent cohorts is needed before its clinical implementation.

Molecular classification is a very important research area since the traditional clinical-pathological factors do not provide accurate prognostic information. The review by Ferrari et al. provides a comprehensive overview of the immunohistochemical expression of biomolecular markers in tongue cancer and their relationships with clinical behavior and prognosis. Pentheroudakis et al. evaluated the prognostic significance of mRNA levels of the EGFR family members HER1-4, the Vascular Endothelial Growth Factors (VEGFs) A, B, C, D, and their receptors VEGFR1, 2, 3 in a small retrospective cohort of HNSCC. The authors reported that high expression of the VEGF-C/VEGFR3 axis in recurrent HNSCC is associated with neck failure (soft tissues/lymph nodes) and inferior survival postrelapse but these findings need to be confirmed in large cohorts.

In addition to EGFR pathway, major research efforts concentrate on the identification of other targets for therapy in HNSCC. Akt expression and hyperactivation is a frequent event in HNSCC and strongly correlates with disease progression. Simons et al. explored the hypothesis that the Akt inhibitor, perifosine (PER), combined with inhibitors of glutathione (GSH) and thioredoxin (Trx) metabolism induces cytotoxicity via metabolic oxidative stress in human head and neck cancer (HNSCC) cells. The authors showed that PER induces oxidative stress and clonogenic killing in HNSCC cell lines that is potentiated with inhibitors of GSH and Trx metabolism. These data provide a biochemical rationale for the use of inhibitors of GSH and Trx metabolism

in combination with PER in combined modality cancer therapies.

Nuclear receptors are implicated in carcinogenesis. Knauer et al. summarize the function, prognostic/ther apeutic value, and, most importantly, ongoing preclinical and clinical studies targeting nuclear receptors in HNSCC. Several lines of evidence support the existence of cancer stem cell subpopulation in solid tumors, including HNSCC. These stem cells account for tumor resistance and aggressive behavior. Chen et al. introduce us to the stem cell concept in HNSCC and its potential application in the treatment of HNSCC patients. The eukaryotic translation initiation factor eIF4E is upregulated in approximately 30% of human cancers including HNSCC and this upregulation correlates with poor prognosis in HNSCC. Culjkovic et al. present the biochemical and molecular properties of the oncogenic potential of eIF4E, the potential strategies for eIF4E targeting in the clinic, and their utility in HNSCC patients. Immunotherapy has been used with limited efficacy in several solid tumors including HNSCC. The comprehensive and updated review by Rapidis et al. summarizes the rationale for immunotherapy in HNSCC and the principal approaches under investigation.

Advances in radiotherapy promise to increase cure rates and reduce acute and late morbidity of patients with HNSCC. Nath et al. provide a detailed overview of image-guided radiotherapy in head and neck cancer patients as well as clinical studies analyzing its use in target delineation, patient positioning, and adaptive radiotherapy.

Accurate staging of HNSCC is essential for developing therapeutic strategies in patients with HNSCC. Al-Ibraheem et al. provide an updated summary on 18F-FDG PET and PET/CT imaging of head and neck cancer Clinical applications of 18F-FDG PET and PET/CT in head and neck cancer include staging, detection of synchronous 2nd primaries, as well as detection of residual or recurrent disease after completion of treatment. Emerging applications are accurate delineation of the tumor volume for radiotherapy treatment planning, monitoring treatment, and prediction in response to targeted therapies. Sentinel node mapping has emerged as a routine procedure for staging of various malignancies, because it can determine lymph node status more accurately. In the review by Vermeeren et al. the sentinel node procedure and its indications in the head and neck region are presented. The authors also discuss the results of SPECT/CT for sentinel node detection and describe how a portable gamma camera may enable intraoperative real-time imaging with improved sentinel node detection.

Advances in molecular biology have offered exciting advances in the treatment of iodine-refractory thyroid cancer. Recent novel and promising findings include additional abnormalities in key pathways associated with thyroid tumorigenesis (RET-Ras-BRAF-MEK, RET-beta-cateinin, TRK-PI3K-AKT, and MDM-p53-PTEN), and gene expression abnormalities. The review by Pinchot et al. provides a comprehensive overview of the vital pathways in Medullary Thyroid Cancer tumorigenesis and focuses on interesting pathways for which targeted drug therapies are currently under development. Patients with multiple recurrences of well-differentiated thyroid carcinoma (WDTC) have significantly worse overall survival compared to those who have ≤ 1 recurrence of their disease. Holler et al. analyzed retrospectively 31 patients with multiple recurrences of WDTC and found that age >45, stage III/IV disease, distant metastasis, vascular invasion, MACIS score >6, and time to recurrence of <12 months were found to be significant predictors for mortality in this subgroup.

We hope that this special issue will inspire interests and new research in the field of head and neck cancer. The development of new targeted therapies the identification of novel predictive and prognostic factors will assist in the development of personalized medicine so that therapy can be tailored and optimized in every patient.

Disclosure: PMH has held laboratory research agreements with industry sponsors developing EGFR inhibitors including Amgen, AstraZeneca, Genentech and ImClone during the last 5 years.

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Review Article

Improving Response Rates to EGFR-Targeted Therapies for Head and Neck Squamous Cell Carcinoma: Candidate Predictive Biomarkers and Combination Treatment with Src Inhibitors

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The epidermal growth factor receptor- (EGFR-) directed antibody, cetuximab, was FDA-approved for the treatment of squamous cell carcinoma of the head and neck (SCCHN) in 2006. Additional EGFR-targeting agents in clinical development for SCCHN include other EGFR-directed antibodies, tyrosine kinase inhibitors and antisense DNA. Although the majority of SCCHN overexpress EGFR, SCCHN clinical responses to EGFR-targeting agents have been modest. Molecular predictors for SCCHN response to EGFR-targeted therapies have not been identified. However, molecular correlate studies in lung cancer and colon cancer, which have EGFR-targeted therapeutics FDA-approved for treatment, may provide insights. We describe candidate predictive markers for SCCHN response to EGFR-targeted therapies and their prevalence in SCCHN. Clinical response will likely be improved by targeted therapy combination treatments. Src family kinases mediate EGFR-dependent and -independent tumor progression pathways in many cancers including SCCHN. Several Src-targeting agents are in clinical development for solid malignancies. Molecular correlate studies for Src-targeting therapies are few and biomarkers correlated with patient response are limited. Identifying SCCHN patients who will respond to combined EGFR- and Src-targeting will require further characterization of molecular correlates. We discuss rationale for EGFR and Src co-targeting for SCCHN treatment and describe recent clinical trials implementing combined Src- and EGFR-targeted therapeutics.

1. Introduction

Ninety-percent of head and neck cancers are squamous cell carcinomas (SCCHN) involving the mucosal surfaces of the oral cavity, pharynx, and larynx. The overall relative 5-year survival rates for cancers of the oral cavity/pharynx and larynx are estimated to be 58.3% and 64.5%, respectively [1]. Morbidities associated with SCCHN and its treatments are significant and include eating and swallowing difficulties. Targeted therapies for SCCHN are under active investigation with the goals of reducing SCCHN morbidity and mortality.

Targeted therapeutics were conceptualized as a means of exploiting specific molecular alterations associated with cancers in order to selectively kill transformed cells and spare normal, healthy tissues. Targeted therapies are anticipated to have fewer associated toxicities than standard chemotherapies, which rely predominately on increased rates of cell division to enhance killing of the tumor cells compared to healthy tissues. For tumors that are treated with radiation and/or surgery, targeted therapies delivered systemically also have the potential to eliminate micrometastases that might not be eliminated with radiation therapy (RT) and/or surgery. In addition to reduced toxicity and treatment of undetected disease, it is hypothesized that effective targeted therapy may interfere specifically with processes that the cancer is dependent upon and be more effective than conventional therapies.

The epidermal growth factor receptor (EGFR) was anticipated to be a good drug target for SCCHN treatment because the majority of SCCHN overexpress EGFR [2, 3], and higher tumor levels of EGFR are associated with poorer clinical outcomes [4, 5]. EGFR participates in SCCHN autocrine

stimulation, and overexpression of EGFR and its primary ligand in humans, transforming growth factor alpha (TGF-α), have been correlated with poor outcomes for patients receiving therapy [5]. Cetuximab (Erbitux; ImClone Systems), a chimeric monoclonal IgG1 antibody directed against EGFR, was FDA-approved for the treatment of SCCHN in combination with RT for locally or regionally advanced disease and as a monotherapy for recurrent or metastatic SCCHN patients who have failed prior platinum-based therapy [6]. In addition to antibodies directed against EGFR, small molecule tyrosine kinase inhibitors (TKI) of EGFR and EGFR antisense agents are currently under active clinical investigation for SCCHN treatment. EGFR-targeted therapeutics delivered as monotherapies for treatment of SCCHN have demonstrated fewer toxicities compared to combined modality treatment regimens but only marginal clinical response (4–10%) [7, 8]. In general responses to EGFR-targeted therapies in SCCHN clinical trials have been modest.

Improving clinical response rates will involve (1) identifying SCCHN patients who are likely to respond to EGFR-targeted therapies, (2) developing effective combinations of targeted therapies, and (3) correctly identifying patients who will respond to specific targeted agents applied alone or in combination. Our understanding of the factors contributing to targeted therapy response now extends beyond the molecular alterations of the tumor to include host genetic variation. In this review, we will summarize molecular data correlated with clinical response to EGFR-targeted therapies and discuss factors that may be considered for identifying responsive SCCHN patients. Preclinical evidence suggests that Src family kinase-targeted agents administered in combination with EGFR-targeted therapies may demonstrate improved clinical response over EGFR-targeted agents alone. Here we also provide rationale for combining EGFR- and Src-targeted therapeutics for treatment of SCCHN, discuss published EGFR- and Src-combination treatment preclinical data, and summarize completed and ongoing clinical trials in solid tumors evaluating Src-targeted therapies in combination with EGFR-targeted therapies.

2. EGFR-Targeted Therapies for SCCHN

There are several EGFR-targeted therapies in clinical development for SCCHN, and these agents are described in Table 1. These inhibitors fall into two primary categories: EGFR-directed antibodies and EGFR tyrosine kinase inhibitors. EGFR-directed antibodies include cetuximab, nimotuzumab (YM Biosciences), panitumumab (Amgen), and zalutumumab (GenMab). EGFR-targeted tyrosine kinase inhibitors include erlotinib (Genetech and OSI Pharmaceuticals) and gefitinib (AstraZeneca). In addition to EGFR-targeted kinase inhibitors, inhibitors with broader target specificities are also in Phase II or III development for SCCHN including lapatinib (GlaxoSmithKline), which is a dual EGFR/HER2 inhibitor, and zactima (AstraZeneca), which targets VEGFR2 and RET in addition to EGFR (Table 1). More recently antisense therapy targeting EGFR has been evaluated in a Phase I clinical trial by our group [9].

Results of clinical trials for EGFR-targeted therapies cetuximab, nimotuzumab, gefitinib, and erlotinib used alone or in combination with conventional treatments for SCCHN have been reviewed by us and others and will not be described in detail here [10–12]. A Phase I study of panitumumab in combination with chemoradiotherapy involving 19-treatment-naïve patients with stage III/IV head and neck cancer reported an 87% complete response rate among the 15 evaluable patients and no grade 3 or 4 chronic toxicities [13]. A Phase I study of lapatinib in combination with chemoradiation in 31 patients with locally advanced SCCHN reported an overall response rate of 81% with radiation-associated mucositis, dermatitis, lymphopenia, and neutropenia as the most common grade 3 or 4 adverse events [14]. Our Phase I study of intratumoral delivery of EGFR antisense DNA in 17 patients with advanced, refractory SCCHN was associated with no grade 3 or 4 or dose-limiting toxicities and a clinical response rate (complete response and partial response by modified RECIST criteria) of 29% [9]. The current phase of clinical development for each of these agents is presented in Table 1.

3. Predictors of Response to EGFR-Targeted Therapies

To date, no molecular marker has been identified to correlate with SCCHN response to EGFR-targeting in patients. SCCHN tumor expression of the truncated form of EGFR, EGFR variant III (vIII), which lacks the ligand binding domain, occurs in up to 40% of SCCHN tumors and confers resistance to EGFR-targeted monoclonal antibodies in SCCHN preclinical models [15]. However, EGFR vIII expression and resistance to EGFR-targeted therapies in SCCHN patients has not been described. Molecular correlates of clinical response/nonresponse to EGFR-targeted therapies have been identified for colon and lung cancers. For example, the treatment of lung cancer with the EGFR tyrosine kinase inhibitor gefitinib demonstrated effective responses in a subset of patients whose lung cancers were subsequently found to harbor EGFR kinase activating mutations [16, 17]. Importantly, EGFR activating mutations do not appear to be frequent in SCCHN [18, 19]. Therefore, some of these molecular correlates, such as the EGFR tyrosine kinase activating mutations are not applicable to SCCHN because the frequency of these mutations is very low and will not be discussed further in this review. However, other molecular correlates of response to EGFR-targeting agents have been described for lung cancer and colorectal cancers including EGFR gene amplification, other somatic tumor mutations and patient genetic variations. These biomarkers have potential utility as predictive markers for SCCHN.

3.1. Tumor EGFR Gene Amplification. EGFR gene amplification occurs in SCCHN, and the rate of reported EGFR gene amplification in SCCHN varies substantially (Table 2). To date, there are no published reports evaluating EGFR gene amplification for association with SCCHN patient response to EGFR-targeted therapies. However, EGFR gene

Table 1: EGFR-targeted therapies in clinical development for SCCHN.

Agent		Sponsor	Class	FDA-approval	Clinical trial phase for SCCHN
Antibodies					
Cetuximab	C225, Erbitux	ImClone Systems	Chimeric IgG1	SCCHN; colorectal cancers	III
Nimotuzumab	h-R3	YM Biosciences	Humanized IgG1	—	IV Advanced disease; II Locally advanced disease
Panitumumab	ABX-EGF; Vectibix	Amgen	Fully human IgG2	Colorectal cancers	III
Zalutumumab	HuMax-EGFR	GenMab	Fully human IgG1	—	III
Tyrosine kinase inhibitors					
Erlotinib	Tarceva; OSI-774	Genetech and OSI Pharmaceuticals	Reversible ATP competive	Lung cancer	III
Gefitinib	ZD-1839; Iressa	AstraZeneca	Reversible ATP competive	Lung cancer, relabeling limits	III
Lapatinib	Tykerb	GlaxoSmithKline	Reversible ATP competitive dual EGFR/Her2	Breast cancer	III
Zactima	ZD6474	AstraZeneca	Reversible ATP competitive VEGFR-2, EGFR and RET	—	II

amplification has been reported to be positively associated with response to EGFR-directed antibody therapies in clinical trials for nonsmall lung cancers (NSCLC) and colorectal cancers. In a phase II study of 229 NSCLC patients with advanced-stage NSCLC treated with cetuximab plus chemotherapy, 76 patient tumors were evaluated for EGFR gene amplification by FISH and disease controls rate (complete response/partial response and stable disease) was found to be significantly higher in patients with FISH-positive tumors compared to FISH-negative tumors (81% versus 55%, $P = .02$). In this same study, median progression-free survival was also significantly longer for patients with FISH-positive tumors compared to FISH-negative tumors (6 months versus 3 months, $P = .0008$) [20]. Several studies have reported positive associations between EGFR gene amplification and metastatic colorectal cancer response to EGFR-directed antibodies [21–23].

EGFR gene amplification in SCCHN has been reported range between 10–58% of SCCHN (Table 2) [24–32]. The range of reported prevalence of EGFR gene amplification may be due to differences in expression by tumor anatomical site. However, several methods were used to assess EGFR gene amplification, including fluorescence in situ hybridization (FISH) and quantitative real-time polymerase-chain reaction- (Q-PCR-) based assays. In addition, different scoring methods were employed in the studies presented in Table 2, some of which included polysomy in the definition for EGFR amplification and others did not. These differences in methodologies likely contribute to the variation in reported rates of EGFR gene amplification in SCCHN. The presence of EGFR gene amplification in a substantial portion of SCCHN and the previously reported associations between EGFR gene amplification and response to EGFR-targeted therapies in other cancers suggest that EGFR gene amplification may be a predictive marker for response to EGFR-targeted therapies in SCCHN. When evaluating EGFR gene amplification for correlation with response to EGFR-targeted agents, it will be important to develop consensus definitions of EGFR gene amplification.

EGFR gene amplification has not consistently been reported to correlate with EGFR protein levels although a plausible mechanism for gene amplification without protein overexpression is lacking [25, 28–30] (Table 2). In NSCLC and colon cancers a positive association between EGFR gene amplification and protein expression has also not been consistently observed [44, 45]. Importantly, EGFR gene amplification status, but not EGFR tumor protein levels, is associated with response to EGFR-targeted therapies in NSCLC and colorectal cancers. These discrepancies likely reflect the semiquantiatitve and variable methods of assessing gene amplification and protein expression levels in various laboratories. The characterization of EGFR gene amplification in SCCHN patients treated with EGFR-directed antibodies and the testing of association with response to therapy will be of interest.

3.2. Tumor KRAS/HRAS Mutations. Ras proteins are small GTPases that regulate signal transduction pathways leading to cell growth, differentiation, and survival. Three RAS genes produce four Ras proteins, KraS 4A, KraS 4B, H-Ras, and N-Ras, that are more than 90% homologous but demonstrate a high degree of tumor-type mutation specificity [46]. KRAS mutations have been reported by several independent groups to be negatively associated with response to EGFR tyrosine kinase inhibitors in lung cancer and EGFR-directed antibodies in colon cancer. A metaanalysis including 17 NSCLC EGFR tyrosine kinase inhibitor clinical studies with 1008 patient tumors and 8 metastatic colorectal cancer (mCRC)

Table 2: Candidate predictive markers for SCCHN response to EGFR-targeted therapies.

Tumor molecular marker	Study/ reference	Tumor type(s)	N tumors assessed	N tumors with molecular marker	Assay method	Positive scoring definition(s)	Associated with EGFR tumor levels
EGFR gene amplification	Sheu et al., 2009 [24]	OSCC	128	22 (17.2%)	FISH	>2.5 EGFR signals relative to Cen7 signal	Yes
	Ch'ng et al., 2008 [25]	SCCHN	39	18 (46%)	FISH	>2 EGFR signals relative to Cen7 signals or ≥15 EGFR copies per cell in ≥10% of cells	No
	Chiang et al., 2008 [26]	OSCC	42	14 (33%)	Q-PCR	≥2 EGFR gene copies relative to LINE1 element	No
	Temam et al., 2007 [27]	SCCHN	134	22 (17%)	Q-PCR ($n = 134$) and FISH ($n = 16$)	Q-PCR: >mean + 1.96 standard deviations of normal WBC EGFR gene copy number normalized to β-globin; FISH: ≥4 gene copies in 40% of cells or gene/chromosome ratio >2 or ≥15 gene copies in ≥10% of cells	No significant correlation between EGFR gene amplification by FISH and EGFR IHC expression
	Chung et al., 2006 [28]	SCCHN	75	43 (58%)	FISH	≥4 gene copies in 40% of cells or gene/chromosome ratio >2 or ≥15 gene copies in ≥10% of cells	No
	Hanawa et al., 2006 [29]	ESCC	106	53 (50%)	FISH	EGFR signal > Cen7 signal	Yes
	Mrhalova et al., 2005 [30]	SCCHN	33	7 (21%)	FISH		No
	Koynova et al., 2005 [31]	Larynx cancers	1080	112 (10.4%)	FISH	≥4 EGFR signals relative to Cen7 in ≥10% of cells	NA
	Freier et al., 2005 [32]	SCCHN	609	12.70%	FISH	≥8 EGFR signals relative to Cen7 in ≥10% of cells	NA
KRAS mutations	Sheu et al., 2009 [24]	OSCC	29	2 (6.9%)	Sequencing	KRAS Q61H mutation	NA
	Lea et al., 2007 [33]	ORAL cancers	122	5 (4%)	GAC database analysis	Somatic missense, nonsense, silent point mutations, frameshift and in-frame deletions and insertions	NA
	Forbes et al., 2008 [34]	Oral, pharynx, larynx cancers	937	24 (3%)	COSMIC database	Datamining of published reports and somatic mutation screening from Cancer Genome Project	NA
HRAS mutations	Forbes et al., 2008 [34]	Oral, pharynx, larynx cancers	686	75 (10%)	COSMIC database	Datamining of published reports and somatic mutation screening from Cancer Genome Project	NA
	Lea et al., 2007 [33]	ORAL cancers	170	19 (11%)	GAC database analysis	Somatic missense, nonsense, silent point mutations, frameshift and in-frame deletions and insertions	NA
	Anderson et al., 1994 [35]	ORAL cancers	35	6 (22%)	PCR and restriction length polymorphism analysis	Presence of appropriately altered restriction enzyme digested DNA fragment	NA

Table 2: Continued.

Tumor molecular marker	Study/ reference	Tumor type(s)	N tumors assessed	N tumors with molecular marker	Assay method	Positive scoring definition(s)	Associated with EGFR tumor levels
PI3KCA mutations	Murugan et al., 2008 [36]	SCCHN	37	2 (5%)	PCR and direct sequencing exons 9 and 20	Somatic missense, nonsense, frameshift, in-frame deletions, and insertions	NA
	Fenic et al., 2007 [37]	SCCHN	33	0 (0%)	PCR and direct sequencing exons 9 and 20	Somatic missense mutations	NA
	Qiu et al., 2006 [38]	SCCHN	38	4 (11%)	PCR and direct sequencing exons 1, 4, 5, 6, 7, 9, and 20	Somatic missense mutations	NA
	Kozaki et al., 2006 [39]	OSCC	108	8 (7%)	PCR and direct sequencing exons 9 and 20	Somatic missense mutations	NA
PTEN mutations	Shin et al., 2002 [40]	OSCC	86	4 (5%)	PCR and exon direct sequencing	Somatic missense, nonsense, silent point mutations, frameshift, in-frame deletions, and insertions	NA
	Poetsch et al., 2002 [41]	SCCHN	52	7 (13%)	PCR and exon direct sequencing	Somatic missense, nonsense, frameshift, in-frame deletions, and insertions	NA
	Mavros et al., 2002 [42]	OSCC	50	0 (0%)	PCR and exon direct sequencing	Somatic missense, nonsense, frameshift, in-frame deletions, and insertions	NA
	Shao et al., 1998 [43]	SCCHN	19	3 (16%)	PCR and exon direct sequencing	Somatic missense, nonsense, frameshift, in-frame deletions, and insertions	NA

Squamous cell carcinoma of the head and neck (SCCHN), oral squamous cell carcinoma (OSCC), esophageal squamous cell carcinoma (ESCC), fluorescence in situ hybridization (FISH), quantitative real-time polymerase chain reaction (PCR), centromere 7 (Cen7), Genetic Alterations in Cancer (GAC) database, and the Catalogue of Somatic Mutations in Cancer (COSMIC) database.

studies of anti-EGFR monoclonal antibody-based therapies in 817 mCRC patients found that KRAS mutations were significantly associated with absence of response to EGFR-targeted therapies for these cancers [47]. KRAS mutations are especially important predictors of unresponsiveness to EGFR-directed antibodies in colorectal cancers with EGFR gene amplification [48]. Reported rates of KRAS mutations in NSCLC range between 8–20% with higher rates reported for adenocarcinoma compared to squamous cell carcinoma histologies [49, 50]. KRAS mutations occur in approximately 30% of colon cancers [51].

KRAS mutations are relatively rare in SCCHN. Only one published report has described KRAS mutations in SCCHN to date, and in this analysis of 29 oral squamous cell carcinoma tumors, 9 (6.9%) were found to harbor KRAS mutations [24]. According to the Catalogue of Somatic Mutations in Cancer (COSMIC) database from the Sanger Institute, KRAS mutations occur in only approximately 3% of all cancers of the oral cavity, pharynx, or larynx while HRAS mutations occur in 10% of these cancers (Table 2) [34]. These rates are similar to data from the Genetic Alterations in Cancer (GAC) database presented in Table 2 [33]. The recent finding that a mouse knock-in model expressing HRAS from the KRAS chromosomal context accumulated HRAS mutations and resulted in increased lung tumorigenicity suggests that tissue-specific expression of KRAS and HRAS likely contributes to tumor-type specificity of mutations in these two genes [52]. To date, only one published study of HRAS mutations SCCHN reported a 22% rate of mutations in oral squamous cell carcinoma (Table 2) [35]. Mutations in HRAS are likely to be more common in SCCHN than KRAS mutations and may be important correlates for lack of response to EGFR-targeted therapies in SCCHN.

3.3. Tumor PI3K-AKT Pathway Mutations. Phosphatidylinositol 3-kinases (PI3Ks) are heterodimeric kinases composed of regulatory and catalytic subunits that are involved in the control of cell proliferation, survival, and motility. The PI3K catalytic subunit, P110alpha (PIK3CA) has been

reported to be somatically mutated and activated in several cancers including SCCHN. Activation of PIK3CA leads to plasma membrane recruitment and activation of Akt and downstream survival mechanisms. PIK3CA mutations have been reported to be associated with resistance to EGFR-targeted monoclonal antibodies in patients with metastatic colorectal cancers (mCRC). In a study involving 110 patients with mCRC, PIK3CA mutations were found to be significantly associated with reduced objective response rates following treatment with cetuximab or panitumumab ($P = .038$) and shorter progression-free survival ($P = .035$) [53]. PIK3CA mutations have been reported to occur in up to 8% of SCCHN as summarized in Table 2 [36–39].

PI3K signaling is inhibited by the activity of the phosphatidylinositol phosphatase, PTEN. PTEN acts as a tumor suppressor by negatively regulating the Akt signaling pathway. PTEN mutations occur in colorectal, lung, and head and neck cancers. Additionally, loss of PTEN expression occurs by mechanisms including promoter methylation and silencing or loss of heterozygosity. In SCCHN, PTEN mutations are not common (Table 2) [40–43], and loss of heterozygocity of PTEN has been reported to occur in approximately 12% of SCCHN [42]. Though the association with response to EGFR-targeted therapy in mCRC and loss of PTEN expression does not appear to be as strongly correlated as response and PIK3CA mutations [53], the consideration of both tumor PTEN expression status and PIK3CA mutation status may contribute to predicting response to EGFR-targeted therapies in SCCHN.

3.4. EGFR Polymorphisms. Several EGFR polymorphisms have been reported to be associated with differential response to EGFR-targeted therapies. In lung cancer, shorter EGFR intron 1 CA repeat polymorphism has been reported to be associated with improved response to gefitinib in two independent studies [54, 55]. In one study involving 70 patients with advanced NSCLC, patients with fewer than 17 CA repeats at either allele had significantly longer survival following treatment with gefitinib than patients having both alleles greater than 16 CA repeats ($P = .039$) [54]. Fewer EGFR intron 1 CA repeats were also significantly associated with mCRC patient response to cetuximab-based treatment in a study involving 110 mCRC patients receiving combined cetuximab-irinotecan salvage therapy [56]. An independent study of 139 NSCLC patients with WHO performance status of 0 or 1 who received gefitinib reported that patients with the EGFR haplotype of −216G/−191C had significantly worse survival with a hazard ratio of 1.85 (95% CI: 1.09 to 3.12) after adjusting for performance status, previous platinum treatment, skin rash, and diarrhea [57]. The EGFR intron 1 CA repeat polymorphism has been reported to affect EGFR basal transcription with higher transcription rates reported in individuals with fewer CA repeats [58, 59]. Differential promoter activity has also been reported for the two most common EGFR haplotypes at the −216G > T and −191C > A with the −216G/−191C haplotype having lower promoter activity and mRNA expression [60, 61]. These studies, therefore, indicate that patient EGFR polymorphisms associated with higher EGFR expression are more likely to respond to EGFR-targeted therapies.

The presence of the EGFR K521R variant has also been found to be associated with significantly improved progression-free survival (PFS) and overall survival (OS) in 32 EGFR-positive mCRC patients treated with cetuximab in combination with irinotecan [62]. Patients with the K521R variant had significantly longer PFS than patients with wild-type EGFR, 5.7 months versus 3.2 months, respectively, ($P = .04$, log rank test) and OS, 20.1 months versus 13.8 months, respectively, ($P = .03$) [62]. This EGFR variant, which resides in the extracellular domain of EGFR, has reduced ligand-binding, growth-stimulation, and kinase activity in vitro for the 521K variant. These findings suggest that EGFR polymorphisms have the potential to be correlated with response to EGFR-targeted therapies in SCCHN.

3.5. FCγRIIa and FCγRIIIa Polymorphisms. Cetuximab, a chimeric monoclonal IgG1 anti-EGFR antibody (Table 1), may exert its antitumor effects via several mechanisms including antibody-dependent cell mediated cytotoxicity (ADCC). The fragment c (Fc) portion of IgG1 antibodies can be recognized by the Fc gamma receptors (FCγR) on immune effector cells to induce ADCC. Polymorphisms in FCγRIIIa have been shown to be associated with differential response to cetuximab in mCRC patients in clinical studies and to SCCHN cell lines in vitro [63–65]. The FCγRIIIa polymorphism-V158F variant 158V was found to have higher cetuximab-mediated ADCC in vitro [64, 65]. The 158V variant was also associated with longer PFS in a study involving 69 mCRC patients treated with cetuximab plus irinotecan [63]. These findings indicate that FCγRIII variants may contribute to response to cetuximab in SCCHN patients.

The ability to correctly predict which patients will respond to which EGFR-targeted therapy will improve clinical response and reduce treatment-associated toxicities for these patients. However, the minority of SCCHN patients have responded to EGFR-targeted therapies in clinical trials, indicating that even if patients likely to respond to EGFR-targeted therapy were identified, they would represent a small portion of SCCHN patients. Even though the majority of SCCHN cancers overexpress EGFR, these tumors are not solely dependent upon EGFR activity. This is likely due to the presence of preexisting or treatment-induced compensatory signaling pathways. Because EGFR is activated in SCCHN and response to EGFR-targeted therapies has been demonstrated in clinical trials, it is reasonable to consider targeted therapies to be used in combination with EGFR-targeted therapeutics. Molecular signaling pathways in SCCHN that can be activated independently of EGFR include pathways initiated by G-protein-coupled receptors, integrins, and other receptor tyrosine kinases. Many of these pathways share Src family kinases (SFK) as downstream mediators of signaling. For these reasons, SFK have been identified as viable candidates for targeting in combination with EGFR. The combination of SFK- and EGFR-targeted agents for treatment of SCCHN is anticipated to have improved clinical efficacy compared to EGFR-targeting agents alone.

Table 3: Src-targeting agents in clinical development.

Agent		Sponsor	Target(s)	SFKs targeted (IC50)	Target site	Irreversible	Solid cancers in phase II or III clinical study∗	FDA approval (Date)	SCCHN clinical trial phase
Dasatinib	BMS-354825	Bristol-Myers Squibb	Src; Abl; c-Kit; PDGFR; others		ATP-binding	No	SCLC, NSCLC, breast, colorectal, head and neck, liver, melanoma, ovarian, pancreatic, sarcoma	Chronic myeloid leukemia (June 2006)	II
AZD0530		AstraZeneca	Src; Abl		ATP-binding	No	SCLC, NSCLC, breast, colorectal, head and neck, melanoma, osteosarcoma, ovarian, pancreatic, prostate	—	II
Bosutinib	SKI-606	Wyeth	Src; Abl		ATP-binding	No	Breast	—	—
KX01	KX2-391	Kinex	Src		Peptide-binding	No	(phase I)	—	—
XL999		Exelixis	Src, VEGFR, PDGFR, FGFR, FLT-3, others		ATP-binding	No	NSCLC, colorectal, kidney, ovarian	—	—

∗ClinicalTrials.gov solid tumors.

4. Src Family Kinases in SCCHN

Eight Src nonreceptor protein tyrosine kinase family members are expressed in humans: c-Src, Blk, Fgr, Fyn, Hck, Lck, Lyn, and c-Yes. c-Src, Fyn, Lyn, and c-Yes are broadly expressed, while Blk, Fgr, Hck, and Lck expression is primarily restricted to hematopoietic cells [66]. Src kinases have been implicated in normal cellular functions including cell adhesion, migration, angiogenesis, survival, proliferation, and differentiation [67, 68]. When these processes are inappropriately regulated, they can contribute to tumorigenesis, tumor progression and metastases. In SCCHN models, Src kinases are activated in response to EGFR stimulation [69], associate with EGFR [69], and have reduced activity following EGFR inhibition in vitro [70]. Src kinases are also upstream activators of EGFR and other receptor tyrosine kinases (RTKs). Following G-protein-coupled receptor (GPCR) stimulation, Src kinases are activated, resulting in the release of RTK ligands [71, 72]. In addition to RTKs and GPCRs, Src kinases are also activated by integrins in SCCHN [73]. Src kinases, therefore, are involved in the autocrine/paracrine stimulation of SCCHN and mediate EGFR-dependent and EGFR-independent signaling events.

Of the Src family kinases (SFK), c-Src is the most studied and most often implicated in cancer. Elevated c-Src protein and/or kinase activity has been reported for cancers of the lung, colon, breast, ovary, and pancreas in addition to head and neck cancers [68, 74]. c-Src is rarely mutated in cancer [74–76]. Therefore, increased activity of upstream signaling components and/or decreased activity of c-Src negative regulators are likely causes of c-Src activation observed in many epithelial cancers.

The expression and activation of specific SFK in SCCHN are less well understood. The SFK c-Src, Fyn, Lyn, and c-Yes are activated in SCCHN cell lines in vitro following stimulation with the EGFR ligand TGF-α [69], and these SFK likely play roles in SCCHN. At least one group has reported differential response of SFK to integrin β_6 signaling following simulation with fibronectin, the integrin β_6 ligand, in oral squamous cell carcinoma cell lines. Integrin β_6, which is neoexpressed in SCCHN, has been found to activate Fyn but not c-Src or c-Yes in SCCHN upon ligation with fibronectin, leading to Fyn-dependent activation of the Raf-ERK/MAPK pathway [73]. The murine knock-out models of specific SFK provide insights into the different roles of the individual SFK. The functions of some of the SFKs are redundant, at least regarding mouse development, as evidenced by lack of phenotype for single knock-out models of c-yes, hck, c-fgr, and blk [66]. The single knock-out murine models of lyn and lck had immune impairments, fyn knock-out mice exhibited defective brain development and impaired memory and immune functions, and c-src

null mice developed a bone remodeling disease with excess accumulation of bone [66]. Therefore, some functions are likely shared between the four SFKs with a subset of functions that may be unique to selective SFK.

5. Src Family Kinases in SCCHN Invasion and Progression

Mortality from SCCHN is usually associated with tumor invasion and locoregional metastases. The major site of SCCHN metastases is locoregional lymph nodes, and presence of neck lymph node metastases is universally accepted as the most important prognostic indicator for SCCHN. The development of metastases requires that cells move from the primary tumor and invade surrounding tissues. Invasion by tumor cells is preceded by the loss of cell adhesion and the gain of mesenchymal features in a process similar to events that occur in development termed epithelial-mesenchymal transition (EMT) [77]. EMT is accompanied by the loss of E-cadherin, which is a principal component of cell adhesion complexes, and the gain of mesenchymal characteristics including expression of vimentin [77].

The activation of Src kinases has been shown to be involved in EMT in cancer [78]. More recently, a study evaluating 50 primary SCCHN tumors for activated phospho-Src (P-Src), E-cadherin, and vimentin expression by immunohistochemical staining found increased P-Src, decreased E-cadherin, and presence of vimentin expression in SCCHN tumors to be significantly associated ($P < .05$) with morphologies associated with aggressive cancers including penetrating invasive fronts, poor or sarcomatoid differentiation, and lymph node metastases [79]. It is important to note that the P-Src antibody used in this study recognizes several activated SFKs and is not specific for P-c-Src. In addition to studies in SCCHN tumors, preclinical studies indicate that SFKs are involved in SCCHN migration and invasion. Our group found that blockage using an Src-specific inhibitor A-419259 resulted in decreased invasion and growth of SCCHN cell lines in vitro following stimulation with a GPCR ligand, gastrin-releasing peptide [72]. An independent group reported that dasatinib (Sprycel, BMS-34825; Bristol-Meyers Squibb), a dual Src/Abl kinase inhibitor (Table 3), inhibited migration and invasion in vitro in all 8 SCCHN cell lines evaluated [80]. Together these studies implicate an important role or roles for SFK in tumor migration and invasion, which are associated with increased mortality in SCCHN. Which SFKs are activated in SCCHN migration and invasion is currently not known. Importantly, epithelial tumor cells including SCCHN that have undergone EMT and acquired mesenchymal characteristics are more resistant to EGFR-targeted therapies than tumor cells that have epithelial characteristics [81]. Combining Src-targeted agents with EGFR-targeted therapies may be more effective than EGFR-targeted therapies alone for the control of SCCHN locoregional metastases.

6. Src-Targeting Agents in Clinical Development

Several small molecule inhibitors of c-Src and SFK are currently in clinical development for solid tumors including dasatinib (Sprycel, BMS), AZD0530 (AstraZeneca), bosutinib (SKI-606; Wyeth), XL999 (Exelixix), and KX01 (Kinex) (Table 3). All of these inhibitors are reversible inhibitors, and all except KX01 are ATP-competitive inhibitors (Table 3). These inhibitors differ primarily in their target specificities. Dasatinib and XL999 target several known kinases in addition to SFK (Table 3), while AZD0530 and bosutinib are dual SFK/Abl inhibitors. Dasatinib, which was FDA-approved for treatment of nonsolid tumors in June 2008, and AZD0530 are in Phase II clinical trials for SCCHN. Bosutinib and XL999 are in Phase II clinical trials for other cancers (Table 3). However, XL999, which inhibits VEGFR, PDGFR and FGFR in addition to Src kinases, was associated with serious cardiovascular toxicities in Phase I and II clinical trials [82–84]. Exelixis suspended new patient enrollment in the ongoing XL999 clinical trials in November 2006. A new addition to Src inhibitors in clinical development includes the c-Src substrate competitive inhibitor, KX01, which is currently being tested in phase I clinical trials. KX01 is exquisitely specific for c-Src whereas other Src-targeting agents inhibit other SFK in addition to c-Src [85–87]. To date there are no reports of Src-targeted therapeutics in SCCHN clinical trials or molecular predictors of response to Src-targeted therapies in patients with solid malignancies.

7. Cotargeting of EGFR and Src Family Kinases in Patients

Combining EGFR- and Src-targeted therapies for SCCHN is supported by results from preclinical studies. Our group reported that combined AZD0530 and gefitinib treatment of SCCHN cell lines in vitro resulted in significantly reduced cell growth and invasion compared to single agent treatments [88]. De novo and acquired resistance to cetuximab are means by which SCCHN patients fail therapy.

SCCHN and NSCLC preclinical models selected for resistance to cetuximab in vitro have been reported to have high levels of activated SFK and to have decreased PI3K/Akt activity following dasatinib treatment [89]. Interestingly, these cetuximab resistant cells were found to be resensitized to cetuximab following treatment with dasatinib [89]. These data in addition to our current understanding that many EGFR-independent cell signaling pathways, including GPCR- and integrin-initiated pathways, are modulated at least in part by SFK provide the rationale for the combined targeting of EGFR and SFK for treatment of SCCHN.

To date, there are no published reports of combined EGFR- and Src-targeted therapies for treatment of patients with solid tumors. Three clinical trials combining EGFR- and Src-targeted therapies for upper aerodigestive cancers are currently ongoing. A Phase I trial combining dasatinib with erlotinib in patients with recurrent NSCLC is ongoing at the H. Lee Moffitt Cancer Center and Research Institute (NCT00444015, ClincalTrials.gov). A Phase I/II study in NSCLC also combining dasatinib with erlotinib is ongoing at M.D. Anderson Cancer Center (NCT00826449, ClinicalTrials.gov). Our group will soon open a Phase 0 biomarker modulation study combining erlotinib with dasatinib for

patients with SCCHN or NSCLC (NCT00779389, ClinicalTrials.gov). Results from these trials are not yet available. Our group recently completed a Phase I trial combining cetuximab with dasatinib for treatment of advanced solid malignancies (NCT00388427, ClinicalTrials.gov). Seventeen of 25 patients enrolled in our Phase I study were evaluable for response, and 9 had stable disease while head ache was a primary toxicity [90]. We have evaluated molecular correlates in these patients and found that P-SFK levels in peripheral blood mononuclear cells were transiently reduced following daily dasatinib dosing (unpublished data). In addition, we found that EGFR, TGF-α, and amphiregulin plasma levels were altered following treatment (unpublished data). A Phase II study combining dasatinib and cetuximab for treating SCCHN patients is planned at the University of Pittsburgh Cancer Institute. Results from molecular correlate studies from this trial and others will be of great importance as the SCCHN medical and research communities work to identify predictive molecular markers of response to these therapies.

8. Summary and Future Directions

Despite the ubiquitous expression of EGFR in SCCHN, clinical responses to EGFR targeting agents, particularly, when administered as single agents, has been modest. Cetuximab was FDA-approved in 2006 for the treatment of newly diagnosed SCCHN in combination with radiation and recently extended to the recurrent/metastatic population in combination with chemoradiotherapy. However, in most of these trials, expression levels of EGFR in the tumor have not correlated with response to cetuximab and no single biomarker to date in baseline tissue has been proven to predict response to EGFR targeting agents. Comprehensive genomic and proteomic studies of baseline tissue are required in the context of clinical trials to begin to identify potential markers of clinical activity. Since EGFR signaling involves intracellular interactions with other oncogenic pathways in SCCHN preclinical models, it is plausible that cotargeting of EGFR in conjunction with blockade of these pathways may be beneficial. Src family kinases represent a potential pathway for targeting, especially given the FDA-approval of the Src kinase inhibitor dasatinib for hematopoietic malignancies. Studies are underway to test this hypothesis in SCCHN patients. Challenges include: (1) the difficulty of testing antiinvasive/antimetastatic agents in clinical trial settings, and (2) the possibility that RECIST criteria may not reflect decreased tumor proliferation, metabolism, or increased apoptosis as evidence by studies that have incorporated PET tracers. More relevant endpoints in EGFR-/Src-targeted trials than tumor shrinkage may include time to progression or overall survival. This may be especially relevant for locoregional recurrence/metastases in SCCHN [91].

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Research Article

MMP9 but Not EGFR, MET, ERCC1, P16, and P-53 Is Associated with Response to Concomitant Radiotherapy, Cetuximab, and Weekly Cisplatin in Patients with Locally Advanced Head and Neck Cancer

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Concomitant administration of radiotherapy with cisplatin or radiotherapy with cetuximab appear to be the treatment of choice for patients with locally advanced head and neck cancer. In the present retrospective analysis, we investigated the predictive role of several biomarkers in an unselected cohort of patients treated with concomitant radiotherapy, weekly cisplatin, and cetuximab (CCRT). We identified 37 patients treated with this approach, of which 13 (35%) achieved a complete response and 10 (27%) achieved a partial response. Severe side effects were mainly leucopenia, dysphagia, rash, and anemia. Tumor EGFR, MET, ERCC1, and p-53 protein and/or gene expression were not associated with treatment response. In contrast, high MMP9 mRNA expression was found to be significantly associated with objective response. In conclusion, CCRT is feasible and active. MMP9 was the only biomarker tested that appears to be of predictive value in cetuximab treated patients. However, this is a hypothesis generating study and the results should not be viewed as definitive evidence until they are validated in a larger cohort.

1. Introduction

Concomitant chemo-radiotherapy, mainly with cisplatin is the standard combined modality approach for the treatment of patients with locally advanced squamous cell carcinoma of the head and neck (SCCHN) region, because it prolongs survival and increases the chance of organ preservation compared to radiotherapy (RT) alone [1–3]. Several potential mechanisms, through which cisplatin acts as a radiosensitizer, have been reported reviewed in [4].

Single-agent cisplatin (100 mg/m^2) administered every 3 weeks concomitantly with RT is widely used since this high dose confers a systemic effect and at the same time acts as a radio-sensitizer [5]. However, the therapeutic benefit derived from the combined modality is counterbalanced in many cases by prohibitive toxicity, mainly neurotoxicity, ototoxicity, emesis, and stomatitis [6]. In order to reduce cisplatin-related toxicity, several investigators tested alternative schedules of cisplatin administration, such as daily or weekly infusions. The use of these different schedules is supported by in vitro data showing that low doses of cisplatin and RT, when combined, act synergistically in cell killing [3]. During the last few years, investigators within the Hellenic Cooperative Oncology Group (HeCOG) had adopted the weekly schedule of cisplatin concomitantly with RT for the treatment of patients with locally advanced SCCHN [7].

It is well documented that epidermal growth factor receptor (EGFR) is overexpressed in 42% to 80% of SCCHN cases [8, 9]. EGFR plays a pivotal role in proliferation and survival of SCCHN cells and its overexpression is associated with advanced stages and poor outcome [10, 11]. In previous studies EGFR expression was proposed as an even stronger predictor of locoregional control than T stage [9]. For this reason EGFR appears to be an attractive target of anticancer drugs. Furthermore, EGFR is an important determinant of response to RT and confers protection of cancer cells from the lethal DNA damage induced by ionizing radiation [12–14].

The main mechanisms through which EGFR confers radio-protection have recently been reviewed [15]. In vitro studies suggest that tumors could be sensitized to irradiation by blocking the radiation-induced nuclear import of EGFR, either through the expression of EGFR tyrosine kinase domain activating mutations or the use of cetuximab (Erbitux, Merck-Serono). Such mutations however, do not commonly occur in head and neck cancer.

Cetuximab is an IgG1 monoclonal antibody against the ligand-binding domain of EGFR. Cetuximab binds EGFR, sequesters the receptor in the cytoplasm and eventually targets it for degradation. It has been demonstrated in vitro that this antibody enhances the radio-sensitivity in SCCHN cells [16, 17] through several processes reviewed in [18, 19].

Because patients with locally advanced SCCHN recur locally more often than in distant sites [20, 21], it seems reasonable for patients with EGFR overexpressing tumors to receive more effective locoregional treatments. One such treatment strategy is the concomitant administration of RT with cetuximab. This rationale is supported by preclinical models, in which cetuximab acts synergistically with RT [22]. In a pivotal randomized phase III trial [23] the concomitant administration of cetuximab and RT improved locoregional control and prolonged survival compared to RT alone in patients with locally advanced SCCHN.

Following the introduction of cetuximab concomitantly with RT for the treatment of locally advanced SCCHN, a number of Greek oncologists used RT with concomitant administration of cetuximab and weekly cisplatin (herein named CCRT), as a treatment strategy for such patients. The background behind this approach was the fact that cetuximab increased both locoregional control and survival of such patients. Therefore, it seems logical to add cisplatin to this active combined therapeutic approach to further improve outcome, especially since this empirical approach is supported by in vitro studies [24].

It has been shown in vitro and in tumor specimens that the expression of the ligand hepatocyte growth factor (HGF) scatter factor and its receptor HGFR (MET) increase during invasive growth of SCCHN and this pathway, by constitutively co-activating other important pathways, may play a critical role in the metastatic process of SCCHN cells [25].

The ERCC1 (excision repair cross-complementation group 1), gene is one of 16 genes encoding for proteins of the nucleotide excision repair complex, which removes cisplatin-induced DNA adducts [26]. ERCC1 was shown in a randomized study [27] to be a significant predictive factor in patients with completely resected non-small-cell lung cancer (NSCLC) treated with cisplatin-based adjuvant chemotherapy. In the above study, only patients with ERCC1 negative tumors had shown benefit from the treatment. Polymorphisms in the 3′-UTR of ERCC1 and in the coding regions of the ERCC2/XPD and XRCC1 genes have been associated with disease prognosis and response to cisplatin in SCCHN patients [28].

Matrix metalloproteinases (MMPs) are a family of zinc-dependent proteinases that play an important role in the destruction and repair of the extracellular matrix and basement membranes in various physiological and pathological processes, including gastrointestinal inflammation and carcinogenesis [29, 30]. Importantly, the activation of the MMPs liberates growth factors from the extracellualr matrix, including EGFR, FGFR and PDGFR ligands [31]. Preclinical studies have demonstrated that MMP9 plays an important role in tumor-induced angiogenesis as well, with tumor-associated inflammatory and stromal cells being the main source of the proteinase. MMP9-mediated release of vascular endothelial growth factor (VEGF) and recruitment of pericytes to the angiogenic vasculature have been postulated to be the major processes involved in MMP9-stimulated angiogenesis [32].

In the present retrospective analysis we report our experience with the use of CCRT in patients with locally advanced SCCHN. To our knowledge this is the first report on the efficacy of this combination in such patients. Furthermore, we evaluated the association of a variety of potential tumor biomarkers with the observed response to CCRT.

2. Patients and Methods

2.1. Eligibility and Treatment. The medical records of 37 patients with newly diagnosed, histologically confirmed locally advanced nonnasopharyngeal SCCHN tumors, treated with CCRT in four centers, in which the aforementioned therapeutic strategy was adopted, were retrospectively reviewed. Patients amenable for this type of treatment had to have an age of >18 years, performance status (PS) 0-1 on the Eastern Cooperative Oncology Group (ECOG) scale and adequate bone marrow, hepatic and renal function to tolerate treatment. According to our standard practice, a written informed consent was obtained from each patient before the acquisition of biological material for research purposes.

All patients were treated with a linear accelerator with the intention to receive definitive RT (70 Gy to the tumor area and 45 Gy to the rest of the neck) concomitantly with weekly cisplatin 40 mg/m^2 and weekly cetuximab 400 mg/m^2, as a loading dose during the first week and 250 mg/m^2 on weeks 2–7. Before treatment administration, all patients were hydrated and given standard premedication. An H3-antagonist was used as antiemetic in all patients.

Drug doses were modified according to the grade of side effects as previously described [7, 33]. Details on the RT technique, as routinely used in our centers, have been previously described as well [7]. All adverse events were graded for this analysis according to the National Cancer Institute Common Toxicity Criteria (NCI-CTC, version 3.0). The radiation Therapy Oncology Group (RTOG) criteria were used to assess RT-related toxicities.

Approximately three months after the completion of CCRT, all patients underwent a work-up including endoscopic examination, chest x-ray, an ultrasound or computer tomography (CT) scan of the liver, and a CT scan or MRI of the head and neck region. In selected patients, especially those with a partial response (PR), an [^{18}F] fluoro-deoxy-D-glucose (FDG) positron emission tomography (PET)/CT scan was also recommended. Baseline and post CCRT scans were retrospectively collected and reviewed by a radiologist (A. K-F.) experienced in head and neck topology and an independent radiologist. Response to CCRT was assessed by the RECIST criteria.

2.2. Tissue Microarray (TMA) Construction. Formalin-fixed paraffin-embedded (FFPE) tumor tissue from 36 patients was used for protein and gene analysis. Representative slides (H&E) from the tissue blocks were reviewed by two experienced pathologists (G. K. and M. B.) for confirmation of the diagnosis, adequacy of material and calculation of the percentage of tumor in each case. Thirty-two specimens were arrayed (2 cores per case, 1.5 mm in diameter) into a recipient paraffin block (Paraplast, McCormick, Saint Louis, MO, USA) using a manual arrayer (Beecher Instruments, Sun Prairie, WI, USA). The TMA block also included tissue cores, in the first and the last column, from skin, tonsil, placental, kidney, thyroid, ovarian, prostate, and urothelial carcinoma that served as positive and negative controls.

2.3. Immunohistochemistry (IHC). Immunohistochemical labelling was performed according to standard protocols with slight modifications [34] on serial 3 μm thick sections, form the original blocks or the TMA block. As previously reported [35], the reproducibility of TMA immunostaining of different proteins compared to that obtained from whole sections of the original paraffin blocks is very high. The deparaffinization, antigen retrieval and staining procedures for EGFR [clone 31G7, Zymed (Invitrogen), Carlsbad, CA, USA; dilution 1 : 50], ERCC1 (clone 8F11, Neomarkers, Fremont, CA, USA; dilution 1:450), p16^{INK4A} (clone SPM304, Spring Bioscience, Fremont, CA, USA; dilution 1 : 150), and p-53 (clone DO-7, Dako, Glostup, Denmark; dilution 1 : 50) were performed using a Bond Max autostainer (Leica, Wetzlar, Germany). The hepatocyte growth factor receptor (HGFR/MET) protein was investigated using an antibody specific for the external domain of the beta chain of the MET protein (clone 8F11, Novocastra, Newcastle Upon Tyne, UK). After deparaffinization and antigen unmasking, the slides were incubated for 1 hour at room temperature with the MET antibody at a dilution of 1 : 50. After washing the primary antibody, the slides were incubated with a nonbiotin polymer detection system (BioGenex, San Ramon, CA) for a total of 40 minutes. The antigen–antibody complex was visualized using diaminobenzidine (BioGenex) as a chromogen. Slides were counterstained with Mayer's hematoxylin for 5 min (Leica), washed in fresh water, dehydrated, and mounted.

The evaluation of all IHC sections was done simultaneously by two pathologists (G. K. and M. B.) blinded as to the patients' clinical characteristics and survival data, according to previously proposed/established criteria with slight modifications. EGFR intensity of reactivity was scored using a four-tier system [36]; 0 (negative), no staining or background staining; 1+, definitive cytoplasmic staining and/or weak discontinuous membranous staining; 2+, moderate complete or incomplete membranous staining; 3+, strong complete membranous staining. Cases were considered positive when more than 10% of tumor cells showed at minimum 1+ staining, while 2+ or 3+ staining was classified as EGFR protein over-expression.

ERCC1 evaluation of nuclear staining was done according to the criteria proposed by Olaussen et al. [27]. The above system was based on a semi-quantitative H score, which combines the stain intensity and the percentage of positive tumor cells. The median of all H scores was chosen as the cut off point for separating positive from negative cases.

HGFR (MET) protein expression was evaluated using an intensity-adjusted scoring system (combining percentage and intensity of staining) according to Nakajima et al. [37]. Briefly, intensity scores ranged from 0 to 3 (0 = no staining, 1 = weakly positive, 2 = moderately positive, and 3 = strongly positive staining), and the staining pattern based on the percentage of positive tumor cells ranged from 0–3 (0 = 0 to 5%, 1 = 6 to 25%, 2 = 26% to 50%, and 3 = 51% to 100%). The localization of staining was either cytoplasmic or cytoplasmic/membraneous. Cases with a total score of at least 2 were considered positive (expressing tumors), whereas cases with a total score of 0-1 were grouped together

and considered to be negative or low expressing tumors. Nuclear and/or cytoplasmic $p16^{INK4A}$ staining in ≥25% of tumor cells was considered positive [38]. For p-53 protein expression, cases were scored as negative or positive, if ≤10% of nuclei or >10% of nuclei were stained, respectively [39].

2.4. Fluorescence in Situ Hybridization (FISH). FISH was performed on 4.5 μm thick TMA sections or whole sections of FFPE archival tissue samples using the LSI EGFR/CEP7 Dual Color Probe, (Abbott Molecular, Des Plaines, IL, USA), the LSI D7S486/CEP7 Dual Color Probe, (Abbott Molecular) and the specific for the HGFR/MET gene at region 7q31, Poseidon Repeat Free MET/SE7 probe (Kreatech Diagnostics, Amsterdam, NL). The EGFR probe, detecting a 300 kbp genomic region spanning the EGFR locus on 7p12, and the LSI D7S486 detecting a 200 kbp genomic region at region 7q31, were labelled with SpectrumOrange, while the centromere 7 specific probe (CEP7) was labelled with SpectrumGreen. The LSI D7S486/CEP7 Dual Color Probe was used to identify deletions in 7q31 that have frequently been detected in SCCHN patients, suggesting the existence of tumor suppressor genes [40]. The HGFR/MET gene probe was directly labeled with PlatinumBright550 and the SE7 (Chromosome 7 Satellite enumeration) probe with PlatinumBright495.

FISH was performed according to the manufacturer's protocol with minor modifications. Briefly, for all probes the deparaffinized tissue sections were incubated in citric acid solution, pH 6.0 for 10 min at 98°C. After washing twice for 2 min in dH_2O, slides were treated with a proteinase K solution for 10 min at 37°C in a hybridizer (Dako), washed for 5 min in 2xSSC solution and 1 min in dH_2O, and dehydrated (75, 85 and 100% ethanol, each for 1 min). Five to 15 μL of the probe mixture were then applied to each slide, slides were covered by cover slips, sealed with fixogum rubber cement, heat denatured for 5 min at 72°C (LSI EGFR/CEP7 and LSI D7S486/CEP7) and 80°C (MET/SE7) on a hot plate, and hybridized for at least 16 h at 37°C in a humidity chamber. After removing the cover slips by incubation in wash buffer (SSC, 0.3% NP-40), slides were washed for 7 min with wash buffer at 72°C. Subsequently, slides were dehydrated in 70%, 90% and 100% ethanol, each for 1 min, air dried protected from light, and finally nuclear counter staining was carried out with DAPI/Antifade solution (ZytoVision).

In 3 cases, due to inadequate material for the FISH assays we perform sequential multilocus fluorescence in situ hybridization (SML-FISH) according to Walch et al., with slide modifications [41]. After image acquisition, the slides previously hybridized with the LSI D7S486/CEP7 were washed by heating the section in SSC solution at 75°C for 16 hours, followed by denaturation at 73°C for 5 minutes in 70% formamide/SSC. Then, the slides were counterstained with DAPI and examined under fluorescence (x100 oil lens) to ensure absence of fluorescent signals. The hybridization, posthybridization and nuclear counterstaining procedure for the MET/SE7 probe was performed as mentioned above.

Slides hybridised with the EGFR/CEP7 probe were analyzed using a Zeiss fluorescence microscope (Axioskop 2 plus HBO 100) equipped with high quality objectives and an appropriate filter set. Slides hybridized with the LSI D7S486/CEP7 and MET/SE7 probes were analyzed using the Nikon 80i fluorescence microscope (Nikon GmbH, Dusseldorf, Germany) with a motorized 4 slide stage, equipped with high quality objectives (all form Nikon), an appropriate four filter set [DAPI, doublePath FIRC/TRITC, ZyGreen that is similar to Abbott Molecular SpectrumGreen and Kreatech's PlatinumBright550, and ZyOrange that is similar to Abbott Molecular SpectrumOrange and Kreatech's PlatinumBright495 (all from Chroma Technology Corp, Rockingham, VT, USA)] and an ultrasensitive black and white camera (QImaging, Surrey, BC, Canada). As a source of fluorescence illumination, the X-cite 120 (EXFO Photonic Solutions Inc, Ontario, Canada) equipped with a long-life 120-watt metal halide short arc lamp was used.

For the assessment of the FISH assays, in the majority of the cases, over 10 fields (x100) were captured by a computer-controlled digital camera and processed by commercially available software systems (FISH Imager Metasystems, Altlussheim, Germany for EGFR/CEP7 and XCyto-Gen, Alphelys, Plaisir, France for LSI D7S486/CEP7 and MET/SE7). For the latter probes, sequential, digital images were captured by a stack motor for the DAPI (1 or 2 planes at 0.5 μm), ZyGreen (5 planes at 0.85 μm or 4 planes at 1.15 μm) and ZyOrange (5 planes at 0.85 μm or 4 planes at 1.15 μm) filter settings, and the resulting images were reconstructed with blue, green and red pseudo-colors. Sixty nonoverlapping intact nuclei from the invasive part of the tumor were evaluated for each case according to morphological criteria using DAPI staining.

The evaluation of the FISH sections was done simultaneously by two observers (G. K and M. B). For each specimen, the absolute and mean copy number per cell of each DNA probe, the total number and percentage of cells with zero, one, two, three, and >4 copies of the respective probe, homozygous and heterozygous deletions, trisomies and polysomies, as well as the gene/CEP7 ratios were calculated.

FISH patterns for the EGFR gene were defined as previously described [42]. The status of the D7S486 locus was evaluated as follows: deletion if >35% of tumor nuclei contained one signal; trisomy/polysomy if >10% of tumor cells showed two or more copies of the D7S486 locus and chromosome 7. HGFR/MET gene status was classified according to Cappuzzo et al. [43] by six FISH strata as follows: (1) disomy (≤2 copies in >90% of the cells); (2) low trisomy (≤2 copies in ≥40% of cells, 3 copies in 10–40% of the cells, ≥4 copies in <10% of cells); (3) high trisomy (≤2 copies in ≥40% of cells, 3 copies in ≥40% of cells, ≥4 copies in <10% of cells); (4) low polysomy (≥4 copies in 10–40% of cells); (5) high polysomy (≥4 copies in ≥40% of cells); and (6) gene amplification (defined by the presence of tight EGFR gene clusters and a ratio of EGFR gene to chromosome of ≥2 or ≥15 copies of EGFR per cell in ≥10% of analyzed cells).

2.5. EGFR, ERCC1 and MMP9 mRNA Expression. For this retrospective study, intact RNA of high quality, as determined by analysis of the housekeeping gene RPL37A, was isolated from 33 FFPE tumour tissue samples employing an experimental method based on proprietary magnetic beads from Siemens Healthcare Diagnostics (Cologne, Germany), as previously described [44]. The number of malignant cells represented at least 30% of all nucleated cells per section, as verified by hematoxylin-eosin staining. Kinetic reverse transcription-polymerase chain reaction (kRT-PCR) was applied for the assessment of messenger RNA (mRNA) expression of EGFR, ERCC1 and MMP9 using the following TaqMan based primer/probe sets:

EGFR Probe CCTTGCCGCAAAGTGTGTAACGGAAT

Forward Primer CGCAAGTGTAAGAAGTGCGAA

Reverse Primer CGTAGCATTTATGGAGAGTGAGTCT

ERCC1 Probe TCCTCGCCTGGAGCCCCGA

Forward Primer AGGAGCTGGCTAAGATGTGTATCCT

Reverse Primer CCAGGTACCGCCCAGCTT

MMP9 Probe CAGGCAGCTGGCAGAGGAATACCTGTAC

Forward Primer CCCTGGAGACCTGAGAACCA

Reverse Primer CCACCCGAGTGTAACCATAGC

RPL37A and GAPDH were used as housekeeping (normalization) genes. Forty cycles of nucleic acid amplification were applied and the cycle threshold (CT) values of the target genes were identified. CT values were normalized by subtracting the CT value of the housekeeping gene RPL37A from the CT value of the target gene (ΔCT). RNA results were then reported as 40-ΔCT values, which correlated proportionally to the mRNA expression level of the target gene.

Human reference total RNA pooled from ten human cell lines (Stratagene, La Jolla, CA) was used as a positive control. RNA-free DNA extracted from tumor tissues was used as a negative control.

2.6. ERCC1, ERCC2/XPD and XRCC1 Gene Polymorphisms. DNA from peripheral blood and FFPE tissues was normalized at 20 ng/uL. The following Taqman SNP genotyping assays were used [Applied Biosystems, Biosolutions, Athens, GR]: C_3145050_10, detecting the ERCC2 Asn312Asp (AAC/GAC) polymorphism [rs1799793]; C_3145033_10, detecting the ERCC2 Lys751Gln (AAG/CAG) polymorphism [rs13181]); C_622564_10, detecting the XRCC1 Gln399Arg (CAG/CGG) polymorphism [rs25487]; and C_2532948_10, detecting the ERCC1 C8092A/CD3EAP Q504K (Gln/Lys) polymorphism [rs3212986]. Of note, the sequence detected by this assay (CACAGGCCGGGACAAGAAGCGGAAG[C/A]AGCA GCA GCA GCC TGT GTA GTC), which matches previous reports [45], includes a polymorphism in the 3′-UTR of the ERCC1 gene, which is simultaneously located at the 3′end of the CD3EAP coding region (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=3212986), since these genes are located in opposite directions at 19q13.3. Thus, CTG>CTT (G/T change) is the forward sequence in ERCC1, corresponding to the reverse CAG>AAG (C/A change) in CD3EAP. Runs were performed in duplicates in 10 μL reactions with 40 ng DNA input, amplified for 40 cycles under standard conditions in an ABI7500 real time PCR system equipped with the SDS v1.4 software keeping the default parameters (Applied Biosystems, Biosolutions, Athens, GR). Negative control did not provide amplification curves, while sample amplification curves were considered for further analysis if the cycle threshold (Ct) for the detected products was <38. Differences of the mean Cts (dCt) for the two alleles detected by each assay were: −1.93 for ERCC1 C8092A, 0.47 for ERCC2 N312D, 1.55 for ERCC2 K751Q, and 1.34 for XRCC1 Q399R, all within the acceptable limits for this type of assays (±2) (Applied Biosystems).

2.7. EGFR and kRAS Mutation Analysis. Genomic DNA was derived from FFPE tumors as previously described [45]. Samples consisting of >75% tumor cells were considered as eligible for DNA extraction and sequence analysis, otherwise macrodissection was performed to increase the tumor cell content to >75%.

We amplified exons 18, 19, 20, and 21 of the EGFR tyrosine kinase domain from genomic DNA (primary tumor tissue DNA) and germline DNA (peripheral blood DNA) that was extracted with the Invisorb Spin Blood Midi Kit (Invitek GmbH, Berlin, Germany) according to the manufacturers instructions. All PCR's were conducted as previously described [46]. All mutations were reconfirmed by PCR amplification and analysis of an independent DNA isolate. Exons 18, 19, 20, and 21 were reconfirmed in all patients identified as harboring mutations. Germline DNA was analyzed on two separate occasions for exons 18, 19, 20, and 21 for all patients with mutations, in order to confirm EGFR mutations as somatic or germline in origin. kRAS mutation analysis of codons 12 and 13 was performed as previously described [47].

All PCR products were purified by solid-phase reversible immobilization chemistry followed by bi-directional dye-terminator fluorescent sequencing. All exons were sequenced with the inner forward and reverse primers used for PCR. Sequences were analyzed by BLAST and chromatograms by manual review, and compared to: EGFR mRNA reference sequence Accession number NM 005228 and/or the EGFR gene sequence Accession number AF288738; RAS mRNA GI 34485723 and/or the RAS gene sequence GI 14277199 (http://www.ncbi.nlm.nci/).

The EGFR exon 21 mutation, L858R, was also analyzed by PCR/RFLP based on the presence of a new *Sau96I* restriction site created by the mutation. Deletions in exon 19 were also analyzed for using high performance gel electrophoresis (>2.5% agarose).

2.8. HPV Detection. Detection of HPV-16 and HPV-18 DNA was performed by one of the authors (A. L.) and was based on amplification of the E6 region as adopted from Ogura et al. [48], with minor modifications. Briefly, each reaction contained 0.2–0.4 μg DNA template in 10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 200 mM dNTPs, 1.5 units *Taq* DNA polymerase (Fermentas), and 100 pM of each of the primers in a total volume of 50 μL. Sense and antisense primer sequences for HPV-16 E6 were 5′-AAGGGCGTAACCGAAATCGGT-3′ and 5′-GTTTGCAGCTCTGTGCATA-3′, respectively. The same sense primer was used for HPV-18 E6. The antisense primer sequence for HPV-18 E6 was 5′-GTGTTCAGTTCCGTGCACA-3′.

The reaction mixure was subjected to PCR amplification using the GeneAmp PCR system 9700 thermal cycler (ABI). PCR cycling conditions consisted of 7 min at 96°C and 1 min at 72°C, followed by 35 cycles, including a denaturation step at 94°C for 30 s, an annealing step at 55°C for 30 s and an elongation step at 72°C for 45 s. The final extension step was carried out at 72°C. To avoid false positive and/or negative results a control (no template DNA) and an HPV positive DNA sample were included.

2.9. Statistical Analysis. Data on selected patient or tumor characteristics, and acute toxicity were obtained from the records. Responses were summarized as number of patients and corresponding percentages. Comparisons of the number of responders according to biomarkers were performed using the Fisher's exact test.

Overall survival (OS) was measured from treatment initiation to patient's death or last contact. Progression-free survival (PFS) was measured from treatment initiation to verified disease progression, death or last contact. In the analysis of PFS, death without prior verified progression was encountered as event. OS and PFS were estimated by the Kaplan-Meier method. For all comparisons, level of significance was set at $a = 0.05$.

3. Results

3.1. Patients' Compliance and Toxicity. Totally, 37 patients fulfilling the eligibility criteria were included in this retrospective analysis. There were 27 men and 10 women with a median age of 60 years (Table 1). Thirty-five patients (95%) completed CCRT. One patient discontinued CCRT after the completion of the 6th week of treatment due to grade 3 thrombocytopenia. One patient, a 74-year-old man, with a history of angina and atrial fibrillation died from acute myocardial infarction during the second week of RT. In the process of reviewing the clinical data, 5 more patients were identified to have had fatal events during the 3-month period post CCRT. More analytically, one of the patients from progressive disease, while a second patient, a 60-year-old man with an unremarkable medical history, from cardiac arrest, one week after the completion of CCRT. Autopsy was refused by his relatives. A third patient, a 46-year-old man, died from massive haemorrhage of the upper aerodigestive truck, 11 weeks post CCRT. Autopsy suggested that the fatal event was attributed to bleeding from a mucosal ulceration on the right pyriform sinus. No evidence of tumor was found. The latter patient, even though a post CCRT scan was not performed, was considered in the present analysis to be complete responder. The fourth patient, a 60-year-old man, alcoholic and heavy smoker, was at the initiation of CCRT on treatment for pulmonary tuberculosis with isoniazid and rifambicin. He died 7 weeks post CCRT. Further medical information about the cause of death could not be obtained. The fifth patient, a 56-year old man with alcoholic cirrhosis died 12 weeks post CCRT from massive bleeding due to the rupture of esopharyngeal varices. The above patients were included in the analysis for response on an "intent to treat" basis. Severe side effects most commonly noticed were leukopenia (70%), dysphagia (62%), skin rash (65%), and anemia (51%) (Table 2).

Table 1: Patient characteristics ($N = 37$).

Age		
Median	59	
Range	36–82	
	N	%
Gender		
Men	27	73
Women	10	27
Performance status		
0	33	89
1	3	8
2	1	3
Primary site		
Oral cavity	12	32
Larynx	11	30
Oropharynx	8	22
Hypopharynx	4	11
Paranasal Sinuses	1	3
Major Salivary Glands	1	3
Stage		
II	2	5
III	6	16
IV	29	78

3.2. Response to CCRT and Survival. Following the completion of CCRT, response was evaluated according to the RECIST criteria for 24 out of 37 patients (Figure 1). For 6 of these patients response was evaluated by PET as well. For one of the patients, response was classified as partial by RECIST, while PET was free of tumor, thus this patient was considered to be a complete responder in the overall response

Table 2: Worst toxicity expressed as *N* (%) during CCRT (RTOG criteria).

	Grade 1	Grade 2	Grade 3	Grade 4
Anemia	12 (32)	6 (16)	1 (3)	0 (0)
Neutropenia	3 (8)	13 (35)	7 (19)	0 (0)
Leucopenia	4 (11)	11 (30)	11 (30)	0 (0)
Thrombocytopenia	4 (11)	3 (8)	2 (5)	0 (0)
Nausea/vomiting	11 (30)	5 (14)	1 (3)	0 (0)
Fatigue	5 (14)	9 (24)	3 (8)	0 (0)
Dysphagia/anorexia	2 (5)	14 (38)	7 (19)	0 (0)
Weight Loss	2 (5)	2 (5)	1 (3)	0 (0)
Dermatitis	3 (8)	7 (19)	2 (5)	0 (0)
Rash	8 (22)	12 (32)	3 (8)	1 (3)
Mucositis	9 (24)	9 (24)	5 (14)	0 (0)
Mouth dryness	6 (16)	7 (19)	0 (0)	0 (0)
Constipation	10 (27)	1 (3)	1 (3)	0 (0)
Diarrhea	0 (0)	1 (3)	1 (3)	0 (0)
Infection	2 (5)	0 (0)	1 (3)	0 (0)
HSR	4 (11)	2 (5)	0 (0)	0 (0)
Otitis	0 (0)	3 (8)	0 (0)	0 (0)
Hoarseness	3 (8)	4 (11)	1 (3)	0 (0)
Peripheral Neuropathy	0 (0)	1 (3)	0 (0)	0 (0)
Nephrotoxicity	0 (0)	1 (3)	0 (0)	0 (0)
Confusion	1 (3)	0 (0)	0 (0)	0 (0)
Dizziness	3 (8)	0 (0)	0 (0)	0 (0)
Pruritus	2 (5)	0 (0)	0 (0)	0 (0)
Bleeding	3 (8)	0 (0)	0 (0)	0 (0)
Pain	1 (3)	1 (3)	0 (0)	0 (0)
Dry skin	1 (3)	0 (0)	0 (0)	0 (0)
Memory loss	1 (3)	0 (0)	0 (0)	0 (0)
Seizure	1 (3)	0 (0)	0 (0)	0 (0)

evaluation. In one additional case, response was evaluated by PET only.

Of the remaining 12 non-evaluable patients, one did not have a CT examination, for 5 patients the CT examinations were not available for central review, while 6 patients died before their response evaluation. However, for one of the latter patients an autopsy was performed and no evidence of tumor was found. This patient is considered to be a complete responder.

Overall, 11 patients (30%, 95% CI 16%–47%) achieved a CR and 11 (30%, 95% CI 16%–47%) a PR. Stable disease was seen in 3 patients (8%, 95% CI 2%–22%) and progressive disease in 5 patients (14%, 95% CI 5%–29%). For one patient the CT examination was not available, and therefore was not evaluated for response. Notably, among three patients with radiological PR that underwent an FDG-PET/CT, one of them had a negative examination. Therefore, this patient was considered as a complete responder in the final analysis. Taking into account the one patient with no evidence of tumor in the autopsy, 13 patients were considered as having achieved a CR (35%, 95% CI 8%–52%) and 10 as having achieved a PR (27%, 95% CI 14%–44%).

After a median follow-up of 21.3 months, 15 patients had a PFS event (10 patients demonstrated disease progression and 5 died of other causes), while a total of 9 patients had died. One-year progression-free and overall survival was 63% and 80%, respectively.

3.3. Immunochemistry and FISH. Individual EGFR, ERCC1, MET, $p16^{INK4A}$, and p-53 IHC and FISH data along with selected patient characteristics and responses are presented in Tables 3 and 4. In summary, thirty-one of 32 tumor samples (97%) were found to be EGFR positive, while in 22 samples (69%) EGFR was overexpressed (Figures 2(a) and 2(b)). No association between EGFR overexpression and complete response was identified (9/22 CRs among patients with EGFR overexpression versus 2/10 CRs among patients without EGFR overexpression; $P = .425$). One sample was EGFR amplified (Figures 3(a) and 3(b)).

The ERCC1 protein was expressed (Figures 2(c) and 2(d)) in 27 out of 33 tumor samples (82%). No association between ERCC1 expression and response was found (9/27 responders among ERCC1 positive patients versus 2/6 responders among ERCC1 negative patients; $P = .999$).

Table 3: Selected patient and tumor characteristics, EGFR, MET, p-53, HPV-16, and p16 status and response to CCRT.

n	Primary site	Gender	Age (years)	Response	EGFR (IHC)	EGFR (FISH)	EGFR (mRNA)	MET (IHC)	MET (FISH)	MET (FISH)	p-53 (IHC)	HPV-16 (DNA)	p16 (IHC)
(1)	Oral cavity	W	69	PR	2+	LLG	H	N	TR	GAIN	5	N	N
(2)	Oral cavity	M	66	PR	2+	DI	H	P	TR	GAIN	>90	N	N
(3)	Oral cavity	M	59	PR	2+	TR	H	N	TR	GAIN	>90	N	N
(4)	Oral cavity	M	82	CR	3+	DI	H	N	LP	GAIN	>90	P	N
(5)	Oral cavity	M	61	NE	3+	TR	L	P	LP	GAIN	>90	P	N
(6)	Oral cavity	M	69	PD	1+	DI	L	N	DI	NORMAL	>90	—	—
(7)	Oral cavity	W	41	PD	3+	DI	L	P	TR	GAIN	80	—	N
(8)	Oral cavity	W	60	CR	3+	TR	H	P	TR	GAIN	0	N	N
(9)	Oral cavity	M	44	CR	3+	TR	H	P	TR	GAIN	30–40	P	P
(10)	Oral cavity	M	60	ED	2+	DI	L	P	TR	GAIN	>90	N	P
(11)	Oral cavity	W	59	PD	3+	DI	H	P	LP	GAIN	20–30	N	N
(12)	Oral cavity	W	55	CR	—	—	Undet.	—	—	—	—	—	—
(13)	Oropharynx	M	57	CR	3+	AMPL	H	P	LP	GAIN	>90	N	N
(14)	Oropharynx	W	36	CR	2+	DI	L	N	LP	GAIN	<5	P	P
(15)	Oropharynx	M	59	CR	3+	DI	L	N	LP	GAIN	0	N	N
(16)	Oropharynx	W	55	CR	3+	DI	L	P	LP	GAIN	30–40	P	P
(17)	Oropharynx	M	69	PR	3+	DI	Undet.	N	DI	NORMAL	—	—	P
(18)	Oropharynx	M	46	CR	NE	—	H	—	—	—	—	N	P
(19)	Oropharynx	M	73	PD	1+	TR	H	N	TR	GAIN	0	N	N
(20)	Oropharynx	M	67	SD	3+	TR	H	P	DI	NORMAL	70–80	N	N
(21)	Hypopharynx	M	46	ED	3+	TR	H	N	HP	GAIN	>90	N	—
(22)	Hypopharynx	W	64	PR	NE	—	L		—	—	—	N	—
(23)	Hypopharynx	M	56	ED	3+	TR	L	P	DI	NORMAL	>90	P	N
(24)	Hypopharynx	W	56	PR	3+	DI	L	N	—	—	0	—	—
(25)	Larynx	M	55	CR	3+	DI	H	N	DI	NORMAL	0	N	N
(26)	Larynx	M	68	PR	2+	DI	L	N	TR	GAIN	>90	N	N
(27)	Larynx	M	60	ED	—	—	Undet.	—	—	—	—	—	—
(28)	Larynx	M	42	PR	3+	DI	L	N	DI	NORMAL	>90	N	N
(29)	Larynx	M	76	CR	3+	LLG	L	N	DI	NORMAL	30–40	N	N
(30)	Larynx	M	74	ED	NE	DI	L	N	—	—	0	N	—
(31)	Larynx	W	46	SD	3+	DI	L	P	TR	GAIN	5–10	N	P
(32)	Larynx	M	74	ED	2+	DI	L	P	TR	GAIN	>90	N	P
(33)	Larynx	M	67	PD	3+	LLG	H	N	TR	GAIN	>90	N	N
(34)	Larynx	M	54	CR	2+	DI	H	N	TR	GAIN	0	N	N
(35)	Larynx	M	54	PR	3+	DI	Undet.	N	DI	NORMAL	—	—	N
(36)	Paranasal Sinuses	M	65	SD	3+	LLG	H	P	TR	GAIN	>90	N	N
(37)	Major Salivary Gland	M	74	PR	3+	TR	L	N	LP	GAIN	>90	N	N

n = sample order number, M = man, W = woman.
CR = complete response, PR = partial response, SD = stable disease, PD = progressive disease, NE = nonevaluable, ED = early death.
LLG = low level gain, DI = disomy, HP = high polysomy, LP=low polysomy, TR = trisomy, AMPL = amplification.
P = positive, N = negative, H = high, L = low, Undet. = undetermined by real time PCR.

The MET protein was expressed (Figures 2(e) and 2(f)) in 14 out of 33 tumor samples (42%). The MET protein was detected as membraneous discontinuous or complete staining and/or cytoplasmic staining. In a small number of cases the endothelial cells of stromal vessels showed mild to moderate staining. No association between MET protein expression and complete response was found (4/14 complete responders among MET positive patients versus 7/19 complete responders among MET negative patients; $P = .719$). However, when considering objective response (CR or PR), a significant association was identified with MET protein expression (5/14 responders among MET positive patients and 15/19 responders among MET negative patients; $P = .029$).

MET gene gain was observed in 23 of 31 cases (74%). More specifically, low trisomy was detected in 16 cases, low polysomy in 6 cases, while high polysomy was identified in 1 case (Figures 3(c) and 3(d)). MET gene status was not

Table 4: Selected patient and tumor characteristics and response to CCRT in comparison to excision repair genes and MMP9 status.

n	Primary site	Gender	Age	Response	ERCC1 (IHC)	ERCC1 (mRNA)	ERCC1 C8092A/ CD3EAP Q504K#	ERCC2-312#	ERCC2-751#	XRCC1-399#	MMP9 (mRNA)
(1)	Oral Cavity	W	69	PR	N	H	A/A	Asn/Asp	Gln/Lys	Arg/Arg	H
(2)	Oral Cavity	M	66	PR	P	H	C/C	Asp/Asp	Lys/Lys	Arg/Arg	H
(3)	Oral Cavity	M	59	PR	P	L	C/C	Asp/Asp	Lys/Lys	Arg/Arg	H
(4)	Oral Cavity	M	82	CR	P	H	C/C	Asp/Asp	Lys/Lys	Gln/Arg	H
(5)	Oral Cavity	M	61	NE	P	L	A/C	Asp/Asp^	Gln/Lys	Gln/Arg	L
(6)	Oral Cavity	M	69	PD	P	L	A/C*	Asn/Asn*	Gln/Gln*	Gln/Gln*	L
(7)	Oral Cavity	W	41	PD	P	L	A/C*	Asn/Asp*	Gln/Lys*	Gln/Arg*	L
(8)	Oral Cavity	W	60	CR	P	H	C/C	Asp/Asp	Lys/Lys	Gln/Arg	H
(9)	Oral Cavity	M	44	CR	P	H	C/C	Asn/Asn	Gln/Gln	Arg/Arg	H
(10)	Oral Cavity	M	60	ED	P	L	C/C	undet.	undet.	undet.	L
(11)	Oral Cavity	W	59	PD	P	L	A/C	Asn/Asp	Gln/Lys	Arg/Arg	H
(12)	Oral Cavity	W	55	CR	Undet.	Undet.	A/C	Asp/Asp	Lys/Lys	Gln/Arg	Undet.
(13)	Oropharynx	M	57	CR	P	H	C/C	undet.	undet.	undet.	H
(14)	Oropharynx	W	36	CR	P	L	C/C	Asn/Asp	Gln/Gln	Gln/Arg	H
(15)	Oropharynx	M	59	CR	P	L	A/C	Asn/Asp	Gln/Lys	Gln/Arg	L
(16)	Oropharynx	W	55	CR	N	H	A/C	Asn/Asp	Gln/Lys	Arg/Arg	H
(17)	Oropharynx	M	69	PR	P	Undet.	A/C	Asn/Asn	Gln/Lys	Gln/Arg	Undet.
(18)	Oropharynx	M	46	CR	N	L	C/C	Asn/Asp	Gln/Lys	Arg/Arg	L
(19)	Oropharynx	M	73	PD	P	L	C/C	Asp/Asp^	Gln/Lys	Gln/Gln	L
(20)	Oropharynx	M	67	SD	P	H	A/A	Asn/Asn	Gln/Gln	Gln/Arg	L
(21)	Hypopharynx	M	46	CR	P	H	A/C	Asn/Asp	Gln/Lys	Gln/Arg	H
(22)	Hypopharynx	W	64	PR	N	H	C/C	Asp/Asp	Lys/Lys	Arg/Arg	L
(23)	Hypopharynx	M	56	ED	P	L	A/C	Asn/Asp^	Lys/Lys	Gln/Arg	L
(24)	Hypopharynx	W	56	PR	P	L	C/C*	Asn/Asp*	Gln/Lys*	Gln/Arg*	H
(25)	Larynx	M	55	CR	N	H	A/C	Asn/Asp	Gln/Lys	Gln/Arg	H
(26)	Larynx	M	68	PR	N	L	A/A	Asp/Asp	Lys/Lys	Arg/Arg	L
(27)	Larynx	M	60	ED	—	Undet.	—	—	—	—	Undet.
(28)	Larynx	M	42	PR	P	N	C/C	Asn/Asn	Gln/Lys	Gln/Gln	H
(29)	Larynx	M	76	CR	P	H	A/C	Asn/Asp	Gln/Gln	Gln/Arg	L
(30)	Larynx	M	74	ED	P	N	C/C	Asn/Asp	Gln/Lys	Arg/Arg	L
(31)	Larynx	W	46	SD	P	N	A/C	Asn/Asp	Gln/Lys	Gln/Arg	L
(32)	Larynx	M	74	ED	N	H	A/A^	Asn/Asp	Lys/Lys	Gln/Arg	L
(33)	Larynx	M	67	PD	N	H	C/C	Asp/Asp	Lys/Lys	Arg/Arg	L
(34)	Larynx	M	54	CR	P	N	C/C	Asp/Asp	Lys/Lys	Arg/Arg	L
(35)	Larynx	M	54	PR	P	Undet.	A/C	Asn/Asp	Gln/Lys	Gln/Arg	Undet.
(36)	Paranasal Sinuses	M	65	SD	P	H	C/C	Asp/Asp	Gln/Lys	Gln/Arg	H
(37)	Major Salivary Glands	M	74	PR	P	H	C/C	Asp/Asp	Gln/Lys	Gln/Arg	H

n = sample order number; M = man; W = woman; H = high; L = low; P = positive; N = negative; # = genotypes from tumor tissue or from matched peripheral blood and tumor tissue samples, unless otherwise specified; ^ = mismatched tumor/peripheral blood genotypes (tumor data are shown); * = peripheral blood data only; Undet. = undetermined by real time PCR.

found to be associated with response (2 responders among 8 patients with normal MET gene status versus 9 responders among 23 patients with MET gene gain, $P = .676$).

The $p16^{INK4A}$ protein was detected in 8 out of 30 cases examined (27%). In addition, in 5 of the 22 negative cases, p16 was highly expressed in the dysplastic squamous epithelium. Two of them showed p16 expression mainly in the dysplastic epithelium and to a small degree in scattered infiltrative neoplastic cells. No association was found between p16 and HPV-16 ($P = .290$). Furthermore, p16 was not found to be associated with response (4 responders among 8 patients with positive p16 status versus 7 responders among 22 patients with negative p16 status, $P = .417$).

The p-53 protein was found to be expressed (Figures 2(g) and 2(h)) in 22 of 33 patients (67%). No significant association with complete response was identified (6/22 complete responders among p-53 positive patients versus

Table 5: Incidence of excision repair genotypes in head and neck cancer patients. Peripheral blood (PB) and tumor tissue (TT) data.

	ERCC1 C8092A/ CD3EAP Q504K (CAG/AAG)			ERCC2-312 Asn/Asp (AAC/GAC)				ERCC2-751 Lys/Gln (AAG/CAG)				XRCC1-399 Gln/Arg (CAG/CGG)			
PB ($n = 26$)	C/C	12	(46.2%)	(G/G)	Asp/Asp	8	(30.8%)	(C/C)	Gln/Gln	4	(15.4%)	(G/G)	Arg/Arg	8	(30.8%)
	A/C	13	(50%)	(A/G)	Asn/Asp	13	(50%)	(A/C)	Lys/Gln	15	(57.7%)	(A/G)	Gln/Arg	15	(57.7%)
	A/A	1	(3.8%)	(A/A)	Asn/Asn	5	(19.2%)	(A/A)	Lys/Lys	7	(26.9%)	(A/A)	Gln/Gln	3	(11.5%)
TT ($n = 33$)	C/C	17	(51.5%)	(G/G)	Asp/Asp	12	(38.7%)	(C/C)	Gln/Gln	5	(16.1%)	(G/G)	Arg/Arg	12	(38.7%)
	A/C	12	(36.4%)	(A/G)	Asn/Asp	15	(48.4%)	(A/C)	Lys/Gln	16	(51.6%)	(A/G)	Gln/Arg	16	(51.6%)
	A/A	4	(12.1%)	(A/A)	Asn/Asn	4	(12.9%)	(A/A)	Lys/Lys	10	(32.3%)	(A/A)	Gln/Gln	3	(9.7%)
	Undet.	0				2				2				2	

Undet. = undetermined with real time PCR.

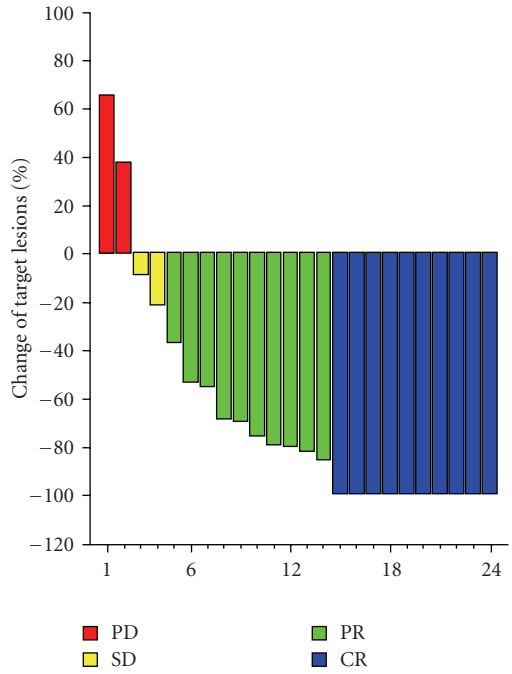

Figure 1: Waterfall for the response of target lesions according to RECIST criteria ($N = 24$).

5/11 complete responders among p-53 negative patients; $P = .437$).

Moreover, no significant association between the status of the D7S486 locus (Figures 3(e) and 3(f)) and response was identified.

3.4. EGFR, ERCC1, and MMP9 mRNA Expression. Individual EGFR, ERCC1 and MMP9 mRNA data along with selected patient characteristics and responses are presented in Tables 3 and 4. For all three genes the median was used as a pre-defined cut-off in order to classify tumors with high (above the median) or low (below the median) mRNA expression. The median normalized EGFR mRNA expression was 34.9 (29.6–39.5). High EGFR mRNA expression, was not found to be associated with complete response (4/17 complete responders among patients with low EGFR mRNA expression, versus 8/16 complete responders among patients with high EGFR mRNA expression; $P = .157$).

Similarly, the median normalized ERCC1 mRNA expression was 34.8 (30.0–39.5), while no association between high ERCC1 mRNA expression and complete response was identified. Specifically, in the group of 17 patients with low ERCC1 mRNA expression 4 patients achieved a complete response, versus 8 complete responders among the 16 patients with high ERCC1 mRNA expression ($P = .157$).

Finally, the median normalized MMP9 mRNA expression was 34.3 (29.5–39.5). Only 4 of the 17 patients with low MMP9 mRNA expression achieved a complete response, while 8 of the 16 patients with high MMP9 mRNA expression demonstrated a complete response to treatment ($P = .157$). Although MMP9 mRNA expression was not found to be significantly associated with complete response, a significant association with the objective response (CR or PR) was identified (6/17 responders among patients with low MMP9 mRNA expression versus 14/16 responders among patients with high MMP9 mRNA expression, $P = .004$).

3.5. ERCC1, ERCC2/XPD, and XRCC1 Gene Polymorphisms. Samples from 36 patients were considered for allelotyping, including 10 from tumor tissue only, 3 from peripheral blood (germline) only and 23 from matched peripheral blood and tumor tissue. The incidence of allelic combinations in germline and tumor tissues is shown in Table 5, while individual data on ERCC1, ERCC2 and XRCC1 gene polymorphisms are presented in Table 4. Briefly, heterozygous polymorphic alleles were common for all targets; concerning homozygous combinations, C8092C was the most frequent genotype for ERCC1, Asp312Asp and Lys751Lys for ERCC2/XPD and Arg399Arg for XRCC1. In 2/10 unmatched tumor tissue samples, allelotyping data could be obtained for ERCC1 but not for ERCC2 and XRCC1, probably due to poor FFPE DNA quality. Overall, the incidence of allelic variants observed in the present study was in accordance with relevant previous data [28].

The germline genotype did not always match the tumor genotype in the same patient, as deduced from the high dCts (5.8, 7.3, and 7.9 in three cases) in the respective tumor

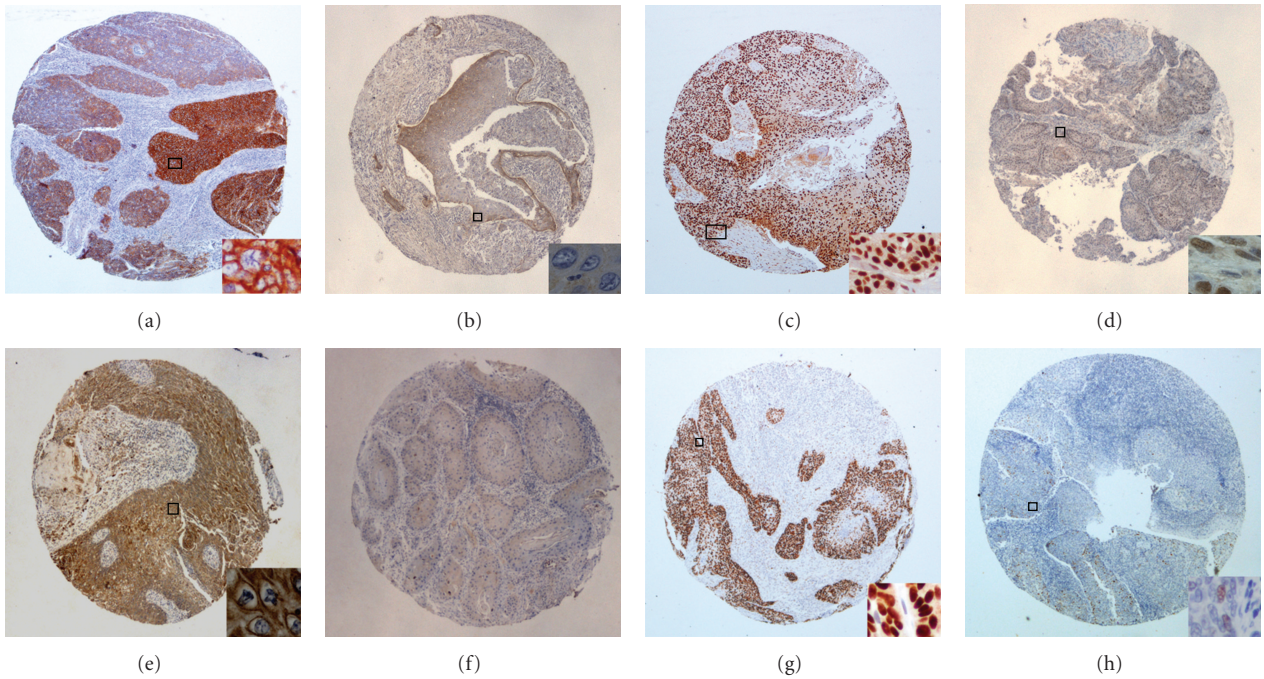

Figure 2: Immunohistochemistry performed on tissue microarrays. (a) EGFR protein expression in all tumor cells with focal intense complete membranous staining (+3); (b) EGFR negative case showing mild cytoplasmic focal staining; (c) ERCC1 protein strong nuclear positivity; (d) ERCC1 protein expression with equal intensity in neoplastic cells and stromal fibroblasts (regarded as negative staining); (e) MET strong cytoplasmic and membraneous protein expression; (f) Lack of MET protein expression in tumor cells; (g) p-53 strong nuclear protein expression; (h) p-53 expression in a small fraction of tumor cells (regarded as negative staining). Original magnification x20; insets (a), (c), and (g) x200; insets (b), (d), (e), and (h) x400.

samples or from the amplification of allele targets that were negative in the matching peripheral blood samples. Changes in tumor genotypes were observed upon repeated testing in 4/23 patients (17%) with matched peripheral blood and tumor samples available for comparison (Table 4). Germline heterozygocity was replaced in one case by homozygocity for the rare A/A allele for ERCC1 C8092A/CD3EAP Q504K, indicating a Lys/Lys genotype for CD3EAP in the tumor. In two additional cases, germline A/G was replaced by G/G for ERCC2-312 (change of Asn/Asp into Asp/Asp in the tumor). In another case, germline ERCC2-312 Asp/Asp (no amplification of the Asn target) was replaced by Asn/Asp (dCt = 1.1) in the matched tumor tissue.

3.6. Mutational Analysis. Only one patient had a somatic EGFR mutation on exon 20, a D770insGF insertion. No patients were found with a kRAS codon 12/13 mutation. Additionally, no patients were identified with an L858R EGFR mutation or codon 19 deletion by alternative methods.

3.7. HPV Detection. We examined the presence of HPV-16 and 18 E6 in 30 patients by PCR. Totally, 6 out of 30 samples (20%) tested were HPV-16 positive (one laryngeal, 3 oral cavity and 2 oropharyngeal tumors). All samples proved to be HPV-18 negative. Interestingly, 4 of the 6 HPV-16 positive patients, who were evaluable for response, achieved a CR post CCRT.

4. Discussion

The present report describes our collective experience with CCRT in patients with locally advanced SCCHN. The CR rate achieved in such a heterogeneous group of patients was 35%. Additionally, 10 patients (27%) were considered as having a PR. Interestingly, one patient with a PR had a negative FDG-PET/CT after the completion of CCRT and was considered as having a CR. It is well known that assessment of response to chemo-radiotherapy in patients with SCCHN is not accurate, since a number of them are considered by radiologists as having partial response, because of residual abnormalities in posttreatment CT scans. During the last few years FDG PET/CT scans had been increasingly used for initial staging and assessment of tumor response in SCCHN [49]. Several investigators have shown that FDG-PET/CT can more accurately predict the lack of residual disease both at the primary site and the neck (negative predictive value 100%, sensitivity 100% and specificity 96%) [50, 51] and it has therefore been considered to be a valuable clinical tool in the management of SCCHN.

The review of our clinical data showed that the treatment was feasible and that the compliance of the patients was satisfactory, since all except two completed RT. It is well known that most patients with SCCHN belong to low social-economic status, are alcoholic, heavy smokers, and bear serious co-morbidities. Furthermore, serious toxic sequelae of chemo-radiotherapy, such as dehydration, infections,

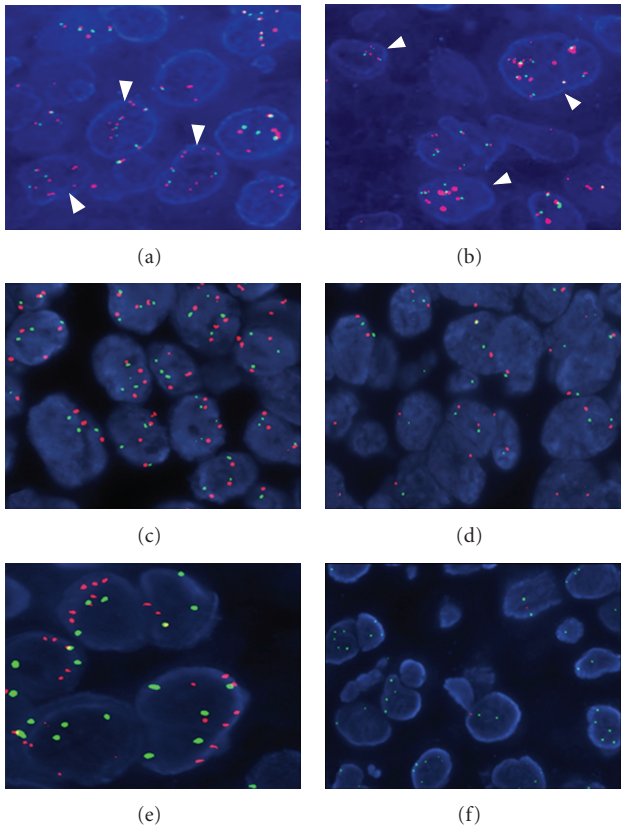

Figure 3: Fluorescence in situ hybridization with gene and centromeric specific probes. (a) and (b) Neoplastic nuclei showing polysomy of chromosome 7 (CEP7, green signals) and EGFR high level gene gain (red signals, arrowheads); (c) Neoplastic nuclei showing trisomy or polysomy of the MET gene (red signals) and SE7 (green signals); (d) Representative area from a case without genetic alterations. The majority of the neoplastic nuclei have 2 copies of the MET gene and SE7; (e) High polysomy of the D7S486 locus (red signals); (f) Deletion of the D7S486 gene locus in tumor cells, as defined by the presence of a single gene locus probe signal (red signals) and two CEP7 signals (green signals), or by the simultaneous lack of both of the gene locus signals and the presence of CEP7 signals (hemizygous and homozygous deletion, resp.).

malnutrition, and excessive weight loss may deteriorate their general heath status and contribute to fatal events. A high incidence of unexpected severe adverse events, including fatal events, was described by Pfister et al. [52] in a phase II study and was confirmed in our retrospective analysis of an unselected SCCHN population. These patients should therefore be closely monitored during CCRT and the immediate period following CCRT.

The discovery of predictive factors in treatments, such as RT concomitantly with cetuximab, is of paramount importance, since this regimen is emerging as the new standard for patients with SCCHN. Unfortunately, to date the identification of such molecular predictors remains elusive. In the present analysis, we evaluated potential associations of EGFR, MET, ERCC1, and MMP9 with response to CCRT.

Even though high EGFR protein expression has been reported to be predictive for increased tumor response in patients with SCCHN treated with conventional fractionated [9] or accelerated [53, 54] RT, this finding has not been confirmed in randomized studies in patients with recurrent and/or metastatic SCCHN treated with gefitinib [55] or cisplatin and cetuximab [56]. Contrary to what would be expected, patients with low to moderate EGFR protein expression demonstrated a higher response rate to the combination of cisplatin and cetuximab than those with high EGFR expression. In our retrospective analysis, we were not able to find a correlation between EGFR protein expression and response to CCRT.

We have also assessed EGFR gene copy number by FISH. We found that in most of the tumors EGFR polysomy but not amplification was evident; however, it was not correlated with response. These findings are in agreement with other trials, showing that the prevalence of EGFR amplification in SCCHN is low [57, 58] and that EGFR gene copy numbers are not correlated with tumor response in patients with recurrent/metastatic SCCHN, who nevertheless responded to the EGFR tyrosine kinase inhibitors (TKIs) erlotinib or gefitinib [59, 60]. It has been reported that in nonsmall cell lung cancer (NSCLC), mutations within the EGFR tyrosine kinase domain, mainly in exons 18, 19 and 21, confer sensitivity to TKIs [61]. However, such mutations are rare in SCCHN, ranging from 1% to 7% in caucasian/white and asian patients, respectively [60, 62, 63]. In a study of 134 SCCHN tumors, direct DNA sequencing could not identify any mutations [58]. In line with these findings, we screened 31 tumors for EGFR mutation in exons 18, 19 and 21 and were able to identify only one patient harboring an EGFR mutation. Apparently, due to the very low prevalence, EGFR mutations cannot be used as predictors of response to anti-EGFR treatment in SCCHN.

Clearly, further studies are needed to fully elucidate the mechanisms of sensitivity and resistance to cetuximab or EGFR TKIs. It is possible that other factors that are further downstream in the EGFR pathway and/or the interplay of the EGFR pathway with other activated pathways are more important than EGFR alone in modulating responses to anti-EGFR treatments.

Additionally, we assessed MET protein expression by IHC and gene copy number by FISH. To our knowledge, this is the first study attempting to correlate MET with response to concomitant RT with cisplatin and cetuximab. MET protein expression was noted in 14 of 33 of tumors studied and the gene was amplified in 5 of the patients. It appears that, as in the case of NSCLC [64, 65], MET gene amplification is an infrequent event in SCCHN as well and is not associated with responses to CCRT.

Interestingly, the present retrospective analysis is one of a few studies that have investigated a potential association between ERCC1 protein expression and response to CCRT in patients with SCCHN. It is noteworthy, that knowledge regarding the role of ERCC1 in SCCHN is very limited. Recently, Handra-Luca et al. [66] reported that low ERCC1 protein expression was associated with higher rates of tumor response (79% versus 56%, $P = .04$) and lower risk

of cancer-specific death (risk ratio 0.42, $P = .04$) in patients with SCCHN treated with cisplatin-based induction chemotherapy. However, this positive association was not confirmed in a similar study recently conducted by our group [67] and in the present analysis. The reasons for this discrepancy, regarding the predictive role of ERCC1, are not clear. Small sample size, differences in the treatment regimens, lack of standardization of the IHC methodology for assessing ERCC1 protein expression, and differences in patient characteristics, stage and tumor location maybe a few, but certainly not the only factors responsible for the conflicting results.

An important finding of the present retrospective analysis was that high MMP9 mRNA expression, assessed by kRT-PCR, was significantly associated with objective response. Positive correlations have been observed between MMP9 mRNA expression levels and metastatic spread of SCCHN tumors [68]. Overexpression by MMP9 may in part be regulated via nuclear factor kappa B (NF-kB) [69]. In addition, inflammatory processes induced by HPV infections could activate MMPs, which would in turn liberate EGFR ligands from the extracellular matrix, thereby promoting HNSCC tumor progression through increased EGFR signalling. It appears therefore, that MMP9 positive tumors could be particularly sensitive to EGFR inhibition. This notion is in complete agreement with our finding that high MMP9 mRNA expression is significantly associated with objective response to cetuximab containing chemotherapy. However, further analysis is needed in noncetuximab treated SCCHN patients, to evaluate whether MMP9 might be a "poor prognosis marker" turned onto an "improved response marker" by the addition of cetuximab to RT or CCRT.

Regarding four commonly studied polymorphic sites in ERCC1, ERCC2/XPD, and XRCC1, it was interesting to identify discordant tumor tissue/peripheral blood genotypes. This may be worthy considering when assessing polymorphisms as prognostic/predictive markers in oncology, since most such available data, including polymorphisms in excision repair genes, derive from determinations in peripheral blood (germline) DNA [28, 45, 70]. As indicated by the diminished presence of one allele with real time PCR, discordant genotypes in 3 out of 4 cases might correspond to loss of heterozygosity (LOH) of the corresponding alleles in the tumor. LOH can be inferred upon SNP-genotyping [71]. This finding needs further validation, while its biological impact, if any, is presently unknown, since LOH in ERCC1 and ERCC2/XPD has not been studied in SCCHN. LOH does not seem to be a common or major event in colorectal carcinogenesis [72]. Nevertheless, other than previously reported [28], we did not observe an association between tumor excision repair gene polymorphisms and patient outcome, possibly due to the small sample size, while the investigated polymorphism in ERCC1 was not related to the corresponding mRNA and protein expression.

Importantly this is the first report on the sensitivity of HPV-associated SCCHN to cetuximab-containing CCRT. There is a large body of molecular evidence suggesting that HPV (mainly HPV-16 and HPV-18) plays an important role in the pathogenesis of SCCHN and particularly of oropharyngeal tumors [73, 74]. HPV-16 is the most prevalent genotype in SCCHN, accounting for more than 90% of positive cases [75]. We assessed the presence of HPV by PCR, since this detection method is probably more sensitive than other methods, such as in situ hybridization [76]. The frequency of the presence of HPV, predominantly the HPV-16 genotype, in Greek patients with oropharyngeal or laryngeal cancer was 43% and 40%, respectively [77, 78]. Finally there are several lines of evidence suggesting that, HPV-associated SCCHN has a better prognosis than SCCHN in HPV-negative patients, possibly due to enhanced radio-sensitivity or the absence of field cancerization [79]. These data are in complete accordance with the findings of our study, in which exclusively HPV-16 DNA was detected in 6 (20%) of our patients. Notably, 4 of these patients were evaluable for response and all of them demonstrated a CR after the completion of CCRT. The observed high responsiveness of the HPV-positive patients might possibly be due to activation of MMP9. All 4 of the above patients exhibiting a CR had high MMP9 mRNA expression. Activation of MMP9 could liberate EGFR ligands from the extracellular matrix, thereby promoting HNSCC tumor progression through increased EGFR signalling. MMP9 positive tumors could therefore be, as discussed earlier, particularly sensitive to EGFR inhibition with cetuximab.

The p16 overexpression reported here was not associated with presence of HPV-16, in contrast to previous studies [80, 81]. As previously shown, p16 overexpression is not limited to HPV-16 positive cases [82], since a small number of cases with HPV negative genotype showed very high p16 expression. Furthermore, the finding of p16 overexpression in HPV-16 negative tumors may be the result of oncogene-driven cellular senescence or infection with other viruses that down-regulate retinoblastoma protein expression [38]. The above combined with the small number of positive cases could explain the lack of association between p16 and HPV-16 positivity reported in our study. However, other contributing factors, such as differences in antibody specificity and limitations of the immunohistochemical and PCR assays cannot be excluded.

As shown in patients with NSCLC, in colorectal or pancreatic cancer patients treated with anti-EGFR targeted treatments reviewed in [83], there is a subgroup of patients that is particularly benefited from such treatments, that is, those who develop the typical acne-like or maculopapular rash [57]. In the present analysis, rash of any grade was not found to be associated with response to CCRT. Likewise, lack of a correlation between the development of rash and response to cetuximab was reported in two other studies in patients with recurrent/metastatic SCCHN [84, 85]. However, in both of these studies, rash was a predictor for survival and in one of them [84] for time to progression (TTP), as well. Whether rash will be found to be significantly correlated with TTP or survival remains to be seen with longer followup. Notably, none of our patients discontinued CCRT due to severe RT-induced dermatitis, which has occasionally been reported in patients with SCCHN treated with RT concomitantly with cetuximab [86]. Nevertheless,

intensive medical treatment should be offered to these patients by experienced dermatologists, since in several cases there is a considerable risk for secondary skin infections.

In conclusion, it appears from the present retrospective analysis that, CCRT is feasible in patients with locally advanced SCCHN. However, extremely close monitoring is required for patients with serious co-morbidities, during CCRT and the 3-month posttreatment period, because such patients are at high-risk for dying from nontreatment related causes. The status of all the genes evaluated in this analysis, except MMP9, was not of predictive value to CCRT. High MMP9 mRNA expression, assessed by kRT-PCR, was found to be significantly associated with objective response. It appears that MMP9 might be of predictive value in SCCHN patients treated with cetuximab. However, it has to be kept in mind that, given the retrospective nature of the present analysis and the relatively small number patients, a selection bias cannot be excluded. Therefore, our findings should by no means be considered as definitive, but rather as hypothesis generating for future prospective trials.

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Review Article

Biomolecular Markers in Cancer of the Tongue

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The incidence of tongue cancer is increasing worldwide, and its aggressiveness remains high regardless of treatment. Genetic changes and the expression of abnormal proteins have been frequently reported in the case of head and neck cancers, but the little information that has been published concerning tongue tumours is often contradictory. This review will concentrate on the immunohistochemical expression of biomolecular markers and their relationships with clinical behaviour and prognosis. Most of these proteins are associated with nodal stage, tumour progression and metastases, but there is still controversy concerning their impact on disease-free and overall survival, and treatment response. More extensive clinical studies are needed to identify the patterns of molecular alterations and the most reliable predictors in order to develop tailored anti-tumour strategies based on the targeting of hypoxia markers, vascular and lymphangiogenic factors, epidermal growth factor receptors, intracytoplasmatic signalling and apoptosis.

1. Introduction

Oral cancer is the most frequent cancer affecting the cervicofacial district, causing about 8000 deaths every year in the United States [1, 2], and cancer of the tongue accounts for approximately 30% of all oral cancers. The most frequent histological type is squamous cell carcinoma (SCC) which mainly affects men in the sixth decade of life [3–6]. The incidence of tongue cancer increased from 1973 to 2001 at the same rhythm as tonsil cancer, and about 10,000 new cases were recorded in the United States in 2007 [7]. According to the Scandinavian registries, the trend towards an increasing incidence only excludes women 65–79 years old [8].

Unlike other studies, survival analyses have demonstrated that survival rates are better among young adults than older patients [9–11], with a 5-year crude survival rate of 65% (95% CI 59–71%) against 45% (95% CI 43–48%) in subjects aged 40–64 years and 33% (95% CI 31–35%) in those aged 65–79 years. Base of the tongue cancer has a poorer prognosis than mobile tongue cancer; according to US National Cancer Database findings, the 5- and 10-year disease-specific survival (DSS) rates for base of the tongue tumours are, respectively, 40.3% and 29.4%, and overall survival (OS) rates are, respectively, 27.8% and 12.2%. An older age (>65 years), low economic income, and advanced stage are independently associated with lower DSS, which is 64.7% for stage I and 30.0% for stage IV [12].

Smoking and alcohol consumption are recognised risk factors for tongue cancer, but are frequently not involved in the case of younger patients [13, 14]. Head and neck cancer (HNC) is heralded by some changes in genetic and epigenetic patterns, with gene inactivation or amplification being the main alterations that can lead to derangements in the molecular pathways involved in regulating cell behaviour [15–23]. Al-Moustafa et al. [24] found that genes encoding for growth factors and cell structure were overexpressed in 0.7% of their cases, and those involved in cell motility and apoptosis were underexpressed in 1%: more specifically, at protein level, Wnt-5a, fibronectin and N-cadherin were upregulated, whereas E-cadherin, claudin-7, the catenins, and connexin 31.1 were downregulated. However, the specific relationships between genes and proteins, the final alteration that may imprint the neoplastic clone and its development, have not yet been ascertained.

Ongoing biological research is attempting to establish whether these proteins can be considered biomarkers that could guide therapeutic choices. SCC of the tongue is characterised by an unpredictable course as some patients with early lesions may develop local recurrence and regional

metastases despite adequate surgery, and so the identification of prognostic markers would enable clinicians to target patients who may benefit from a specifically tailored treatment strategy.

This review will concentrate on the most recent advances in the rapidly evolving field of biomarker research in this tumour type.

2. Viral Infections

Two viruses are commonly associated with HNC. The integration of Epstein-Barr virus (EBV) into mucosal cells is the most important pathogenetic factor in the development of nasopharyngeal carcinoma, which is endemic in geographical areas such as the Middle East and South-East Asia, the Arctic area, and Northern Africa [25–30]. EBV is transmitted through saliva, but its cell source is controversial, although putative reservoirs include the oral epithelium and salivary glands. Frangou et al. [31] observed EBV replication in 1.3% of tongue mucosal samples, but no latent infection was found, and EBV infection was not detected in the tongue carcinomas. It is, therefore, reasonable to argue that EBV replication occurs infrequently in tongue epithelial cells, and that EBV is probably not involved in the pathogenesis of tongue cancer.

Oropharyngeal cancer is closely associated with human papilloma virus (HPV), whose growing incidence in young adults accounts for a proportional increase in the incidence of tonsil cancer. Subtypes 16 and 18 are commonly involved in the pathogenesis of oropharyngeal carcinoma [32], and are suspected of increasing the risk of tongue cancer by 3–5 times [33–35]. The prevalence of HPV in tongue cancer varies considerably but, when it is present, the median copy numbers of E6 DNA in nontonsillar specimens is approximately 80,000 times lower than in tonsillar specimens [36]. Kantola et al. [37] found that none of 105 mobile tongue cancer patients harboured HPV, and two studies have reported HPV frequencies in oral tongue cancer of 2.3% and 1.96%, thus confirming its small etiopathogenetic role, at least in the mobile portion of the tongue [38, 39]. Liang et al. [39] reported a higher incidence of HPV in base of the tongue cancer (51.5%), and Dahlgren et al. [38] stated that mobile and base of the tongue SCC are different diseases, with HPV being present in 40% of the patients affected by the latter and, as has been observed in the case of tonsillar cancer, the presence of HPV in base of the tongue cancer positively influenced survival ($P = .0159$). Interestingly, the HPV-positive base of the tongue cancer patients still had an advantage over those who were HPV-negative in terms of 5-year DSS ($P = .0362$), whereas tumour stage at the time of diagnosis no longer had an impact ($P = .0863$) [38]. The presence of HPV is, therefore, clearly associated with a better prognosis, and outweighs the predictive value of disease stage.

3. Hypoxia: Follow-Up (a)

Deranged vascular architecture and necrotic changes within neoplastic tissue are responsible for tumour hypoxia, which is associated with a poor outcome in HNC patients [40, 41]. Poorly oxygenated tumours have a poor prognosis as they may be resistant to radio- and chemotherapy, and favour malignant progression [42–45]. Tumour cells harbouring genetic alterations survive longer than normal cells in a hypoxic environment and are more likely to transmit genomic instability as a consequence of selective pressure, after which the neoplastic clone can easily grow, increase angiogenesis and motility, and finally spread through the lymphatic system or blood vessels [45–48].

It is thought that hypoxia upregulates some highly expressed proteins that are easily recognised immunohistochemically and may act as endogenous biomarkers in HNC [45]. Hypoxia-inducible factor 1a (HIF-1α) is a partner in a dimer that acts as a transcription factor by binding a specific DNA sequence and activating gene transcription. Under hypoxic conditions, HIF-1α levels increase and activate genes coding for growth and angiogenesis factors, as well as glycolytic enzymes. It has been demonstrated that such genes, particularly, carbonic anhydrase IX (CA-9), vascular endothelial growth factor (VEGF), and erythropoietin (EPO), are highly expressed in HNC [49, 50] but transferring immunohistochemical results to the clinical setting in order to identify their real prognostic value and impact on clinical practice is difficult.

Roh et al. [51], retrospectively, studied T2 tongue cancer using monoclonal antibodies against HIF-1α, HIF-2α, CA-9, the glucose transporter (GLUT-1) and EPO receptors (EPORs), and found that only GLUT-1 was related to nodal stage and could, therefore, be used as a potential predictor of nodal metastates. Univariate analysis showed that HIF-1α and EPOR expression significantly correlated with DSS ($P < .05$), but not with other clinicopathological variables such as tumour thickness, nodal involvement, and resection margin status, and multivariate analysis showed that only EPOR expression remained a significant predictor of DSS ($P = .030$). However, the small number of patients and the fact that they all had T2 tongue cancer makes it difficult to draw any definite conclusions.

The role of exogenous hypoxia markers is beyond the scope of this review, but it is worth mentioning the role of pimonidazole, a marker of exogenous hypoxia in human SCC of the cervix and head and neck [52–54]. The pimonidazole binding assay is a direct indicator of tumour hypoxia, which has been proved to be significantly associated with locoregional control and disease-free survival (DFS) [55]. Patients with hypoxic tumors show a worse initial response to treatment and have more locoregional recurrences during the first 15 months of followup, thus suggesting that their worse outcome mainly depends on early locoregional failures.

4. VEGF

Vascular endothelial growth factors (VEGFs) are a family of proteins with specific angiogenic properties that increase vessel permeability, and endothelial cell growth, proliferation,

migration, and differentiation [56, 57]. VEGF-A/vascular permeability factor and VEGF-C have been recently recognised as lymphangiogenic/angiogenic factors that induce lymph and blood vessel hyperplasia and facilitate tumour progression and metastases [58, 59]. VEGF-A consists of four isoforms with a different molecular mass (121, 165, 189, and 206 amino acids) and different biological activity [60]. VEGF-C is structurally very similar to VEGF-D, and both of these and their major receptors (VEGFR-2 and VEGFR-3) are expressed in many cancer cells and may regulate lymphangiogenesis by facilitating the signalling network between endothelial and cancer cells [61–66]. A significant correlation has been demonstrated between VEGF-A and VEGF-C expression and lymph node metastases [67, 68], and patients overexpressing these two factors tend to show decreased survival. On the contrary, VEGF-D has been found to be underexpressed in HNC cells, and it is thought that it has an antagonistic effect on other VEGFs and may play a role in the late process of neoangiogenesis stabilisation.

Kishimoto et al. [69] investigated the association between VEGF-C expression and regional lymph node metastases in oral squamous cell carcinoma (OSCC) by examining its immunohistochemical expression in biopsy specimens obtained from 62 patients. In the early stages (T1 and T2), VEGF-C expression closely correlated with lymph node metastases ($P < .001$), but there was no significant correlation in the advanced stages (T3 and T4). These findings indicate that VEGF-C expression in biopsy specimens could be used as a reliable predictor of regional lymph node metastases, particularly in early OSCC, and may become an important factor when choosing the most appropriate treatment.

The limited data concerning tongue cancer are conflicting. Kim et al. studied the expression of VEGF and metalloproteinase-2 and -9 in 38 oral tongue cancer patients, and found a significant correlation between VEGF expression and the extent of tumour invasion ($P = .002$). Furthermore, the tumour-free survival of the VEGF-positive patients was significantly worse than that of the VEGF-negative patients ($P = .019$) [70]. However, Faustino et al. did not find a a similar correlation in early stage OSCC: 60 out of 87 patients (68.9%) were affected by tongue cancer and it was found that VEGF-C expression did not predict occult lymph node metastases in T1-T2N0 tumours [71]. In another study, Cho et al. found high VEGF expression in 20 out of 33 specimens of resected tongue cancer (60.6%), but no correlation between it and recurrence ($P = .33$) [72]; the expressions of maspin, an inhibitor of angiogenesis and tumour suppressant [73, 74], and mutant-type p53 were also evaluated but did not correlate with recurrent disease. We studied 56 patients undergoing radical surgery for tongue cancer and found that the expression of VEGF-C and its receptor VEGFR-2 correlated with DFS but not OS (unpublished data). Although there is some evidence that VEGF-C plays a role in causing more aggressive tongue cancer, further studies of larger patient series are needed.

5. Tight Junctional Proteins

There are three main types of intercellular junctions: tight, adherens, and gap junctions. The most apical components of the junctional complexes are tight junctions (TJs), which play a major role as paracellular barriers to the transport of ions, water, and proteins, and are also believed to be involved in the signalling cascades controlling cell growth and differentiation. Together with desmosomes, they form part of cell-to-cell adhesion apparatuses, and strongly regulate the invasion of cancer cells [75–81]. TJs are involved in the neoplastic process because they couple the extracellular milieu to intracellular signalling pathways and the cytoskeleton [82]. Deranged TJ permeability may increase the diffusion of nutrients and other factors that promote tumour growth and/or survival [83].

Claudins and occludin are tight junctional proteins whose expression has been studied in various tumour types [84], and tentatively correlated with tumour proliferation as a result of a mechanism involving the activity of matrix metalloproteinases [85]. Claudin 7 is known to be underexpressed in HNC [24], but only Bello et al. have described its expression in tongue cancer. They analysed the distribution of claudins (1, 4, 5, and 7) and occludin in 97 patients with superficial and invasive front of tongue cancer, and found that claudins 1 and 7 were strongly expressed, claudin 4 moderately expressed, and claudin 5 the least expressed; occludin staining was irrelevant. Cause-specific survival analysis showed that, in comparison with intermediate immunoreactivity, high and low claudin 7 immunoreactivity tended to be associated with decreased survival [86]. The authors suggested that claudin 7 levels could be used for prognostic purposes, but the subjective nature of the immunohistochemical evaluation requires caution.

6. ErbB2-Ki-67: Tyrosine-Kinase (b)

ErbB2 (HER-2/neu) is a tyrosine kinase transmembrane receptor that belongs to the family of epidermal growth factor receptors (EGFRs), like ErbB1/HER-1, ErbB3/HER-3, and ErbB4/HER-4. It can be activated by means of heterodimerisation with the other members of the family, and is involved in cell proliferation and differentiation. It also plays a major role in tumour invasion via mitogen-activated protein kinase (MAPK) or phosphatidylinositol 3-kinase-(PI3K) AKT-activated pathways [87, 88]. Many (but not all) authors have demonstrated ErbB2 overexpression and gene amplification in oral SCC, and that they are associated with early recurrence, local, and distant metastases, or shorter survival [89–94]. Fatty acid synthase (FAS) is located in the cytosol and is responsible for the endogenous synthesis of saturated long-chain fatty acids. Its expression is upregulated in a number of human epithelial malignancies, including OSCC [95–100]. It has been shown that the overexpression of human ErbB2 in mouse fibroblasts stimulates FAS protein expression through a PI3K-dependent pathway. FAS is essential for cell proliferation, and its specific inhibition

reduces cell growth, blocks DNA replication, and promotes apoptosis in various cancer cell lines [101, 102].

Silva et al. have shown that intracytoplasmatic ErbB2 expression correlates with the 10-year survival of tongue cancer patients, which was 24.1% in the case of high expression, and 53.4% in the case of weak or negative expression (log-rank test, $P = .0096$). The proliferation index, evaluated by means of Ki-67, significantly predicted both OS (log-rank test, $P = .0001$) and DFS (log-rank test, $P = .0047$); however, it did not correlate with the cell surface coexpression of FAS and ErbB2, thus indicating a favourable prognosis in both cases [103]. The same group has also studied the microscopic characteristics of tongue cancer, and found that histological grade ($P < .05$), lymphatic permeation ($P < .001$), perineural infiltration ($P < .05$), and nodal metastases ($P < .02$) are all associated with FAS status. High FAS expression correlates with aggressive histological features and may be important for tumour progression [104]. Different results have been obtained in tongue cancer using the Hercept test, which demonstrated that the expression of Erb B2 does not correlate with clinicopathological parameters and is not useful in treatment decision making [105].

7. p-53

The p53 tumour suppressor gene is located on the short arm of human chromosome 17 and encodes for a phosphoprotein that has dual activity on normal cells: it inhibits cell proliferation by arresting it at the G1-phase after DNA damage, and it induces apoptosis after genotoxic damage. It is thought that both mechanisms also suppress tumour growth [106].

The expression of p53 is strikingly important in the response to irradiation or cytotoxic drugs, and it has been shown that an alteration in the p53 gene may cause treatment failure in cancer patients as it prevents the triggering of the apoptotic pathway [107, 108]. Many environmental factors can alter p53 function, as has been demonstrated in the case of cigarette smoking and asbestos exposure in lung cancer [109, 110]. Exogenous factors can easily and directly act on cells in the oral cavity, but the role of cigarette smoking in deregulating p53 protein is still unclear [111, 112].

Atula et al. [113] have studied p53 mutations and protein expression in tongue cancer, and found mutations in 54% of the samples by means of single-stranded conformation polymorphism (SSCP) analysis, which correlated with tumour size (41% in T1-2 versus 90% in T3-4; Fisher's exact test, $P < .01$) and grading (75% of grade 2-3 versus 32% of low-grade cancers; chi-squared test, $P < .01$). Although experimental models have demonstrated that p53 mutations precede and favour the appearance of metastases [114, 115], this study found no correlation between metastases and p53 mutations or protein expression, a finding that can be explained on the grounds of the progressive accumulation of mutations during the course of cancer or viewed as an early event contributing to more aggressive behaviour. Tongue cancer develops as a sum of several environmental and genetic factors affecting the same cell, thus leading to its progressive malignant transformation and metastatic dissemination [116].

The expression of p53 in oral leukoplakia is higher than in cancer of the tongue and should probably be considered an early event in tumour progression [117]. Nagler et al. studied 116 patients with tongue cancer, and found the 5-year probability of OS was 55%, and better for mobile tongue than base of the tongue cancer (70% versus 32%, $P = .0008$). Immunohistological analysis of p53, the antiapoptotic protein Bcl-2 and c-erbB-2, and an assessment of the rate of apoptosis by means of terminal dUTP nick-end-labelling (TUNEL), in 55 specimens, revealed a significant correlation between p53 and TUNEL staining, but the link with prognosis needs to be studied further [118]. It has also been found that p53 positivity is not a reliable means of selecting patients for elective neck dissection in the management of N0 oral tongue cancer [119].

8. Osteopontin

Osteopontin (OPN) is a calcium binding protein that binds alpha rather than beta integrin and CD44 receptors, and activates intracellular signalling pathways associated with cell adhesion and migration [120–122]. It is expressed and secreted by many kinds of cancers, and has been associated with tumour progression and invasion [123–126]. It can also be induced by VEGF and is involved in vessel angiogenesis and endothelial cell survival [127–129]. Matsuzaki et al. [130] failed to demonstrate a correlation between OPN expression and lymphatic metastases and survival in T1-4 tongue cancer, but OPN expression has also been studied in T1-2 tongue cancer using a different means of immunohistochemical evaluation [131]. Thirty out of 94 patients (31.9%) expressed OPN and this significantly correlated with a more advanced T stage (T2 versus T1) ($P = .004$), positive lymph nodes ($P < .001$), the presence of tumor necrosis ($P = .016$), and greater tumour thickness ($P < .001$). Interestingly, the patients expressing OPN showed a significantly lower DFS rate (63.4% versus 92.8%; log-rank test, $P < .001$).

Using the method developed by Matsuzaki et al. [130], OPN expression still significantly related to the expression of VEGF and CD105 (both $P < .001$), tumour invasion depth ($P = .001$), and regional nodal metastases ($P < .001$), and Chien et al. [131] confirmed the relationship between OPN and VEGF, thus suggesting their importance in the development of new vessels in early tongue cancer. Hypoxia can also contribute to the increased expression of OPN and the activation of other important angiogenetic factors. These data argue in favour of a role of OPN in predicting a poor prognosis, and, therefore, possibly in influencing the decision to adopt more aggressive therapy.

9. Survivin: OSCC (c)

A large number of cancer cells acquire resistance to treatment by evading apoptosis, and a family of inhibitors of apoptosis proteins (IAPs) can interfere with programmed

cell death [132, 133]. The ultimate effectors of the apoptotic machinery are the intracellular proteases called caspases [134]. Caspase-8 and -9 trigger the activation of more caspases that execute the cell death program. It is believed that survivin, which is encoded by the gene BIRC5, blocks caspase-mediated death by forming a stable complex with X-IAP, which has a synergistic inhibitory action on apoptosis [135]. Survivin is highly expressed in many cancer types and has been associated with a more aggressive phenotype and poor outcome in oral SCC [136]. In their study of OSCC in Taiwan, Lin et al. found no significant correlation between survivin expression and patient age, gender, oral habits, cancer location, or TNM status, but the patients with high survivin expression, an advanced stage, a larger tumour size or positive lymph node metastases had a significantly shorter OS than the others ($P = .014$, .012, .005, and .011, log-rank test) [137]. Survivin protein expression may thus be considered an important early event in oral carcinogenesis and predicts an unfavourable prognosis for OSCC. On the other hand, Freier et al. found no statistical difference between tumours with a gain in *BIRC5* gene copy number and those with a balanced *BIRC5* locus ($P > .05$) in terms of the prevalence of high survivin expression, and high survivin expression predicted longer OS in a subgroup of patients with advanced tumours treated by radiotherapy [138]. The authors concluded that the additional *BIRC5* copies were probably biologically inactive, that another distinct molecular mechanism might be responsible for high survivin expression in OSCC, and that survivin might be used to define better the patients who may benefit from radiation therapy.

The difference in these results may also have been due to the evaluation system used, because the latter study used a score that took into account both nuclear and cytoplasmic cells. A study of nuclear staining alone in breast and colon cancer found a correlation with better survival, and so it is reasonable to imagine that Freier's finding of an impact on survival was due to the nuclear expression of survivin.

Unfortunately all of these studies involved OSCC series that included only a minority of patients affected by tongue cancer. In our own recent series of tongue cancer patients, immunohistochemical analysis of survivin did not correlate with DFS or OS (unpublished data).

10. EGFR

Epidermal growth factor receptor (EGFR) is a 170-kDa transmembrane glycoprotein whose gene is located on chromosome 7p12. It is a member of the family of tyrosine kinase (TK) growth factor receptors, a group of proteins whose aberrant activity plays a key role in cell growth and neoplastic progression [139, 140]. A number of extracellular growth factor ligands, including epidermal growth factor (EGF) and transforming growth factor alpha (TGF-α), bind to EGFR and thus lead to the downstream activation of ras, which ultimately leads to cell cycle progression, decreased apoptosis, as well as increased angiogenesis and metastatic properties [141, 142]. EGFR and its ligand TGF-α are overexpressed in nearly all HNC [143, 144], and its expression is typically associated with greater radio- chemoresistance and shorter DFS and OS [145, 146]. EGFR expression has been reported to be 29% and 50% in hypopharyngeal and oral cavity cancers [147], and ranging from 42% to 80% in other types of HNC [148, 149].

Few data are available concerning the expression and prognostic value of EGFR in tongue cancer. EGFR mutations are not frequent (they have been found in 14% of investigated cases) and, unlike in nonsmall cell lung cancer (NSCLC), they do not correlate with prognosis [150]. Treatment with tyrosine kinase inhibitors is less effective in HNC than NSCLC [151, 152] although the types of mutations are very similar [153]. Mahmoud et al. studied HNC specimens for EGFR mutations and expression in a Japanese population and found a silent mutation in only one case, thus reflecting the low incidence reported in previous studies, whereas overexpression (+2, +3) was found in 68% of the tumours. EGFR overexpression was significantly associated with poor tumor differentiation ($P = .02$) and a positive nodal stage ($P = .032$) [154].

As in the case of Western patients, mutations are rare in Japanese HNC [155, 156], and protein overexpression rather than mutation might be responsible for activating the EGFR pathway. Ulanovski et al. studied 27 patients who underwent surgery for SCC of the tongue. EGFR and erb-B2 were expressed in 34% and 17% of the specimens, but the authors could not demonstrate any association between EGFR expression or erbB2, and tumour depth, lymph node status, extracapsular invasion, recurrence, or survival [157].

11. Conclusions

Cancer of the tongue is frequent and has a poor prognosis, with a 5-year survival rate of less than 50%. Treatments should be individualized on the basis of the biological characteristics of the tumour with the aim of improving locoregional control, preventing distant metastases, and lengthening survival. The role of EBV and HPV is very slight, although the latter may indicate a better prognosis. Among hypoxia markers, only the expression of EPOR and pimonidazole correlates with locoregional control and DFS, but these findings are based on a small number of patients. VEGF, tight junction proteins, and p53 expression hardly correlate with poor prognostic features, and the survivin findings are also controversial although it may be useful to select a subpopulation of patients who may benefit from radiation therapy. The intracytoplasmic expression of erbB2 and the ki-67 proliferation index are associated with OS, and FAS expression is related to aggressive histological features. OPN is a VEGF-inducible factor, that is, overexpressed in cases of aggressive cell behaviour, and is associated with decreased DFS, at least in T1-2 tumours. EGFR mutations are seldom found in tongue cancer and do not play a significant prognostic role; likewise, EGFR overexpression correlates with nodal stage but not DFS or OS.

In this era of targeted therapies tailored on the biological characteristics of tumours, the results, as far as tongue cancer is concerned, are still poor and conflicting, and new insights are eagerly expected with the aim of offering the best possible treatment to each patient.

Antiangiogenetics, anti-EGFR, tyrosine-kinase inhibitors and proapoptotics are all factors deserving further evaluation in order to improve outcomes in patients affected by cancer of the tongue.

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Research Article

Transcriptional Activity of Human Epidermal Growth Factor Receptor Family and Angiogenesis Effectors in Locoregionally Recurrent Head and Neck Squamous Cell Carcinoma and Correlation with Patient Outcome

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Locoregional recurrence is the most common failure pattern in patients with head and neck squamous cell carcinoma (HNSCC). We retrospectively identified 41 HNSCC patients with locoregional relapse and used kinetic reverse transcription-polymerase chain reaction (kRT-PCR) in order to study fresh-frozen tumour messenger RNA (mRNA) levels of the Human Epidermal growth factor family members HER1-4, the Vascular Endothelial Growth Factors (VEGFs) A, B, C, D, and their receptors VEGFR1, 2, 3. High VEGF-C and VEGFR3 tumour mRNA expression correlated with relapse beyond the primary locus (neck nodes or soft tissues, $P < .05$). Tumours with regional nodal involvement at diagnosis more often exhibited high transcriptional activity of VEGFR1 and VEGFR3 at the time of relapse ($P < .05$). At a median follow-up of 52 months from the time of locoregional recurrence, patients with high VEGF-C tumours at relapse had significantly poorer postrelapse progression-free survival (R-PFS, 5 versus 47 months, log-rank $P = .052$) and a trend for inferior postrelapse overall survival (R-OS, 22 versus 44 months, log-rank $P = .076$) in comparison to low VEGF-C tumours. Similar association with dismal outcome was seen for its receptor, VEGFR3 tumoural mRNA levels (log-rank $P = .060$). In contrast, suppressed tumour transcription of VEGF-D was associated with poorer post-relapse survival, though statistical significance was not reached. Active transcription of the VEGF-C/VEGFR3 axis in recurrent HNSCC is associated with failure at neck soft tissues/lymph nodes and inferior survival post-relapse.

1. Introduction

Locoregional recurrence is the most common pattern of failure after definitive treatment of head and neck squamous cell carcinoma (HNSCC), despite increasing use of combined modality approaches incorporating chemotherapy, radiotherapy, and surgery as initial management of patients with locally advanced tumours [1]. Failure to achieve control of locoregional disease increases the likelihood of distant metastases and compromises patient survival and quality of life. Even in patients succumbing to distant metastatic disease, uncontrolled cancer at the primary site or neck is seen in 90% of the cases [2]. In several large series and multi-institutional trials, the rate of locoregional relapse ranged from 20% to 57%, the most important predictors for failure being involved resection margins, regional nodal metastases, advanced T stage, high grade, neurogenic/vessel invasion, and p53 gene mutations [3]. In the occurrence of

isolated locoregional recurrence, long-term disease control is achieved in a minority of patients (10%–25%), namely, those able to undergo surgical salvage and/or re-irradiation. Clinicopathological parameters that predict outcome of patients with HNSCC locoregional recurrence have been reported in a number of studies and included time interval from diagnosis to relapse, bulk, site, and resectability of recurrence, ability to re-irradiate at doses >60 Gy, and performance status [4, 5]. However, no data are available on molecular tumour biomarkers of potential prognostic/predictive significance for the outcome of patients with locoregionally recurrent HNSCC. Several investigators have reported overexpression of Human Epidermal growth factor Receptor (HER) family members and active angiogenic activity in HNSCC, with important implications since therapeutic compounds targeting these cellular pathways are available. In view of the above, we studied the tumour transcriptional activity of HER and vascular endothelial growth factor (VEGF/VEGFR) pathways at the occurrence of locoregional recurrence, retrospectively examined associations with clinicopathological characteristics and analyzed their utility for predicting patient outcome following relapse.

2. Patients and Methods

Patients with localized stage I-III HNSCC managed between January 2002 and August 2004 at the ENT Department of the Aristotle University of Thessaloniki with potentially curative surgery and/or radical external beam irradiation and subsequently experiencing isolated locoregional recurrence were retrospectively identified. Isolated locoregional recurrence was defined as one occurring in the primary site, neck nodes or neck soft tissues in the absence of distant metastases. This constituted the criterion for patient identification and for the study of HER/VEGF pathways in fresh tumour tissue biopsies obtained at the time of locoregional recurrence and snap-frozen at $-80^{\circ}\mathrm{C}$. A waiver of consent for the use of biologic material was provided by the Bioethics Committee of the Aristotle University of Thessaloniki.

Intact RNA of high quality as determined by analysis of the housekeeping gene RPL37A was isolated from 41 fresh-frozen tumour tissue samples with tumour cellularity of at least 70%. Approximately 50 mg of fresh-frozen tumor tissue were crushed in liquid nitrogen. RLT-Buffer (QIAGEN, Hilden, Germany) was added and the homogenate was centrifuged through a QIAshredder column (QIAGEN). From the eluate, total RNA was isolated using the RNeasy Kit (QIAGEN) according to the manufacturer's instructions. RNA yield was determined by UV absorbance, and RNA quality was assessed by analysis of ribosomal RNA band integrity on an Agilent 2100 Bioanalyzer RNA 6000 LabChip kit (Agilent Technologies, Palo Alto, CA). Kinetic reverse transcription-polymerase chain reaction (kRT-PCR) was applied for the assessment of messenger RNA (mRNA) expression of HER1 (EGFR), HER2, HER3, HER4, VEGF-A (all isoforms), VEGF-B, VEGF-C, VEGF-D, VEGFR1 (FLT1), VEGFR2 (KDR), and VEGFR3 (FLT4) using the following TaqMan-based primer/probe sets:

VEGF-A Probe CACCATGCAGATTATGCGGATCAA-ACCT
Forward Primer GCCCACTGAGGAGTCCAACA
Reverse Primer TCCTATGTGCTGGCCTTGGT
VEGF-B Probe CACATCTATCCATGACACCACTTTCCT-CTGG
Forward Primer TGGCAGGTAGCGCGAGTAT
Reverse Primer CCCTGTCTCCCAGCCTGAT
VEGF-C Probe TTGAGTCATCTCCAGCATCCGAGGAAA
Forward Primer CCACAGATGTCATGGAATCCAT
Reverse Primer TGCCTGGCTCAGGAAGATTT
VEGF-D Probe TGACATTGAAACACTAAAAGTTATAGA-TGAAGAATGGCA
Forward Primer ACTAGGTTTGCGGCAACTTTCT
Reverse Primer TCTCTAGGGCTGCACTGAGTTCT
FLT1 Probe TGCTGTCGCCCTGGTAGTCATCAAACA
Forward Primer CATGGGAGAGGCCAACAGA
Reverse Primer AACCTTTGAAGAACTTTTACCGAATG
KDR Probe TCTTGGCATCGCGAAAGTGTATCCACA
Forward Primer TTCCAAGTGGCTAAGGGCAT
Reverse Primer CGTGCCGCCAGGTCC
FLT4 Probe TGCCTGCTTCCCTGGGTAGTCCC
Forward Primer GCACCCACTTACCCCGC
Reverse Primer GAGTTTAACTCAGGTGTCACCTTTGA

Forty cycles of amplification were applied, and the cycle threshold (CT) values of the target genes were identified. CT values were normalized by subtracting the CT value of the housekeeping gene RPL37A from the CT value of the target gene (ΔCT). RNA results were then reported as 40-ΔCT values, which would correlate proportionally to the mRNA expression level of the target gene. Human reference total RNA pooled from ten human cell lines (Stratagene, La Jolla, CA) was used as a positive control. RNA-free DNA extracted from tumor tissues was used as a negative control.

We sought to study the distribution of biomarker values, the correlation of biomarkers to various clinicopathological parameters at first diagnosis and at the time of recurrence, the association of biomarkers with time from diagnosis to relapse (relapse-free interval, RFI), and their predictive significance for relapse-related progression-free survival (R-PFS) and overall survival (R-OS). RFI was measured from initial diagnosis until the time of isolated locoregional recurrence, R-PFS from the time of isolated locoregional relapse until verified disease progression, and death or last contact and R-OS from locoregional relapse until death from any cause or date of last contact. Disease progression (R-PFS event) was considered to be an increase in tumour maximal diameter of >20% or appearance of new lesions despite salvage therapy. Both R-OS and R-PFS were estimated using the Kaplan-Meier product-limit method, and comparisons were performed using the log-rank test.

Categorical data were presented as counts and corresponding percentages, while the continuous variables were summarized using the medians and ranges. Distributional studies of gene mRNA expression values confirmed the absence of natural cut-offs in frequency histograms, while the small sample size further supported the use of the median as the optimal cut-off. Gene mRNA expression was considered low or negative when below the median of all

measured mRNA values and high or positive when above the median and was used as a categorical variable in the analysis. Comparisons between mRNA expression and categorical variables were performed using the Fisher's exact test. The level of significance for all statistical tests was $\alpha = 0.05$. Analysis was conducted using the SPPS for Windows, version 15.

3. Results

3.1. Clinicopathological Characteristics. Forty-one male patients, mostly heavy smokers and consumers of alcohol, initially presented at a median age of 65 with hoarseness and dysphagia. Diagnostic work-up led to diagnosis of squamous cell carcinoma of the larynx predominantly (90% of cases), mostly stage T1-3 (88% of cases), more often node-negative (85%), and moderately-well to well differentiated (61%). Initial management consisted of surgical resection of the tumour by either local excision (24%), segmental (19%), or total (24%) laryngectomy, whereas in one-third of the cases only a bioptic procedure was done and radical external beam radiotherapy was administered. Adjuvant chemotherapy was not administered, with the exception of one patient. Locoregional relapse occurred after a median of 15 months in the primary site (66%), neck lymph nodes (15%), or neck soft tissues (19%) and was managed by means of surgical resection (65% of patients) and/or irradiation (24%) and chemotherapy (24%). At the time of relapse, 46% of patients were managed with surgery only and 19% with resection followed by irradiation or chemotherapy. Among the 24% of patients who received radiotherapy at relapse, 17% had external beam radiotherapy only and 7% concurrent chemoradiation. No patients received re-irradiation. Among five patients who had chemotherapy administered and available data, three were treated with paclitaxel/liposomal doxorubicin, one with paclitaxel/gemcitabine, and one with weekly methotrexate. Clinicopathological characteristics at first diagnosis and at locoregional relapse are summarised in Table 1.

3.2. Association of Biomarkers with Clinicopathological Parameters. High versus low mRNA expression of HER1-4 genes, VEGF-A, B, C, D genes, and receptors VEGFR1, R2, R3 were examined for associations with alcohol consumption, tobacco consumption, age, and nodal status at initial diagnosis, relapse-free interval, site, size, and grade at relapse.

High VEGF-C transcription correlated significantly with tropism for relapse beyond the primary site: 50% of relapsing patients with high tumoural VEGF-C mRNA expression relapsed in lymph nodes or soft tissues versus only 15% of those who harboured tumours with low VEGF-C (test, $P = .009$). The same association was observed for tumoural transcription of VEGFR3 and the receptor of VEGF-C: tumours with high mRNA expression of VEGFR3 relapsed in neck nodes or soft tissues in 53% of the recurrent cases, while those with low expression relapsed in only 10% ($P = .017$). Tumoural VEGF-C/VEGFR3 mRNA expression may be a marker of predilection for relapse in regional lymph nodes/soft tissues rather than the primary site.

Tumours with regional nodal involvement at diagnosis more often exhibited high transcriptional activity of VEGFR1 or VEGFR3 at the time of relapse (test, $P < .05$). Among tumours profiled with high mRNA expression of VEGFR1 or VEGFR3 at relapse, regional nodal involvement had occurred in approximately 20% of the cases at initial presentation. In sharp contrast, no nodal metastases had been present at initial diagnosis in cases where tumoural VEGFR1 or VEGFR3 mRNA expression at relapse was low. This preliminary finding deserves further investigation, as it appears that profiling of VEGFR1 and VEGFR3 in HNSCC patients at initial diagnosis may be of potential value for predicting nodal involvement or locoregional relapse.

In addition, a trend was found for high VEGFR1 tumoural expression at relapse to be associated with tropism for nodal or soft tissue failure (test, $P = .056$), and for high VEGF-B with a history of high alcohol consumption ($P = .075$). No other clinically or statistically significant associations of studied biomarkers with clinicopathological characteristics were seen. Table 2 summarizes the biomarkers with the most significant associations with clinicopathological data, while all associations of the HER family genes with clinicopathological data are shown in Table 3.

3.3. Predictive Significance for RFI. Transcriptional activity of any of the studied biomarkers was not significantly associated with occurrence of early or late locoregional relapse (RFI of less versus more than 12 months). Moreover, transcription of the studied biomarkers could not predict the timing of relapse, even when the latter was examined as a continuous time variable (Mann-Whitney U test, $P > 0.1$).

3.4. Predictive Significance for R-PFS. At a median follow-up of 52 months from the time of locoregional recurrence (range 8–53 months), transcriptional activity of HER and VEGF/VEGFR family members was examined for predictive significance for survival from relapse until progression or death (R-PFS). High mRNA expression of VEGF-C in the tumour at the time of locoregional recurrence was significantly associated with shorter progression-free survival (log-rank, $P = .052$). Patients who harboured tumours with low VEGF-C mRNA expression had a median R-PFS of 47 months versus a median R-PFS of only 5 months for the patients with tumours expressing high VEGF-C (Figure 1). Moreover, mRNA expression levels of its receptor, VEGFR3, were related to patient outcome with a trend for statistical significance (log-rank, $P = .060$). Patients with high tumour transcription of VEGFR3 at relapse reached a median R-PFS of only 12 months, in contrast to those harbouring tumours with low VEGFR3 mRNA expression, in whom the median R-PFS had not been reached yet at the time of the analysis (Figure 2). An association of tumour VEGF-D expression and R-PFS was speculated, though no statistical significance was observed (log-rank, $P = 0.41$). Low tumour VEGF-D mRNA expression was associated with a median R-PFS of

Table 1: Clinicopathological characteristics at initial diagnosis and locoregional relapse.

	$N = 41$			
	At diagnosis		At recurrence	
Age				
Median (range)	65 (45–77)			
Relapse-free interval (months)				
Median (range)			15 (5–221)	
Size (cm)				
Median (range)	2 (0.3–6)		2.6 (0.6–10)	
	N	%	*N*	%
Gender				
Male	41	100		
Family history				
No	29	71		
Yes	12	29		
Smoking history				
No	2	5	28	68
Yes	39	95	13	32
Pack years				
Median (range)	52.5 (0–125)			
Alcohol consumption				
Low	13	32		
Moderate	16	39		
High	12	29		
Symptoms				
Hoarseness	26	63		
Dyshphagia	10	24		
Dyspnoea	1	2		
Sore mouth	2	5		
Ulceration	1	2		
Lymphadenopathy	1	2		
Primary site				
Glottic	26	63		
Supraglottic	10	24		
Transglottic	1	2		
Oropharynx	3	7		
Unknown primary	1	2		
Site of recurrence				
Local			27	66
Lymph nodes ± local			6	15
Other			8	19
T stage				
T1	16	39		
T2	12	29		
T3	8	20		
T4	4	10		
Unknown	1	2		
N stage				
N0	35	85		
N1	3	7		
N2	1	2		
Unknown	2	5		

Table 1: Continued.

	$N = 41$			
	At diagnosis		At recurrence	
Grade				
I	7	17	9	22
II	18	44	16	39
III	5	12	7	17
IV	1	2	2	5
In Situ	1	2	0	0
Verrucous	1	2	1	2
Unknown	8	20	6	15
Surgery				
Biopsy	13	32	14	34
Total laryngectomy ± nodal resection	10	24	19	46
Hemilaryngectomy or segmental resection	8	19	1	2
Local resection	10	24	7	17
Radiotherapy (RT)				
No	20	49	30	73
Yes	21	51	10	24
Unknown	0	0	1	2
RT dose (Gy)				
Median (range)	66 (64–74)		69 (40–72)	
Chemotherapy (CT)				
No	40	98	30	73
Yes	1	2	10	24
Unknown	0	0	1	2
CT duration (months)				
Median (range)			3.7 (1.8–5.0)	
Radiotherapy only	20	49	7	17
Chemotherapy only	0	0	7	17
Paclitaxel + gemcitabine			1	
Paclitaxel + liposomal doxorubicin			3	
Methotrexate			1	
Missing data			2	
Surgery only	19	46	19	46
Chemoradiotherapy	1	2	3	7
Paclitaxel + gemcitabine			1	
Paclitaxel + liposomal doxorubicin			1	
Missing data			1	

only 10 months, while high VEGF-D with a median R-PFS of 47 months (Figure 3).

3.5. Predictive Significance for R-OS. Among all studied biomarkers, only VEGF-C tumoural transcription at recurrence exhibited a trend for a statistically significant association with survival of relapsed patients (log-rank, $P = .076$). Those patients who harboured tumours with high VEGF-C at relapse had a median R-OS of 22 months, whereas patients with low-level tumour VEGF-C had a median survival of 44 months (Figure 4). Of note, high tumour expression levels of VEGF-D at locoregional recurrence were associated with an improved patient outcome, albeit not statistically significant (log-rank, $P = .15$), as had been the case with R-PFS. In cases with low tumour VEGF-D levels, the median R-OS was only 17 months, in contrast to cases with high VEGF-D tumour mRNA expression, in which the median survival had not been reached yet, at a median follow-up of 52 months (Figure 5).

4. Discussion

The impact of locoregional recurrence in patients with HNSCC is devastating in several aspects: function, cosmesis, quality of life, and most importantly, survival. Standard

Table 2: Association of VEGF-C, VEGFR1 (FLT1), and VEGFR3 (FLT4) mRNA expression with clinicopathological parameters.

	VEGF-C			VEGFR1 (FLT1)			VEGFR3 (FLT4)		
	Low	High	*P*	Low	High	*P*	Low	High	*P*
Alcohol consumption			.168			.324			.999
Low	4 (20)	9 (45)		8 (40)	5 (25)		7 (35)	6 (32)	
Moderate	10 (50)	5 (25)		5 (25)	10 (50)		8 (40)	7 (37)	
High	6 (30)	6 (30)		7 (35)	5 (25)		5 (25)	6 (32)	
Site of relapse			**.009**			.056			**.017**
Local only	17 (85)	10 (50)		17 (85)	10 (50)		18 (90)	9 (47)	
Lymph nodes ± Local	3 (19)	3 (15)		2 (10)	4 (20)		1 (5)	5 (26)	
Other	0 (0)	7 (35)		1 (5)	6 (30)		1 (5)	5 (26)	
Size at 1st relapse			.712			.110			.999
<2 cm	3 (15)	4 (20)		6 (30)	1 (5)		4 (20)	3 (16)	
2–4 cm	10 (50)	9 (45)		11 (55)	10 (50)		11 (55)	9 (47)	
>4 cm	5 (25)	2 (10)		2 (10)	5 (25)		4 (20)	3 (16)	
Unknown	2(10)	5 (25)		1 (5)	4 (20)		1 (5)	4 (21)	
Lymph nodes at diagnosis			.342			**.047**			**.041**
N0	19 (95)	16 (80)		20 (100)	15 (75)		20 (100)	14 (74)	
N1-N2	1 (5)	3 (15)		0 (0)	4 (20)		0 (0)	4 (21)	
Unknown	0 (0)	1 (5)		0 (0)	1 (5)		0 (0)	1 (5)	
Differentiation grade at relapse			.697			.697			.697
Well or moderate	14 (70)	10 (50)		14 (70)	10 (50)		14 (70)	10 (53)	
Poor or undifferentiated	4 (20	5 (25)		4 (20)	5 (25)		4 (20)	5 (26)	
Unknown	2 (10)	5 (25)		2 (10)	5 (25)		2 (10)	4 (21)	
Pack years exposure			.341			.341			.751
<52.5	9 (45)	13 (65)		9 (45)	13 (65)		10 (50)	11 (58)	
>52.5	11 (55)	7 (35)		11 (55)	7 (35)		10 (50)	8 (42)	
Age			.527			.999			.999
<65	9 (45)	12 (60)		11 (55)	10 (50)		10 (50)	10 (53)	
>65	11 (55)	8 (40)		9 (45)	10 (50)		10 (50)	9 (47)	
Diagnosis to recurrence interval			.333			.748			.748
<12 months	6 (30)	10 (50)		7 (35)	9 (45)		9 (45)	7 (37)	
>12 months	14 (70)	10 (50)		13 (65)	11 (55)		11 (55)	12 (63)	

clinical and pathological factors of established prognostic significance for patient outcome have been reported: resection margins, regional nodal metastases, advanced T stage, high grade, and neurogenic/vessel invasion [6, 7]. Still, 20%–30% of the patients with localised T1-T2 disease managed with negative margin resection, nodal clearance, and postsurgery irradiation eventually recur in the neck [1, 2]. EGFR (HER1), HER2, HER3, and HER4 transmembrane receptors are essential for proliferation, motility, and invasion of the malignant cell, with the former two having been studied more extensively. The rate of HNSCC tumours presenting immunohistochemical (IHC) protein overexpression was found to be 80%–90% for EGFR and 4%–39% for HER2 [8, 9]. Although EGFR and HER2 IHC protein expression was shown to be of prognostic value for inferior clinical outcome, they were unreliable predictors of benefit from targeted therapeutic agents [10]. Especially EGFR is expressed in almost all HNSCC tumours, in keeping with the squamous cell phenotype, while its immunohistochemical protein staining is a subjective assay lacking the dynamic range of quantitative evaluation. EGFR and other HER family members form heterodimers upon ligand binding and activate intracellular signalling cascades that regulate survival, proliferation, motility, and angiogenesis of the malignant cell cluster. Recent large phase III trials showed overall survival benefit from the combination of the anti-EGFR monoclonal antibody cetuximab with radiotherapy or chemotherapy in patients with locally advanced or metastatic HNSCC [11, 12]. This clinical breakthrough makes imperative the need for the identification of biomarkers that would predict tumour response or resistance to EGFR-modulating agents.

VEGF protein overexpression assessed by IHC was found in 90% of HNSCC tumours, associated with a 2-fold higher risk of death at two years [13]. The five VEGF ligands (VEGF-A, B, C, D, and E) interact as dimers with the three types of VEGF receptors (VEGFR1, 2 and 3) found on endothelial and tumour cells. Receptor homo- or heterodimerisation initiates complex intracellular signalling mechanisms leading to formation of new tumour blood vessels (VEGFR1 and

Table 3: Association of mRNA expression the HER family genes with clinicopathological parameters.

	EGFR			HER2			HER3			HER4		
	Low $N = 21$	High $N = 20$	P	Low $N = 18$	High $N = 18$	P	Low $N = 21$	High $N = 20$	P	Low $N = 20$	High $N = 19$	P
Alcohol Consumption			.577			.404			.259			.239
Low	5 (24)	8 (40)		8 (44)	5 (28)		9 (43)	4 (20)		9 (45)	4 (21)	
Moderate	9 (43)	7 (35)		7 (39)	6 (33)		6 (29)	10 (50)		6 (30)	10 (53)	
High	7 (33)	5 (25)		3 (17)	7 (39)		6 (29)	6 (30)		5 (25)	5 (26)	
Site of relapse			.999			.501			.812			.545
Local only	14 (67)	13 (65)		13 (72)	13 (72)		13 (62)	14 (70)		12 (60)	15 (79)	
Lymph nodes ± Local	3 (14)	3 (15)		2 (11)	4 (22)		4 (19)	2 (10)		3 (15)	2 (11)	
Other	4 (19)	4 (20)		3 (17)	1 (6)		4 (19)	4 (20)		5 (25)	2 (11)	
Size at 1st relapse			.425			.256			.145			.716
< 2 cm	4 (19)	3 (15)		3 (17)	4 (22)		4 (19)	3 (15)		3 (15)	4 (21)	
2–4 cm	9 (43)	13 (65)		8 (44)	10 (56)		9 (43)	13 (66)		11 (55)	10 (53)	
> 4 cm	5 (24)	2 (10)		5 (28)	1 (6)		6 (29)	1 (5)		5 (25)	2 (10)	
Unknown	3 (14)	2 (10)		2 (11)	3 (17)		2 (10)	3 (15)		1 (5)	3 (16)	
Lymph nodes at diagnosis			.999			.999			.999			.999
N0	18 (86)	18 (90)		16 (89)	16 (89)		18 (86)	18 (90)		17 (85)	17 (89)	
N1-N2	2 (10)	2 (10)		2 (11)	2 (11)		2 (10)	2 (10)		2 (10)	2 (11)	
Unknown	1 (5)	0 (0)		0 (0)	(0)		1 (5)	0 (0)		1 (5)	0 (0)	
Differentiation grade at relapse			.240			.417			.999			.448
Well or moderate	13 (62)	12 (60)		13 (72)	9 (50)		12 (57)	13 (65)		12 (60)	13 (81)	
Poor or undifferentiated	2 (10)	7 (35)		3 (17)	5 (28)		4 (19)	5 (25)		6 (33)	3 (19)	
Unknown	6 (29)	1 (5)		2 (11)	4 (22)		5 (24)	2 (10)		2 (10)	3 (16)	
Pack years exposure			.999			.999			.999			.205
< 52.5	11 (52)	11 (55)		10 (56)	9 (50)		11 (52)	11 (55)		8 (40)	12 (63)	
> 52.5	10 (48)	9 (45)		8 (44)	9 (50)		10 (48)	9 (45)		12 (60)	7 (37)	
Age			.217			.094			.538			.752
< 65	13 (62)	8 (40)		12 (67)	6 (33)		12 (57)	9 (45)		11 (55)	9 (47)	
> 65	8 (38)	12 (60)		6 (33)	12 (67)		9 (43)	11 (55)		9 (45)	10 (53)	
Diagnosis to recurrence Interval			.530			.305			.341			.748
< 12 months	7 (33)	9 (45)		9 (50)	5 (28)		10 (48)	6 (30)		9 (45)	7 (37)	
> 12 months	14 (67)	11 (55)		9 (50)	13 (72)		11 (52)	14 (70)		11 (55)	12 (63)	

2) or lymph vessels (VEGFR3) [14]. Therapeutic agents targeting the VEGF ligands or receptors inhibit neoplastic angiogenesis, optimise remaining vasculature, decrease interstitial fluid pressure, and synergistically kill tumour cells when given in combination with chemotherapy or radiotherapy in preclinical models [15]. Bevacizumab, a monoclonal antibody that binds VEGF, and tyrosine kinase inhibitors of the VEGF receptors are currently being evaluated in HNSCC patients, along with biomarkers that could predict for benefit from such targeted therapies. Seiwert et al. recently reported that the ratio of phosphorylated VEGFR2 to total VEGFR2, measured by immunofluorescence, predicts for response in patients with recurrent or metastatic HNSCC receiving bevacizumab/erlotinib combination therapy [16].

Gene transcriptional profiling of messenger RNA by means of real time kRT-PCR provides a quantitative evaluation method that is not affected by observer variability or the widely known IHC technique limitations. In order to screen for molecular predictors of outcome of patients with

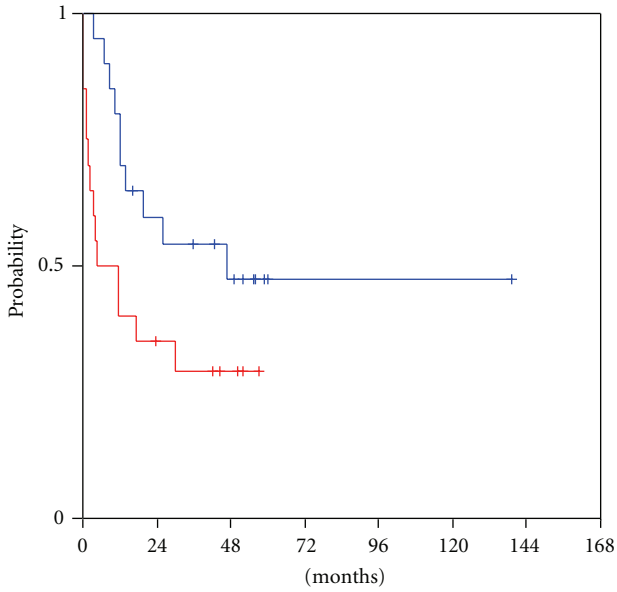

Figure 1: Relapse-related PFS in patients with low (blue line) and high (red line) tumour VEGF-C mRNA expression.

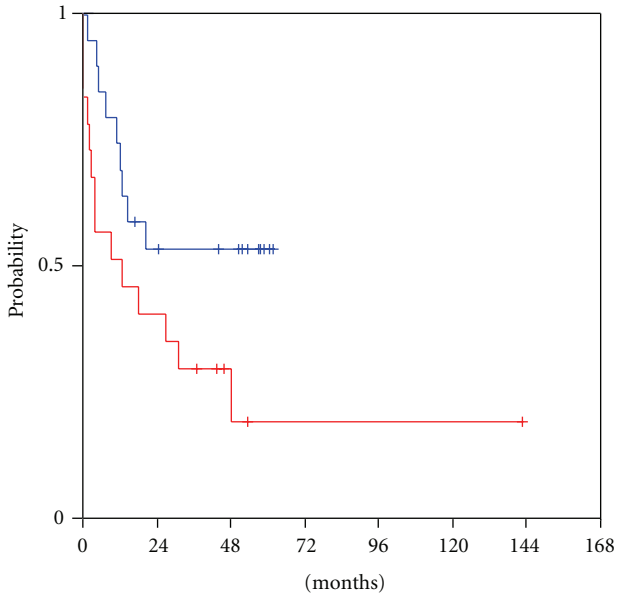

Figure 2: Relapse-related PFS in patients with low (blue line) and high (red line) tumour VEGFR3 mRNA expression.

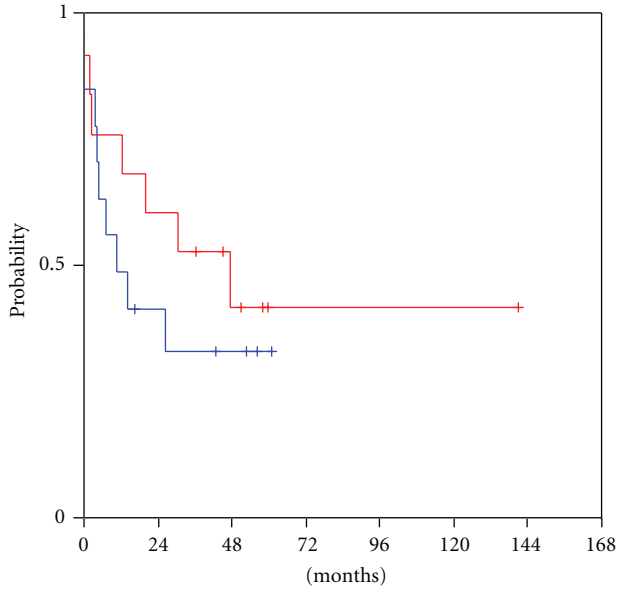

Figure 3: Relapse-related PFS in patients with low (blue line) and high (red line) tumour VEGF-D mRNA expression.

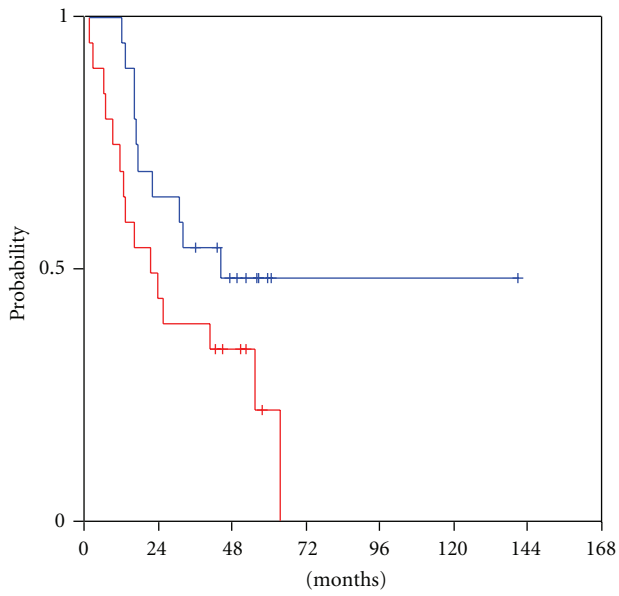

Figure 4: Relapse-related OS in patients with low (blue line) and high (red line) tumour VEGF-C mRNA expression.

recurrent HNSCC, we studied fresh-frozen tumours from 41 patients with locoregional recurrence of relatively low-risk disease at presentation: the median tumour size was 2 cm, 68% of cases being T1-2, 85% N0, and 61% well to moderately well differentiated. Despite the small sample size, transcriptional activation of the VEGF-C/VEGFR3 axis at relapse was associated with recurrence outside the primary site (neck nodes or soft tissues) and inferior progression-free and overall survival from relapse at a marginal statistical significance. Moreover, tumours that were node-positive at presentation had higher VEGFR1 and VEGFR3 mRNA expression levels at relapse. Despite the preliminary nature of these findings, in a small retrospective cohort, the emergence of statistically significant associations of angiogenesis effectors with outcome, in patients initially presenting with low-risk tumours, hints for the presence of clinical significance and a more robust correlation, should the sample size had been larger.

Our observation incriminating tumoural VEGF-C/VEGFR3 signalling in nodal/soft tissue relapse and poor

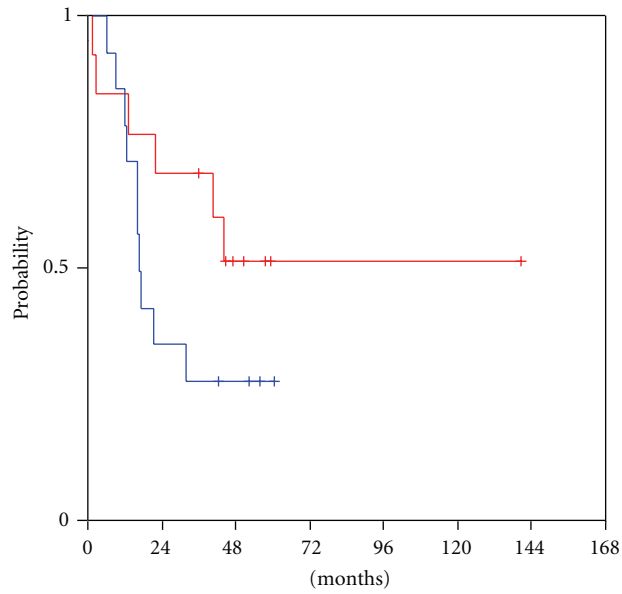

Figure 5: Relapse-related OS in patients with low (blue line) and high (red line) tumour VEGF-D mRNA expression.

post-relapse outcome is in keeping with recent published evidence. Several investigators reported association of protein or mRNA expression of VEGF-C with lymphatic metastases and invasion in HNSCC, gastric, prostate, and breast cancer cell lines and small patient series [17–19]. Of note, Tanigaki et al. found tumoural VEGF-A/VEGFR1 and 2 transcription correlated to development of distant metastases, while VEGF-C/VEGFR3 to locoregional recurrence [20]. Moreover, O-Charoenrat et al. reported that in contrast to other VEGF ligands, VEGF-D mRNA was suppressed in HNSCC tumours. Preclinical data have shown that active HER1/2 signalling upregulates VEGF-A and C and downregulates VEGF-D transcription in lung adenocarcinoma and HNSCC cell lines [21]. We observed inferior R-PFS and R-OS in patients harbouring tumours with low VEGF-D mRNA expression compared to those with high VEGF-D, though statistical significance was not reached. VEGF-D may exert an antagonistic effect on neoplastic neovascularisation, forming heterodimers with VEGF-A, B, and C and modulating the activity of the VEGFR1, 2, and 3 along with the neuropilin receptors. This phenomenon of counter-regulation is probably extremely important for the fine-tuning of angiogenesis. Recently, VEGF-Ab, a splice variant of the powerful proangiogenic VEGF-A ligand, was shown to exert antiangiogenic effects in normal tissues and a variety of solid tumours [22].

The mechanism of the adverse prognostic impact of VEGF-C/VEGFR3 signalling may include dissemination of tumour cells in the systemic circulation and arrest in lymph nodes/distant sites, direct enhancement of lymph-angiogenesis, and creation of a permissive environment for tumour progression by the induction of adhesion molecules, growth factors, and proteolytic enzymes. In contrast to VEGF-C, HER signalling was not significant for predicting patient outcome, despite in vitro data emphasizing its key role in the control of cell cycle, invasion, and the induction of VEGF-A and C-mediated angiogenesis. Indeed, in an HNSCC patient series, protein expression of EGFR or HER2 could not predict benefit from chemotherapy or targeted therapies [8–11]. Only EGFR gene amplification activating EGFR gene mutations and the presence of the truncated form of the EGFRvIII protein correlated with clinical benefit or patient outcome [10]. Although our mRNA methodology could not screen for these biomarkers, Agulnik et al. found excess EGFR gene copy numbers in only 4 out of 37 patients, and Willmore-Payne et al. reported HER1/2 mutations in less than 10% of patients with HNSCC [23, 24]. In contrast, Chung et al. observed EGFR gene amplification in 58% of 75 recurrent or metastatic HNSCC patients and reported its association with poor outcome [25]. However, EGFR copy number status did not correlate with protein or mRNA expression. This could explain our inability to find any prognostic significance for EGFR mRNA levels in our study. Indeed, HER1/HER2 gene amplification may be an early oncogenic event, with most gene copies becoming transcriptionally inactive later. Alternate splicing of EGFR transcripts, not detected by our mRNA probes, could also offer another explanation [26]. Moreover, the EGFR/HER2 genes may carry prognostic information not associated with their amplification status per se but rather act as surrogate markers of genetic instability or of other coamplified genes [27]. Of note, Seiwert et al. reported that endothelial but not tumour cell EGFR protein levels correlated with response to bevacizumab + erlotinib [16]. Moreover, the combination reduced VEGFR2 and EGFR protein expression in neoplastic endothelia but not tumour cells. Our mRNA analysis, though based on frozen sections with ≥70% tumour cellularity, would not discriminate between tumour cells and neoplastic vessel endothelial cells.

In conclusion, VEGF-C/VEGFR3 mRNA expression at relapse may be of potential value as a new biomarker predicting nodal/soft tissue regional relapse and poor outcome after recurrence in HNSCC patients. It should be stressed that molecular profiling of primary tumours is necessary in order to obtain prognostic information at diagnosis. Comparison of the molecular profiles of primary and matched recurrent tumors is required to derive safe conclusions and was not done in our study. Still, our findings may serve as hypothesis-generating data and, if validated in larger prospective series, may justify more aggressive neck management at presentation and treatment of HNSCC patients exhibiting high VEGF-C mRNA expression with targeted therapies (anti-VEGF-C antibodies, VEGFR3 tyrosine kinase inhibitors), either upfront or at recurrence, in order to optimise their outcome.

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Research Article

Inhibition of Glutathione and Thioredoxin Metabolism Enhances Sensitivity to Perifosine in Head and Neck Cancer Cells

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The hypothesis that the Akt inhibitor, perifosine (PER), combined with inhibitors of glutathione (GSH) and thioredoxin (Trx) metabolism will induce cytotoxicity via metabolic oxidative stress in human head and neck cancer (HNSCC) cells was tested. PER induced increases in glutathione disulfide (%GSSG) in FaDu, Cal-27, and SCC-25 HNSCCs as well as causing significant clonogenic cell killing in FaDu and Cal-27, which was suppressed by simultaneous treatment with N-acetylcysteine (NAC). An inhibitor of GSH synthesis, buthionine sulfoximine (BSO), sensitized Cal-27 and SCC-25 cells to PER-induced clonogenic killing as well as decreased total GSH and increased %GSSG. Additionally, inhibition of thioredoxin reductase activity (TrxRed) with auranofin (AUR) was able to induce PER sensitization in SCC-25 cells that were initially refractory to PER. These results support the conclusion that PER induces oxidative stress and clonogenic killing in HNSCC cells that is enhanced with inhibitors of GSH and Trx metabolism.

1. Introduction

Growing evidence exists that cancer cells are under increased intrinsic metabolic oxidative stress compared to normal untransformed cells due in part to mitochondrial dysfunction [1–3]. Studies have shown that defects in mitochondrial respiration led to activation of the Akt (protein kinase B) pathway, which may be an important mechanism by which cancer cells use to survive under conditions of chronic oxidative stress [4]. Akt is a serine-threonine protein kinase, which has been shown to have a role in angiogenesis, cell cycle progression, differentiation, and cell growth [5, 6]. Akt is hyperactivated in many cancer types including breast, colorectal, ovarian, and especially head and neck cancer (HNSCC) compared to normal tissue [7, 8], which led to the hypothesis that metabolic oxidative stress may be causally related to the increased Akt activity observed in cancer cells. Given that the Akt pathway is critical for cell survival, and cancer cells have been suggested to demonstrate increased intracellular hydroperoxide production compared to normal (untransformed) cells [2, 9–11], we propose that tumor cells may increase Akt activity to compensate for increased intracellular hydroperoxides and oxidative stress caused by defects in mitochondrial respiration. Furthermore, we propose that therapeutic interventions designed to inhibit Akt activation and hydroperoxide detoxification combined with manipulations that increase prooxidant production would preferentially kill tumor cells versus normal cells via oxidative stress.

Perifosine (Octadecyl-(1,1-dimethyl-piperidinio-4-yl)-phosphate (PER)) is a bioavailable alkylphospholipid which is currently being tested in phases 1 and 2 clinical trials [12–17] and is a member of a larger group of membrane permeable single-chain antitumor alkylphosphocholines (APCs) [18–20]. PER has shown significant antiproliferative activity in in vitro and in vivo tumor models including breast, colon, prostate and HNSCC [18, 21]. Akt is a specific target of PER by targeting the plekstrin homology (PH) domain of Akt and preventing its translocation to the plasma membrane to be activated [21]. The aim of the

present study is to determine if PER induces oxidative stress and if disrupting thiol antioxidant metabolism pathways further enhances sensitivity to PER-induced clonogenic cell killing in HNSCC cells.

2. Materials and Methods

2.1. Cells and Culture Conditions. FaDu, Cal-27, and SCC-25 human head and neck squamous carcinoma cells were obtained from the American Type Culture Collection (Manassas, VA). FaDu and Cal-27 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 4 mM L-glutamine, 1 mM sodium pyruvate, 1.5 g/L sodium bicarbonate, and 4.5 g/L glucose with 10% fetal bovine serum (FBS; Hyclone, Logan, UT). SCC-25 cells were maintained in a 1 : 1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium containing 1.2 g/L sodium bicarbonate, 2.5 mM L-glutamine, 15 mM HEPES, 0.5 mM sodium pyruvate, 4.5 g/L glucose, and 400 ng/mL hydrocortisone with 10% fetal bovine serum. Cultures were maintained in 5% CO_2 and humidified in a 37°C incubator.

2.2. Drug Treatment. N-acetyl cysteine (NAC) and L-buthionine-[S,R]-sulfoximine (BSO) were obtained from Sigma Chemical Co. (St. Louis, MO). Auranofin (AUR) was obtained from ICN Biochemicals (Aurora, OH). Perifosine (PER) was obtained from Cayman Chemical (Ann Arbor, MI). All drugs were used without further purification. Drugs were added to cells at final concentrations of 1–10 μM PER, 0.5 μM AUR, 20 mM NAC, and 1.0 mM BSO. PER and AUR were dissolved in ethanol and dimethyl sulfoxide (DMSO), respectively, and then diluted with 0.9% sodium chloride (Hospira, Lake Forest, IL), NAC was dissolved in 1 M sodium bicarbonate (pH 7.0) and BSO was dissolved in PBS. The required volume of each drug was added directly to complete cell culture media on cells to achieve the desired final concentrations. All cells were placed in a 37°C incubator and harvested at the time points indicated.

2.3. Detection of Activated Akt and Total Akt Levels. Cells were harvested after 24 hours drug exposure, washed with PBS, and then lysed with cell lysis buffer. Cell lysates were standardized for protein content and resolved by SDS-PAGE. Nitrocellulose blots were probed with rabbit antiAkt, antiphospho-Akt Ser^{473} (Cell Signaling Technologies, Beverly, MA), or antiGAPDH (Cell Signaling) antibodies.

2.4. Glutathione Assay. Cell pellets were thawed and homogenized in 50 mM PO_4 buffer (pH 7.8) containing 1.34 mM diethylenetriaminepentaacetic acid (DETAPAC) buffer. Total glutathione content was determined by the method of Anderson [22]. Reduced glutathione (GSH) and glutathione disulfide (GSSG) were distinguished by addition of 2 μL of a 1 : 1 mixture of 2-vinylpyridine and ethanol per 30 μL of sample followed by incubation for 1 hour and assayed as described previously [23]. All glutathione determinations were normalized to the protein content of whole homogenates using the method of Lowry et al. [24].

2.5. Clonogenic Cell Survival Experiments. Cells were treated with PER for 24 hours prior to each clonogenic survival experiment. In the indicated experiments, cells were treated with NAC, BSO, or AUR for 1 hour before and during PER exposure. Attached cells from experimental dishes were trypsinized with 1 mL trypsin-EDTA (CellGro, Herndon, VA) and inactivated with DMEM containing 10% fetal bovine serum (Hyclone). The cells were diluted and counted using a Coulter counter. Cells were plated at low density (150–1000 per plate), and clones were allowed to grow in a humidified 5% CO_2, 37°C environment for 14 days in complete medium, in the presence of 0.1% gentamicin. Cells were fixed with 70% ethanol and stained with Coomassie blue for analysis of clonogenic cell survival as previously described [25].

Individual assays were performed with multiple dilutions with at least four cloning dishes per data point, repeated in at least 3 separate experiments.

2.6. Thioredoxin Reductase Assay. Thioredoxin reductase (TrxRed) activity was determined spectrophotometrically using the method of Holmgren and Bjornstedt [26]. Enzymatic activity was determined by subtracting the time-dependent increase in absorbance at 412 nm in the presence of the TR activity inhibitor, aurothioglucose from total activity. One unit of activity was defined as 1 μM TNB formed/(min·mg protein). Protein concentrations were determined by the Lowry assay [24].

2.7. Statistical Analysis. Statistical analysis was done using GraphPad Prism version 4 for Windows (GraphPad Software, San Diego, CA). To determine differences between 3 or more means, one-way ANOVA with Tukey posttests were performed. Error bars represent the standard error of the mean. All statistical analysis was performed at the $P < .05$ level of significance.

3. Results

3.1. Effect of Perifosine on Akt Expression. To determine the effect of PER on Akt expression in HNSCC, FaDu, Cal-27, and SCC-25 cells were treated with 5 μM PER for 24 hours then harvested for the detection of activated Akt (pAkt) and total Akt. Activated Akt was detected in all 3 cell lines but varied in their expression levels (Figure 1). PER completely inhibited the expression of pAkt in FaDu and Cal-27 cells and partially inhibited pAkt expression in SCC-25 cells (Figure 1). Total Akt was also inhibited by PER in Cal-27 cells (Figure 1). These results confirm that PER inhibited pAkt activity in HNSCCs.

3.2. Effect of Perifosine on Cells Growth and Survival. To investigate the effect of PER on human head and neck squamous carcinoma cell growth, FaDu, Cal-27, and SCC-25 cells were treated with increasing doses of PER (1–10 μM) and then counted at 0, 24, 48, and 72 hours after treatment. PER at 1, 5, and 10 μM inhibited FaDu cell growth after 72 hours (Figure 2(a)) while only 5 and 10 μM PER inhibited

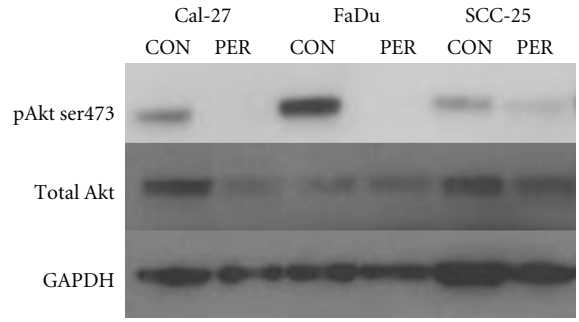

Figure 1: Effect of perifosine (PER) on Akt expression. Akt phosphorylation in FaDu, Cal-27, and SCC-25 cells was assayed by Western blots (50 μg protein loaded in each well) for Akt and phosphor-Akt Ser473 in the presence or absence of 5 μM PER for 24 hours. GAPDH was used as a loading control.

Cal-27 growth after 72 hours (Figure 2(b)). SCC-25 cells did not respond to any of the PER doses over a 72-hour period (Figure 2(c)). When we analyzed clonogenic survival after 24 hours of treatment with PER, we observed that 1 μM PER did not affect survival in any of the cells lines but 5 and 10 μM PER significantly decreased survival in FaDu and Cal-27 cells compared to control ($P < .01$, Figure 2(d)). SCC-25 cells were again resistant to PER treatment with 10 μM PER causing a slight but nonsignificant decrease in SCC-25 cells compared to control (Figure 2(d)). These results show that PER causes growth arrest and cytotoxicity in HNSCC cells but these affects are dose dependent and vary by cell type.

3.3. Perifosine Induced Disruptions in Glutathione Metabolism Consistent with Oxidative Stress. We examined if oxidative stress could be contributing to the growth inhibitory and cytotoxic effects of PER by measuring glutathione (GSH/GSSG) levels in the cells. The GSH/GSSG redox couple represents a major small molecular weight thiol-disulfide redox buffer in cells [27]. The amount of total GSH that was oxidized (GSSG) was used to calculate percentage GSSG (%GSSG). Consequently, an increase in %GSSG is believed to signify a shift towards a more highly oxidizing intracellular environment indicative of oxidative stress [27]. We analyzed GSH/GSSG levels in FaDu, Cal-27, and SCC-25 cells after treatment with 5 μM PER for 24 hours. We chose to further analyze the effects of 5 μM PER in FaDu and Cal-27 cells because it was a clinically achievable dose and well under mean steady state plasma concentrations (16.2 μM) achieved in patients with solid tumors after a maximum tolerated dose of 200 mg PER per day [12]. We analyzed the effects of 10 μM PER in SCC-25 cells because this was the only dose that showed slight cytotoxicity in the clonogenic cell survival assay (Figure 2(d)). PER caused an increase in total GSH levels in Cal-27 and SCC-25 cells compared to control which suggests that PER induced the synthesis and accumulation of total GSH in an attempt to maintain the redox buffering capacity of the intracellular reducing environment (Figure 3(a)). PER did not significantly change total GSH levels in FaDu cells (Figure 3(a)). Increases in GSSG were observed when all cell lines were treated with PER although this effect was only significant in FaDu cells (Figure 3(b)). When we calculated the effect of PER on %GSSG, all 3 cell lines demonstrated significant increases in %GSSG compared to control cells with FaDu showing the greatest increase in %GSSG and SCC-25 showing the least (Figure 3(c)). These results support the hypothesis that the toxicity of PER may be in part mediated by disruptions in GSH and/or thiol metabolism consistent with causing oxidative stress.

3.4. PER-Induced Disruptions in Glutathione Metabolism and Cytotoxicity Are Inhibited by NAC. To further analyze the involvement of oxidative stress in PER-induced cytotoxicity, FaDu, Cal-27, and SCC-25 cells were treated with 20 mM of the thiol antioxidant N-acetyl cysteine (NAC) for 1 hour before and during exposure to PER, then analyzed for GSH/GSSG levels and clonogenic survival. Treatment with NAC alone significantly increased GSH levels in SCC-25 cells and also increased GSH in the presence of PER in SCC-25 cells (Figure 3(a)). Additionally, NAC suppressed the PER-induced increase in GSSG in all 3 cell lines with FaDu and Cal-27 reaching significance (Figure 3(b)). However, PER-induced increases in %GSSG were significantly suppressed by NAC in all 3 cell lines which were comparable to NAC alone (Figure 3(c)). When we analyzed the effect of NAC on PER-induced cytotoxicity, NAC partially but significantly rescued the cytotoxicity induced by the combination of PER (36%: NAC + PER versus 83%: PER, $P < .05$) in FaDu cells (Figure 3(d)). NAC did not significantly rescue Cal-27 and SCC-25 cells from PER-induced cytotoxicity (Figure 3(d)). Taken together, Figure 3 supports the hypothesis that PER induces disruptions in thiol metabolism consistent with oxidative stress, which was reversed by NAC, and PER-induced cytotoxicity in FaDu cells may be due in part to increases in oxidative stress.

3.5. PER-Induced Cytotoxicity Is Enhanced by Buthionine Sulfoximine. To determine if GSH depletion would further enhance the cytotoxicity induced by PER, Cal-27, and SCC-25 cells were treated with 1 mM BSO, which is an inhibitor of GSH synthesis, for 1 hour before and during treatment with PER for 24 hours then analyzed for clonogenic survival and GSH/GSSG. We did not study the effect of BSO on PER-induced cytotoxicity in FaDu cells since PER caused such extensive cell killing as a single agent in this cell line. The results indicate that the cell killing observed with PER in Cal-27 was significantly enhanced by BSO (77%: BSO + PER versus 56%: PER, $P < .05$, Figure 4(a)). Additionally, SCC-25 cells which were initially resistant to PER treatment (Figure 2(d)) became sensitized to PER in the presence of BSO (44%: BSO + PER versus 11%: PER, $P < .05$, Figure 4(a)), but this sensitization was not comparable to the cytotoxicity induced by PER in FaDu (83%) and Cal-27 cells (56%, Figure 2(d)). BSO alone and in the presence of PER significantly depleted total GSH to less than 10% of control cells in Cal-27 but only depleted 35% of total GSH in SCC-25 cells (Figure 4(b)). Furthermore, BSO alone and in combination with PER significantly increased %GSSG in Cal-27 cells compared to control cells which was not

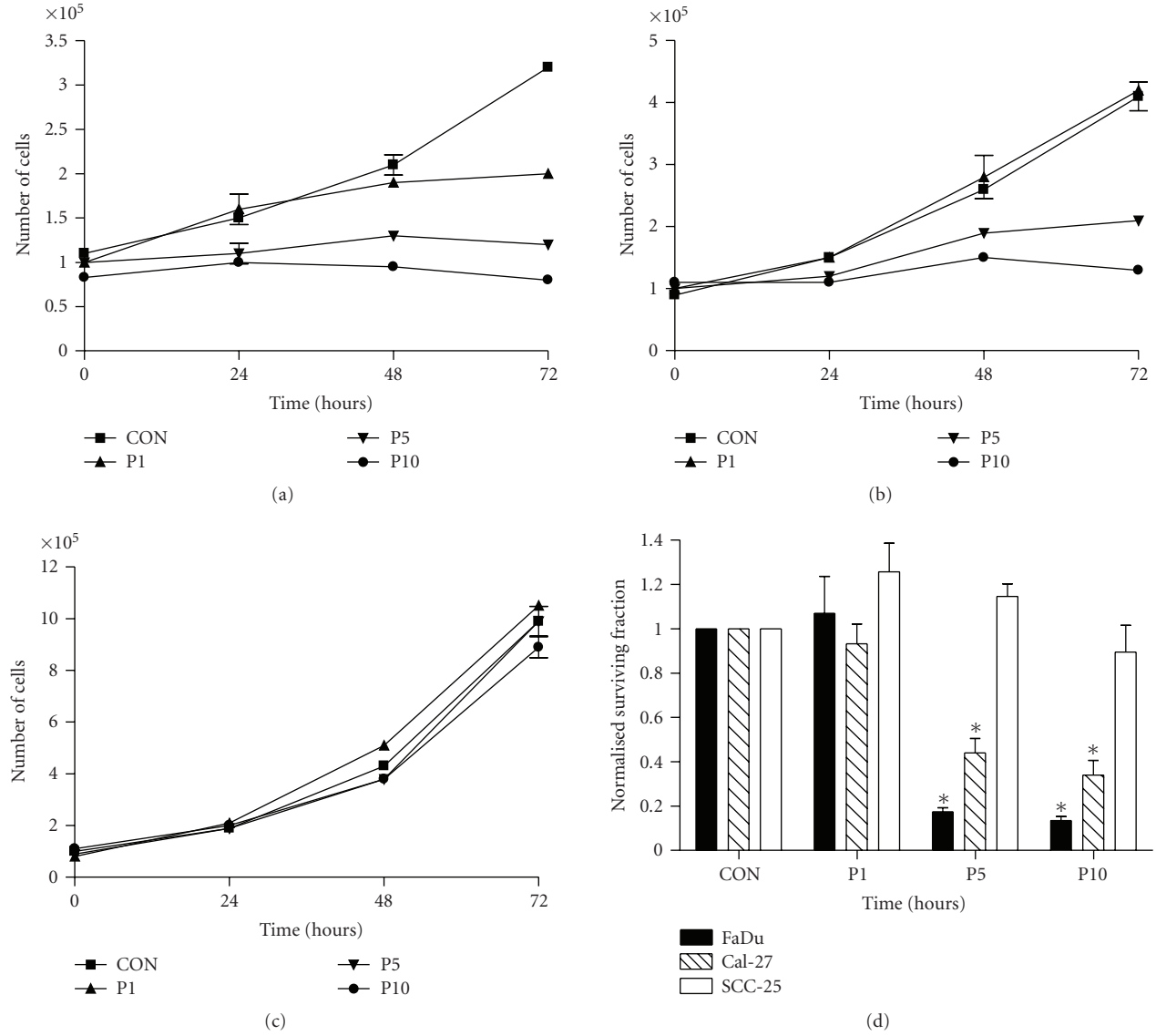

Figure 2: Effect of perifosine (PER) in head and neck cancer cell growth and survival. FaDu (a), Cal-27 (b), and SCC-25 (c) cells were treated with 1, 5, and 10 μmol/L PER and grown for 72 hours. (d) Cells were treated with 1–10 μM PER for 24 hours then plated for clonogenic survival. Clonogenic cell survival data were normalized to control (CON). Error bars represent ± 1SD of $N = 4$–6 experiments performed on different days with at least 2 cloning dishes taken from 1 treatment dish. $*$, $P < .05$ versus control.

observed in SCC-25 cells (Figure 4(c)). These data show that significant depletion of glutathione (GSH) with BSO enhances the cytotoxicity observed with PER and the extent of sensitization may be due to the degree of GSH depletion and induction of GSSG.

3.6. PER-Induced Cytotoxicity Is Enhanced by Auranofin. Since BSO did not significantly enhance PER-induced cell killing in SCC-25 cells to that of PER-induced cell killing observed in FaDu and Cal-27 (Figure 2(d)), we determined if inhibition of thioredoxin (Trx) metabolism would enhance PER-induced cell killing in SCC-25 cells and compared the results to those of Cal-27. We pretreated cells with 0.5 μM AUR for 1 hour before and during exposure to PER (Figure 4). We chose this dose of 0.5 μM AUR because it was shown to inhibit thioredoxin reductase (TrxRed) activity by approximately 50% in Cal-27 and SCC-25 cells compared to control cells (Table 1). AUR caused about 30% cell killing in both cell lines compared to control (Figure 4). Interestingly, AUR did not further enhance PER-induced cell killing in Cal-27 cells but AUR did sensitize SCC-25 cells to PER-induced cell killing which was now comparable to PER-induced cell killing in Cal-27 cells (56%: AUR + PER [SCC-25] versus 56%: PER [Cal-27], Figure 4). Table 1 shows that AUR alone and in combination with PER did in fact inhibit TrxRed activity in Cal-27 and SCC-25 cells. PER alone did not affect TrxRed activity in any of the cell lines (Table 1). The data in Figure 4 and Table 1 suggest that inhibition of GSH and Trx metabolism pathways enhance sensitivity to PER.

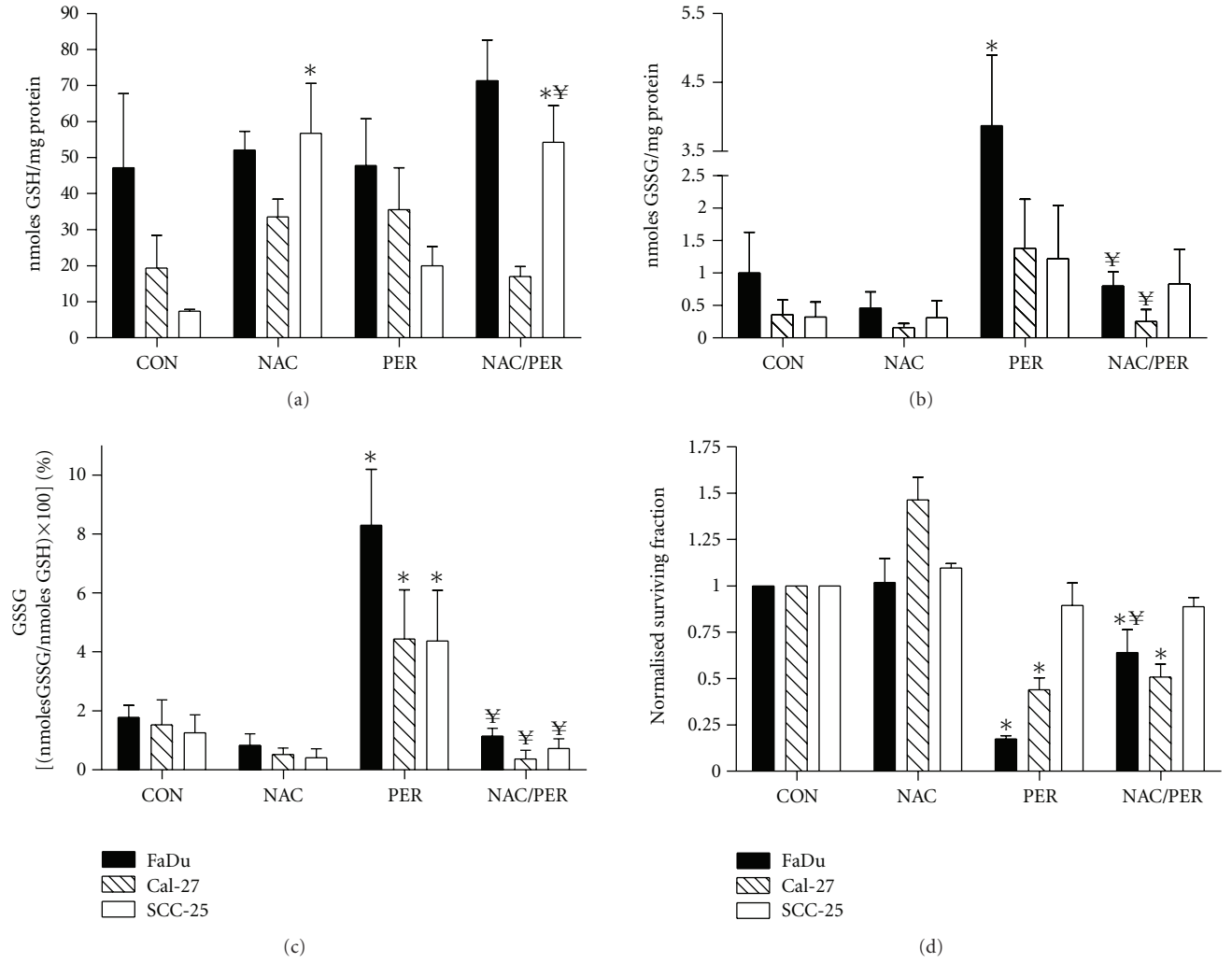

Figure 3: Effect of perifosine (PER) and N-acetyl-cysteine (NAC) on total glutathione (GSH) levels (a), glutathione disulfide (GSSG) levels (b), percentage glutathione disulfide (%GSSG) levels (c), and cytotoxicity (d) in head and neck cancer cells. Cells were treated with 5 μM PER (FaDu and Cal-27) or 10 μM PER (SCC-25) for 24 hours with or without treatment with 20 mM NAC for 1 hour before and during PER exposure. (a)–(c) Cells were harvested for glutathione analysis using the spectrophotometric recycling assay. Error bars represent ± 1SD of $N = 4$ experiments. (d) Cells were analyzed for clonogenic survival and the data were normalized to control (CON). Error bars represent ± 1SD of $N = 3$ experiments performed on different days with at least 2 cloning dishes taken from 1 treatment dish. ∗, $P < .001$ versus control; ¥, $P < .001$ versus respective treatment without NAC.

Table 1

	Thioredoxin reductase activity (U/mg protein)			
Cell line	CON	AUR	PER	AUR/PER
Cal-27	0.05 ± 0.02	0.03 ± 0.01	0.07 ± 0.01	0.01 ± 0.001
SCC-25	0.10 ± 0.003	0.05 ± 0.04	0.09 ± 0.02	0.04 ± 0.04

3.7. Thiol Antioxidant Status Predicts Response to Perifosine. Since each head and neck cell line evaluated in these studies varied in their response to PER (Figures 1 and 2), and we demonstrated that disrupting GSH or Trx antioxidant pathways sensitized certain cell lines to PER (Figure 4), we determined if GSH or TrxRed activity predicted response to PER. We plotted the surviving fraction of each cell line in response to PER as a function of TrxRed activity at control levels (Figure 5(a)) and total GSH content at control levels (Figure 5(b)). The results showed that TrxRed activity significantly correlated with PER-induced cytotoxicity, with FaDu, which demonstrated the greatest cell killing in response to PER (Figure 2(d)), having the least TrxRed activity (Figure 5(a)), and SCC-25 cells which were resistant to PER (Figure 2(d)), having the highest level of TrxRed activity ($r^2 = 0.8995$, Figure 5(a)). Additionally, total GSH levels also correlated with PER-induced cytotoxicity, with FaDu cells having high GSH levels compared to the other cell lines and SCC-25 cells having low GSH levels ($r^2 = 0.7286$, Figure 5(b)). These data suggest that cells with high TrxRed activity and low total GSH levels may be resistant to PER and cells with low TrxRed activity and high GSH content may be sensitive to PER.

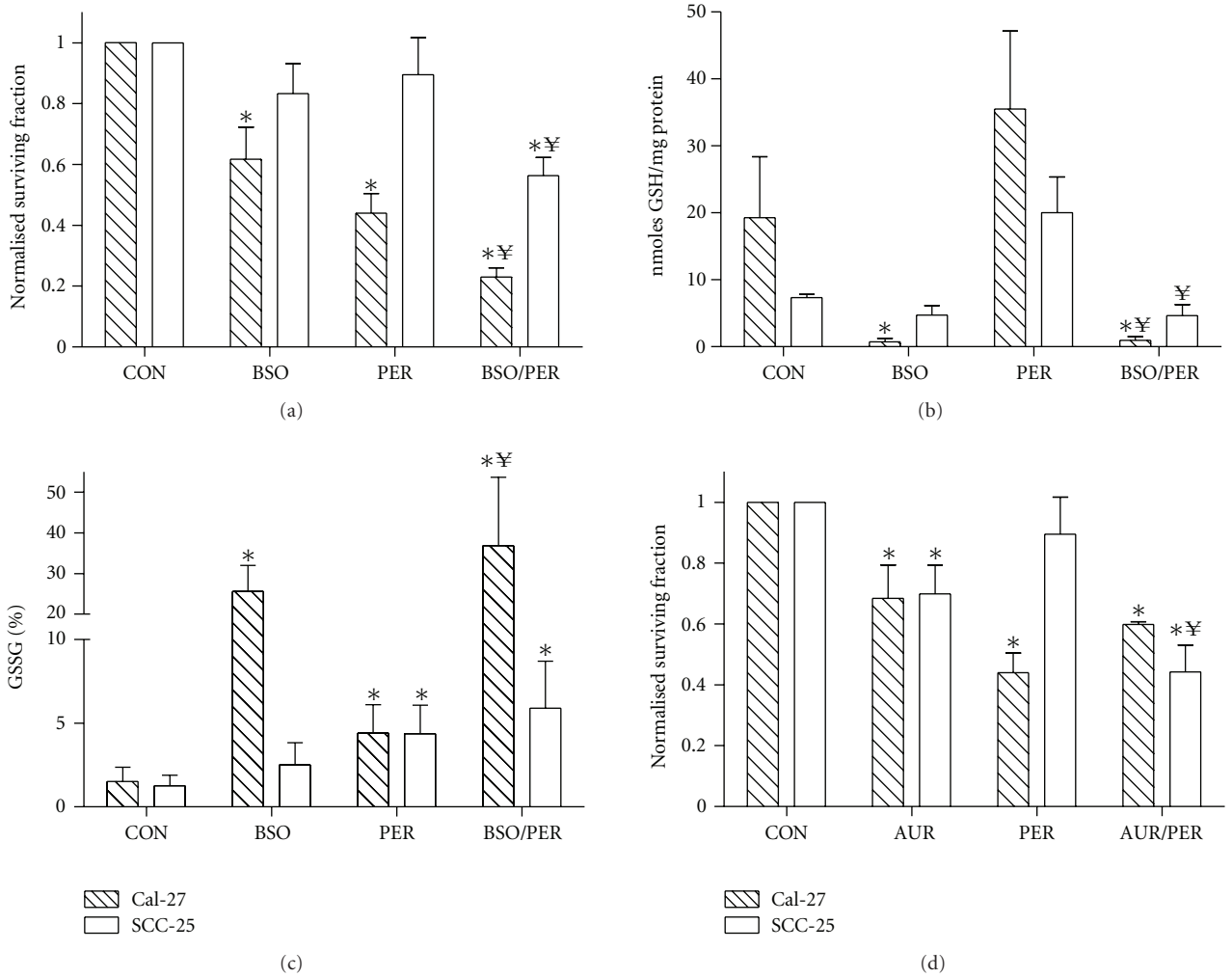

Figure 4: Effect of inhibitors of glutathione and thioredoxin metabolism on perifosine (PER) toxicity in head and neck cancer cells. (a) Cal-27 and SCC-25 cells were treated with 5 μM PER (Cal-27) or 10 μM PER (SCC-25) for 24 hours with or without treatment with 1 mM buthionine sulfoximine (BSO) for 1 hour before and during PER exposure. (b)-(c) Cells were treated as stated above and harvested for total glutathione (GSH) levels (b), and percentage glutathione disulfide (%GSSG) levels (c) using the spectrophotometric recycling assay. Error bars represent ± 1SD of $N = 3$ experiments. (d) Cal-27 and SCC-25 cells were treated with 5 μM PER (Cal-27) or 10 μM PER (SCC-25) for 24 hour with or without treatment with 0.5 μM Auranofin (AUR) for 1 hour before and during PER exposure. Clonogenic cell survival data were normalized to control (CON). Error bars represent ± 1SD of $N = 3$ experiments performed on different days with at least 4 cloning dishes taken from 1 treatment dish. ∗, $P < .001$ versus control; ¥, $P < .05$ versus respective treatment without BSO or AUR.

4. Discussion

PER as a single agent has shown favorable responses in patients with advanced soft tissue sarcomas [14] and Waldenstrom macroglobulinemia [28]. However, responses to PER in patients with common solid tumors have been disappointing and have not justified the further investigation of PER as a single agent. In this study we investigate the role oxidative stress plays in the mechanism of PER and show that sensitivity to PER may be enhanced by disrupting thiol antioxidant metabolism pathways and increasing oxidative stress in head and neck cancer cells.

Akt expression and hyperactivation is a frequent event in HNSCC and strongly correlates with disease progression [8, 29]. The HNSCC cell lines used in this study, FaDu, Cal-27, and SCC-25 all expressed the activated form of Akt (pAkt, Figure 1), which was inhibited by 24 hours treatment with 5 μM PER. Although pAkt was inhibited in all cases, sensitivity to PER varied among the cell lines with FaDu exhibiting the greatest growth inhibition and cytotoxicity and SCC-25 exhibiting no response (Figure 2(d)). Nevertheless, these data support findings by Kondapaka et al. (2003), who showed that 5 μM PER for 24 hours inhibited pAkt expression and growth in PC-3 prostate carcinoma cells [21] and Patel et al. who showed antiproliferative effects with 0.6–8.9 μM PER in head and neck cancer cells [30]. Additionally we observed that pAkt was expressed at different levels in the 3 cell lines with FaDu showing the highest expression and SCC-25 showing the least (Figure 1). Since FaDu cells with the highest expression of pAkt were the most

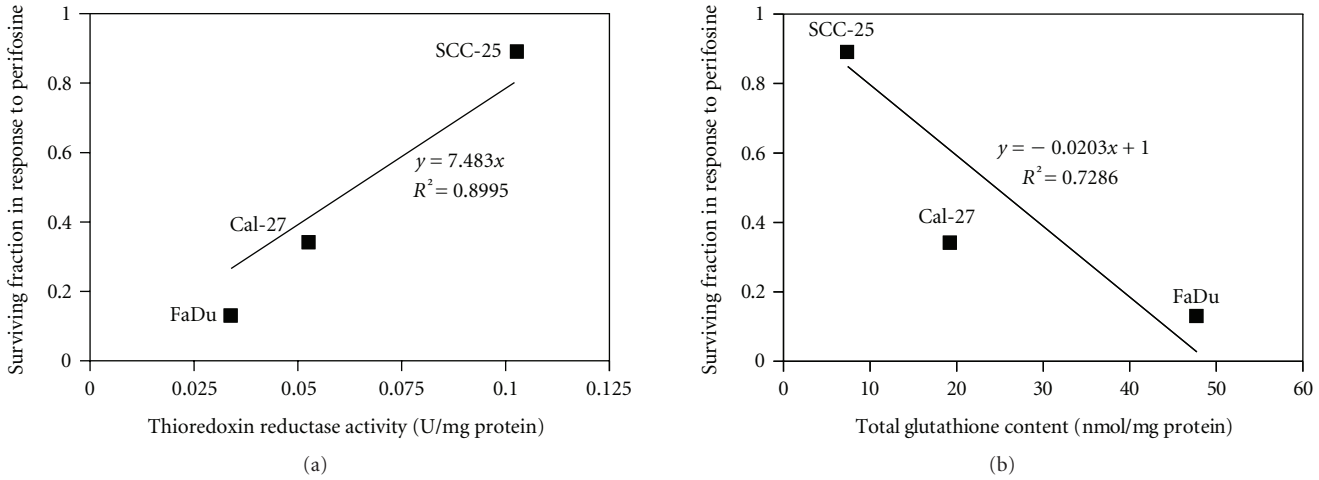

Figure 5: Association of perifosine-(PER-) induced cytotoxicity with glutathione (GSH) content and thioredoxin reductase activity (TrxRed) in head and neck cancer cells. FaDu, Cal-27, and SCC-25 cells were analyzed for mean baseline TrxRed activity (a) and mean total GSH content (b) and compared to their respective mean surviving fraction in response to 5 μM PER (FaDu and Cal-27) or 10 μM PER (SCC-25).

sensitive to the Akt inhibitor PER and SCC-25 cells with relatively low pAkt expression were resistant to PER, it is possible that tumors with high pAkt expression are more susceptible to Akt pathway inhibitors than tumors with low pAkt expression.

Activation of the Akt pathway is crucial for cell survival and cellular redox status is involved in the reversible activation and inactivation of this pathway [4, 31, 32]. For example, moderate levels of ROS activate Akt pathway signaling and promote cell survival, but high or chronic oxidative stress inhibits this pathway resulting in apoptosis [4, 31–34]. Since cancer cells are under increased metabolic oxidative stress compared to normal cells and the Akt pathway may be activated for survival under these oxidizing conditions, we proposed that inhibition of the Akt pathway with PER would increase oxidative stress to such an extent that would render cancer cells sensitive to further increases in oxidative stress.

We investigated the effects of PER on oxidative stress by analyzing glutathione (GSH/GSSG) levels. The glutathione system is a major intracellular redox buffer in the cell and is involved in the detoxification of H_2O_2 and organic hydroperoxides [27] and the ratio of GSH to GSSG can be used as a reflection of intracellular redox status [27]. PER induced significant increases in %GSSG in all 3 cell lines compared to control cells (Figure 3(c)) which indicated an increase in oxidative stress and suggests that inhibition of Akt may be involved in increasing oxidative stress. To further support this idea, the thiol antioxidant NAC was able to completely suppress the increase in %GSSG in all 3 cell lines (Figure 3(c)). Additionally, NAC was able to partially but significantly reverse the cytotoxicity induced by PER in FaDu cells suggesting that increased oxidative stress was involved in PER-induced cytotoxicity in this cell line (Figure 3(d)).

To further probe the role of GSH in the effects of PER, we used BSO, an inhibitor of glutamate cysteine ligase, which is believed to be the rate-limiting enzyme in the synthesis of GSH [35, 36] in Cal-27 and SCC-25 cells. Previous studies in our laboratory have shown that BSO significantly depleted GSH pools in breast and head and neck cancer cells while sensitizing cancer cells to chemotherapy agents [37, 38]. BSO has also been used in clinical trials for cancer therapy to enhance the cytotoxicity of chemotherapeutic agents [39]. In the present studies, BSO was found to significantly increase the cytotoxicity induced by PER in Cal-27 cells (Figure 4(a)). As expected, BSO significantly decreased total GSH levels and increased %GSSG in Cal-27 cells as a single agent and in combination with PER (Figure 4(b)), which suggests that inhibition of GSH synthesis further enhanced the oxidative stress induced by PER and further sensitized these cells to the toxicity of PER in Cal-27 cells.

Overall SCC-25 cells were more resistant to PER treatment than FaDu and Cal-27 cells (Figures 1 and 2). We also observed that BSO was not as effective in SCC-25 cells as in Cal-27 cells at sensitization to PER (Figure 4(a)), which was evident in the lack of significant GSH depletion in this cell line (Figure 4(b)). Although there was a trend toward an increase in %GSSG in BSO + PER compared to PER alone in SCC-25 cells (Figure 4(c)), this increase was not significant and was not nearly as great as the BSO + PER-induced increase in %GSSG observed in Cal-27 cells (Figure 4(c)). These observations suggest that PER-induced cytotoxicity in SCC-25 cells is less dependent on the glutathione/glutathione peroxidase system than in Cal-27 cells. Preliminary experiments in our laboratory support these observations and show that PER induced an increase in GPx activity in Cal-27 cells but not SCC-25 cells (data not shown). Therefore, we propose that other antioxidant systems, such as the thioredoxin (Trx) system may be involved in PER-induced cytotoxicity in SCC-25 cells.

The Trx system is a highly conserved ubiquitous system comprised of thioredoxin reductase (TrxRed), thioredoxin (Trx), thioredoxin peroxidases (a.k.a., peroxiredoxins), and NADPH [40]. The Trx system plays an important role in the redox regulation of multiple intracellular processes and

resistance to cytotoxic agents that induce oxidative stress [41, 42]. TrxRed is a selenocysteine-containing protein that catalyzes the reduction of Trx using NADPH as a reducing agent [40]. TrxRed has been shown to initiate signaling pathways in response to oxidative stress that play a role in protecting the cell from oxidative stress and is therefore believed to be a potential target for cytotoxic agents that induce oxidative stress [40, 43, 44].

To investigate the role of Trx metabolism in SCC-25 cells we used Auranofin (S-triethylphosphinegold(I)-2,3,4,6-tetra-O-acetyl-1-thio-b-Dglucopyranoside (AUR)), which is a relatively specific inhibitor of TrxRed (Table 1). AUR belongs to the gold(I)-based drug class utilized in the treatment of rheumatoid arthritis [45] and has been shown to stimulate the mitochondrial production of hydrogen peroxide [46, 47]. AUR significantly sensitized SCC-25 cells to PER (Figure 4(d)) to a greater extent than BSO (Figure 4(a)), which was comparable to the PER-induced cytotoxicity seen in Cal-27 (Figure 2(d)). Interestingly, AUR did not significantly sensitize Cal-27 cells to PER (Figure 4(d)). This further supports the idea that the Trx pathway was more involved in PER-induced cytotxicity in SCC-25 cells than Cal-27 cells, in which the GSH pathway appeared to be more important.

We expected that BSO or AUR would sensitize Cal-27 and SCC-25 cells to PER to a greater extent than what we observed in Figures 4(a) and 4(d) based on the fact that both BSO, AUR and PER all induce oxidative stress as single agents and should be mechanistically linked. However, we do acknowledge that more extensive analysis of dose responses for BSO + PER and AUR + PER is needed (i.e., isobologram analysis) and our data shown in Figure 4 does not address this issue of mechanistic linkage. On the other hand, our data strongly suggests the opposite, in that BSO or AUR in combination with PER may be mechanistically unlinked since BSO + PER (in Cal-27 and SCC-25) and AUR + PER (in SCC-25) appear to be additive (Figures 4(a) and 4(d)). BSO inhibits the synthesis of GSH, AUR inhibits TrxRed activity and PER inhibits Akt pathway signaling, therefore, the mechanism of action of these agents is not linked. However, BSO, AUR and PER all have significant effects on oxidative stress which further justifies the need for more extensive dose response analysis of these drugs to determine additivity or synergy.

It is important to note that SCC-25 cells possess twice as much TrxRed activity as Cal-27 cells and that Cal-27 cells possess about twice the amount of total GSH compared to SCC-25 cells (Table 1, Figure 4). This led us to propose that total GSH and TrxRed activity may predict response to PER in head and neck cancer cells. To gain some insight into this relationship we plotted TR activity and total GSH of each cell line at control levels against the surviving fraction in response to PER (Figure 5). The results suggested that high TrxRed activity and low total GSH (as in SCC-25 cells) rendered the cells resistant to PER treatment, while low TrxRed activity and high total GSH (as in FaDu cells) rendered cells sensitive to PER (Figure 5). These results support data by Ceccarelli et al. [48], who showed that lung cancer cells with high Trx expression levels had a more aggressive phenotype, but were more sensitive to Trx inhibition than cells with low Trx expression levels. These results also may account for why TrxRed is upregulated in many tumor cells and supports the speculation that drug-resistant cells may be more susceptible to the inhibition of TrxRed to promote cytotoxicity and susceptibility to other chemotherapeutic drugs. Additionally, our preliminary experiments have shown higher GPx activity in FaDu cells compared to Cal-27 and SCC-25, and GPx activity appeared to correlate with sensitivity to PER similar to that shown in Figure 5(b) (data not shown). We are currently repeating these experiments, but these encouraging results further suggest that there may be a higher level of GSH metabolism in FaDu cells compared to SCC-25 cells.

Overall, the data provided here support the conclusion that PER induces oxidative stress in HNSCC cells and disrupting thiol antioxidant metabolic pathways enhances susceptibility to PER via oxidative stress. These data also support the speculation that TrxRed activity and total GSH levels may help to predict response to PER. Finally, these data provide a biochemical rationale for the use of inhibitors of GSH and Trx metabolism in combination with PER to enhance susceptibility of HNSCC cells to clonogenic cell killing by PER in combined modality cancer therapies.

Abbreviations

PER: Perifosine
AUR: Auranofin
BSO: L-buthionine-[S,R]-sulfoximine
NAC: N-acetyl cysteine
GSH: Glutathione
GSSG: Glutathione disulfide
TrxRed: Thioredoxin reductase
Trx: Thioredoxin.

Acknowledgments

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Review Article

Prognostic and Therapeutic Potential of Nuclear Receptors in Head and Neck Squamous Cell Carcinomas

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Head and neck squamous cell carcinomas are among the most common neoplasms worldwide and characterized by local tumor aggressiveness, high rate of early recurrences, development of metastasis, and second primary cancers. Despite modern therapeutic strategies and sophisticated surgical management, overall survival-rates remained largely unchanged over the last decades. Thus, the need for novel treatment options for this tumor entity is undeniable. A key event in carcinogenesis is the uncontrolled modulation of genetic programs. Nuclear receptors belong to a large superfamily of transcription factors implicated in a broad spectrum of physiological and pathophysiological processes, including cancer. Several nuclear receptors have also been associated with head and neck cancer. This review will summarize their mode of action, prognostic/therapeutic relevance, as well as preclinical and clinical studies currently targeting nuclear receptors in this tumor entity.

1. Introduction

Most malignancies of the upper aerodigestive tract (Figure 1), comprising the naso-, oro-, hypo-, and laryngopharynx, are squamous cell carcinomas. Head and neck squamous cell carcinomas (HNSCCs) are the primary tumor type in head and neck cancer (HNC), characterized by local tumor aggressiveness, high rate of early recurrences, metastasis, and development of second primary tumors, which are the major cause of morbidity and mortality in HNSCC (details in [1–4]). More than 90% of HNC cases are induced by chronic exposure to carcinogens enclosed in all forms of tobacco, synergized by heavy alcohol consumptions and poor diet (see [5, 6]). It is estimated that about 5%–10% of suspicious lesions arising in the mucous membranes of the mouth, pharynx, and larynx undergo malignant transformation. Cure rates of early disease (stage I and II) range between 70% and 80%, and chemoprevention strategies seem promising to control potentially malignant oral lesions (reviewed in [1–3]). However, long-term survival rates, especially for advanced HNC, have not improved significantly over the last decades. Despite modern therapeutic strategies and sophisticated surgical management of the tumor, the estimated five-year survival rate for advanced disease (30%–40%) remains poor ([1–3] and references therein). Currently, rational therapeutic strategies targeting growth factor receptors by specific antibodies or kinase inhibitors have gained increasing clinical relevance in particular for the treatment of locally advanced cancer with the intent of preserving speech and swallowing (see [1–3]). Thus, developing new therapeutic strategies and defining novel target proteins for the treatment of advanced HNC is of particular importance.

In this respect, nuclear receptors (NRs) are transcription factors implicated in cancer development and are recently attracting major interest as therapeutic targets (see [7, 8]). As NRs modulate cell proliferation, apoptosis, invasion, and migration, clearly representing hallmarks of cancer cells, several highly successful cancer drugs target this receptor family [8–11]. Since several NRs have been shown to be expressed also in head and neck cancer cells, NRs are most likely also contributing to HNSCC development and progression [12, 13].

NRs belong to a large superfamily of transcription factors and based on sequence comparison are currently classified into seven subfamilies (Table 1). These transcription factors

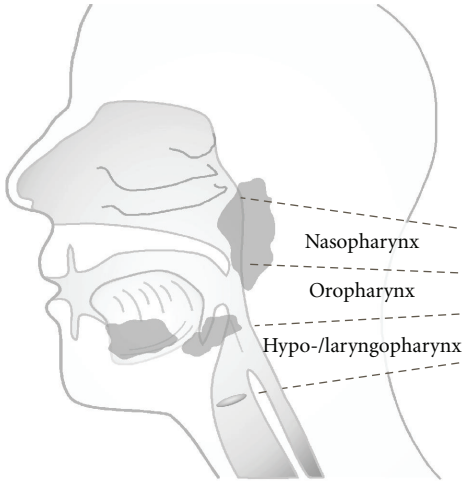

Figure 1: *Schematic anatomy of the head and neck region.* Head and neck cancer includes different types of malignancies that can develop in the mouth, nose and throat.

are able to modulate transcription of a variety of target genes by several distinct mechanisms, including both transcriptional activation and repression [7, 8, 14, 15]. Transcriptional regulation can either be ligand-dependent or -independent, genomic or nongenomic, allowing NRs to mediate gene repression or its release, gene activation, or gene *trans*-repression (details in [7, 8, 16]). In particular, the large group of so-called orphan nuclear receptors, for which natural ligands are still unknown, do not exist at all (true orphans), or have only recently been identified (adopted orphans) is adding additional complexity to the field (Table 1) ([8, 17], and references within).

In contrast to cell surface growth factor receptors, such as the epidermal growth factor receptor (EGFR), which activate genetic programs through complex intracellular signaling cascades, NRs are able to directly bind to specific DNA-sequences, so-called hormone response elements (HREs). Thus, NRs are composed of an N-terminal regulatory domain (activation function 1 = AF1), followed by a DNA-binding domain (DBD), a ligand-binding domain (LBD), and another C-terminal regulatory domain (activation function 2 = AF2) (Figure 2) [7, 8]. Despite their conserved structural organization, the biological functions of NRs are highly diverse. Nevertheless, two major modes of NR action can be assigned, depending on their intracellular steady-state localization in the absence of ligands (Figure 3). One group of NRs is confined to the cytoplasm within multiprotein-complexes in the absence of ligand. Upon ligand binding, they actively enter the nucleus and bind to HREs as homo- or heterodimers (Figure 4, details in [7, 8]). Other NRs already reside in the nucleus in a complex with corepressor proteins, while ligand binding triggers corepressor dissociation allowing the recruitment of coactivators [18, 19]. However, in order to fulfill multiple biological tasks minor to major deviations from these two modes of NR action exist [7, 8].

NRs are not only implicated in a broad spectrum of physiological processes but are associated with many human diseases including metabolic and cardiovascular disorders as well as cancer. Beside their proven clinical relevance for hormone regulated malignancies, there is rather limited information on their pathophysiological role as well as their prognostic and therapeutic potential for head and neck cancer [7, 8, 12, 20–22]. Most studies were investigating members of two classes of the NR superfamily, the thyroid hormone receptor-like and the estrogen receptor-like receptors (Table 1). Thus, we will focus on relevant members of these subfamilies, summarize their potential diagnostic/prognostic value, and discuss their therapeutic potential.

2. Thyroid Hormone Receptor-Like Receptors

2.1. Peroxisome Proliferator-Activated Receptors. Within the thyroid hormone receptor-like receptor subfamily, the peroxisome proliferator-activated receptors (PPARs) show the highest disease relevance for HNSCC. To date, three isoforms of the PPAR ($\alpha, \beta/\delta$, and γ) have been identified, all able to form heterodimers with retinoid X receptors (RXRs) (see [23, 24]). PPARs are expressed in different cell types and activate the transcription of several genes involved in a variety of biological processes, including lipid metabolism and insulin sensitivity (see [23, 24]). Furthermore, a role in limiting inflammation has also been reported [24, 25]. As tumor cell metabolism and inflammation appear to be critical for tumorigenesis and clinical outcome, NRs may thus directly or/and indirectly modulate malignancies [26, 27]. As such, PPARγ is overexpressed in many epithelial malignancies [22, 28, 29] including oral squamous cell carcinoma [30].

In the absence of ligand, PPARs are complexed with corepressor proteins, thus acting as transcriptional repressors. Ligand binding induces conformational changes facilitating heterodimerization with RXR, thus leading to the attraction of transcriptional coactivators (Figures 3 and 4) (see [19, 24]). Natural and synthetic ligands for PPARs include lipophilic molecules such as fatty acids and eicosanoids as well as thiazolidinedione (TZD) drugs and derivates thereof (overview in [7, 24, 31]). PPARγ ligands seem to exert their effects in a dosage-dependent manner [32], although the detailed mechanism is currently not yet resolved. The postulated cancer modulating mechanisms are diverse, including effects on Wnt signaling, inhibition of NFκB, as well as the modulation of cell cycle regulators and pro- and antiapoptotic proteins, which have been linked with head and neck cancer (see [4, 23]).

Clinical Aspects of Peroxisome Proliferator-Activated Receptors in HNSCC. In HNSCC, overexpression on the protein level has been convincingly demonstrated for PPARβ and PPARγ [12, 30, 33]. Agonist binding to PPARβ can induce cell differentiation, growth arrest, and apoptosis of cancer cells [34]. Additionally, such activating PPARβ ligands were shown to exert antiproliferative on human colon and breast cancers (details in [23, 24]) and were also suggested as potential chemopreventive agents for oral carcinogenesis [12, 35, 36]. Of note, since at least 1.6 million patients

Table 1: Current classification of the NR superfamily into subfamilies according to sequence homology. Trivial abbreviations are given in brackets. NRs implicated in head and neck tumorigenesis are given in bold; asterisks indicate orphan receptors.

Subfamily	Full name	Subfamily members (trivial abbreviation)
Subfamily 1	Thyroid hormone receptor-like receptors	
Subgroups	**Peroxisome proliferator-activated receptors**	**Peroxisome proliferator-activated receptor (PPAR) $\alpha, \beta/\delta, \gamma$**
	Retinoic acid receptors	**Retinoic acid receptor (RAR) α, β, γ**
	Retinoic acid receptor-related orphan receptors	Retinoic acid receptor-related orphan receptor (ROR) α, β, γ
	Rev-ErbA*	Rev-ErbA (EAR1) α, β
	Thyroid hormone receptors	Thyroid hormone receptor (TR) α, β
	Liver X receptor-like receptors*	Liver X receptor (LXR) α, β; Farnesoid X receptor (FXR)
	Vitamin D receptor-like receptors	Vitamin D receptor (VDR); Pregnane X receptor (PXR); Constitutive androstane receptor (CAR)
Subfamily 2	Retinoid X receptor-like receptors	
Subgroups	Hepatocyte nuclear factor-4	Hepatocyte nuclear factor-4 (HNF-4) α, γ
	Retinoid X receptors	Retinoid X receptor (RXR) α, β, γ
	Testicular receptors*	Testicular receptor 2, 4 (TR2/4)
	Tailless-like receptors*	Human homologue of the Drosophila tailless gene (TLX); Photoreceptor cell-specific nuclear receptor (PNR)
	Chicken ovalbumin upstream promoter-transcription factor-like receptors*	Chicken ovalbumin upstream promoter-transcription factor (COUP-TF) I, II; V-erbA-related (EAR2)
Subfamily 3	Estrogen receptor-like receptors	
Subgroups	**Estrogen receptors**	**Estrogen receptor (ER) α, β**
	Estrogen related receptors*	Estrogen-related receptor (ERR) α, β, γ
	3-Ketosteroid receptors	**Androgen receptor (AR); Progesterone receptor (PR);**Glucocorticoid receptor (GR); Mineralocorticoid receptor (MR);
Subfamily 4	Nerve growth factor IB-like receptors	
	Nerve Growth factor IB/ Nuclear receptor related/ Neuron-derived orphan receptor*	Nerve Growth factor IB (NGF-IB); Nuclear receptor related 1 (NURR1); Neuron-derived orphan receptor 1 (NOR1)
Subfamily 5	Steroidogenic factor-like receptors	
	Steroidogenic factor/Liver receptor homolog*	Steroidogenic factor 1 (SF1); Liver receptor homologue-1 (LHR1)
Subfamily 6	Germ cell nuclear factor-like receptors	
	Germ cell nuclear factor*	Germ cell nuclear factor (GCNF)
Subfamily 0	Miscellaneous receptors	
	Dosage-sensitive sex reversal, adrenal hypoplasia critical region/Small heterodimer partner*	Dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1 (DAX); Small heterodimer partner (SHP)

take antidiabetic drugs that function as PPARβ ligands, epidemiological data on their long-term effects on tumor prevention would therefore be of value to rationally design cancer chemoprevention trials.

Paradoxically, not only PPARγ agonists are considered as potential therapeutic agents in cancer therapy but also antagonists were studied in this respect [30]. PPARγ inhibition was shown to induce apoptosis and anoikis and inhibit tumor cell invasion in squamous cell carcinomas [30]. Moreover, the results of several studies indicated that the growth-inhibiting activity of PPARγ ligands in OSCC may be PPARγ independent [37]. Others showed that the observed effects were strongly dependent on PPARγ-expression [12, 38] as well as on the type and concentration of the agonist [39].

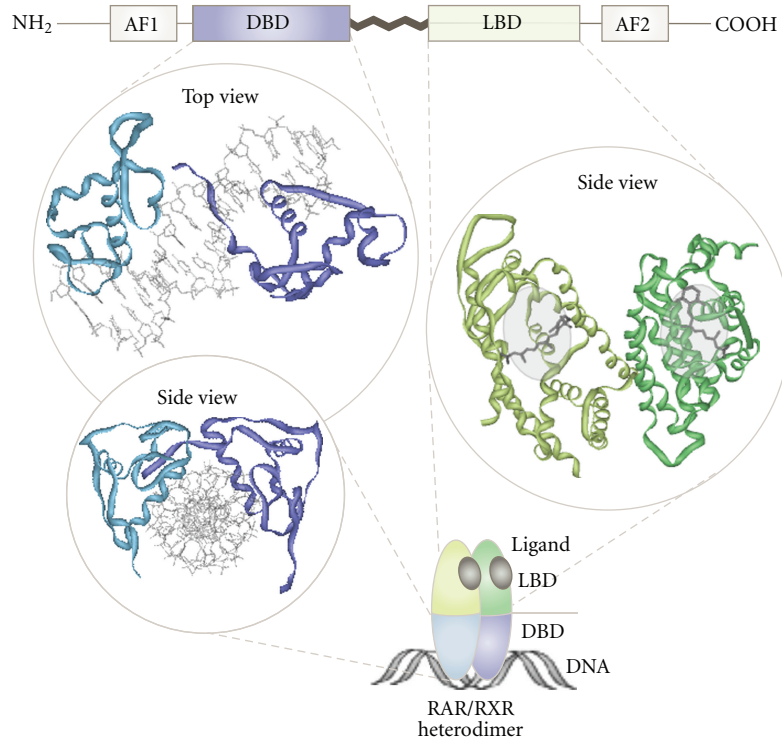

Figure 2: *Domain organization and structural binding modes of NRs.* Upper panel: NRs are composed of an N-terminal regulatory domain (activation function 1 = AF1), followed by a DNA-binding domain (DBD), a ligand-binding domain (LBD), and a C-terminal domain (activation function 2 = AF2). Left panel: 3D model illustrating how the DBDs of the RAR/RXR heterodimer (PDB 1DSZ) interact with their target DNA-sequence. Right panel: solid ribbon representation illustrating the LBD of the RAR/RXR heterodimer (PDB 1DKF) complexed with the ligands 9-*cis*-RA for RXR (PDB 3LBD) and ATRA for RAR (PDB 2LBD). PDB files are taken from the RCBS Protein Data Bank (http://www.pdb.org).

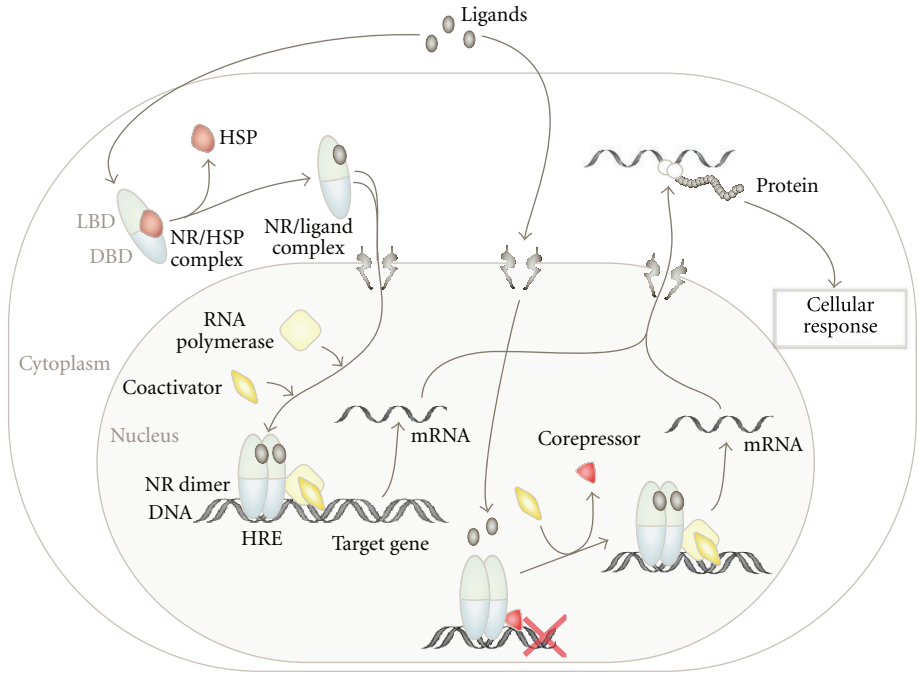

Figure 3: *Simplified model illustrating the two major modes of NR activation.* Natural or synthetic ligands diffuse through the cell membrane and bind to cytosolic or nuclear NRs. Ligand binding to cytoplasmic NRs triggers conformational changes resulting in dissociation of heat shock proteins (HSPs) and receptor dimerization, allowing active nuclear import and transactivation by binding to HREs. Other NRs are constitutively nuclear and complexed with corepressors in the absence of ligands. Ligand binding induces conformational changes resulting in the recruitment of coactivators to activate transcription of target genes.

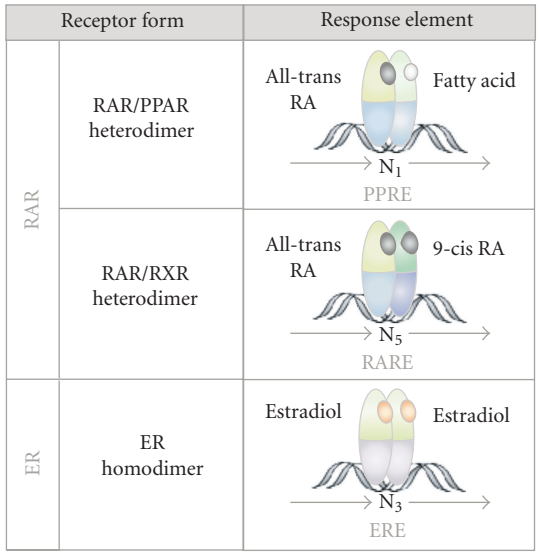

Figure 4: *DNA-binding modes of NRs implicated in HNSCC.* RAR can heterodimerize with PPARs, which can be activated by lipophilic ligands. Alternatively, RARs are able to heterodimerize with RXRs, which are activated by 9-*cis* RA. Such heterodimers can bind to specific half-site retinoic acid (RARE) or peroxisome proliferator response elements (PPREs) direct repeats in the DNA of target genes. Estradiol binding induces estrogen receptor homodimerization and binding to palindromic half-site estrogen response element (ERE) inverted repeats. N: Any nucleotide occurring within the specific response element.

In the majority of OSCC cases, PPARγ mRNA could be detected by RT-PCR [37]. By immunohistochemical analysis of primary tumors, PPARγ was often found in low-grade tumors, especially in tumor endothelium [38], and a favorable impact of PPARγ expression on relapse-free survival of the patients could be demonstrated [40].

The beneficial effects of PPARγ ligands on malignancies were tested in several clinical trials, but outcomes proved to be highly diverse. Some trials revealed 40% partial response rates, whereas others could not show any significant beneficial effect [41, 42].

Moreover, one may speculate that the tumor modulating effects of PPARγ ligands are mediated indirectly by affecting the tumor microenvironment, such as cancer-associated fibroblasts or tumor endothelial cells [43]. In fact, PPARγ ligands have been shown to affect endothelial cell proliferation and migration and hence to regulate angiogenesis [44]. Also hypoxia-induced angiogenesis appears to be affected by PPARγ ligands in cancer therapy, even if the precise mechanisms still remain unclear [45]. As angiogenesis is a crucial aspect for tumor development, therapy resistance and metastasis and inhibition of angiogenesis may hence have contributed to the clinical benefit observed.

In sum, PPARγ ligands appear to be of clinical benefit for the treatment of head and neck cancer, in particular for OSCC. Nevertheless, a more detailed molecular knowledge on PPARγ biology is clearly required. Increasing knowledge about the mode of action, specificity, and dosage-dependence of PPAR agonistic and antagonistic ligands will hopefully allow a better modeling of PPAR receptor function and thus lead to a more effective design of combinatorial application schemes for cancer treatment and cancer prevention in the future.

2.2. Retinoid Acid Receptors. Another group of thyroid hormone receptor-like receptors implicated in HNSCC is the retinoid acid receptors (RARs). RARs are characterized by their activation via vitamin A derivatives. Upon activation, RARs are able to heterodimerize with retinoid X receptors (RXR) and to bind to specific hormone response elements (HREs), thereby modulating transcription of target genes (Figures 3 and 4) [8, 26, 46]. To date, a variety of coactivator- and corepressor-proteins have been identified, allowing the fine-tuning of target gene transcription, ranging from repression to full activation [18]. However, the molecular details are just beginning to be uncovered [8, 26, 46]. RAR activation often leads to differentiation [47], cell-cycle arrest [48], or apoptosis [49], culminating in the inhibiting of tumor growth. Hence, its ligand retinoid acid (RA) or derivates thereof are currently tested as therapeutic agents in several cancer types (Table 2) [50]. Paradoxically, in some malignancies RA rather promotes cell survival, which may be due to the ability of RA to also activate PPARs, and as a consequence expression of prosurvival genes is induced [46]. Schug et al. could also show that the channeling of RA between these two nuclear receptor heterodimers is mediated by the cytoplasmic RA transporters CRABP2 and FABP5 and thus is strongly depending on the FABP5/CRABP2 ratio [46]. Thus, the channeling of RA to different receptor heterodimers appears to be crucial for the regulation of cell-proliferation, positively or negatively affecting tumor growth [46]. Interestingly, both proteins were found differentially expressed in metastatic and HPV-associated HNSCC, but their biological and clinical effects remain to be investigated [51, 52].

An additional way of biological regulation is epigenetic modulation playing an important role in cancer development (reviewed in [53]). Gene silencing caused by aberrant hypermethylation of CpG islands has not only been detected in promoter regions of several tumor suppressor genes [53], but several studies show hypermethylation of the RARβ promoter in colon, breast, and lung cancers [54, 55]. In head and neck carcinogenesis, hypermethylation of the RARβ promoter was found to be indeed associated with RARβ downregulation and hence appears to be biologically relevant [56].

Clinical Aspects of Retinoid Acid Receptors in HNSCC. As outlined above, a rationale for the use of retinoids in chemoprevention and cancer therapy was provided experimentally by different cellular [57] and animal models [58]. Moreover, this strategy was supported by epidemiological data as well as by clinical trial outcomes [26, 59–61]. Several clinical chemoprevention trials including patients with increased risk for developing cancer have shown that treatment with retinoids resulted in the suppression of

Table 2: Overview of current clinical trials in the field of HNSCC targeting NRs. The NCI protocol ID is given in bold (for further details see: http://www.cancer.gov/CLINICALTRIALS).

NR	Clinical trial / identifier	Drug	Tumor entity	Phase
PPAR	Pioglitazone in Preventing Head and Neck Cancer in Patients With Oral Leukoplakia/NCT00099021	Pioglitazone	Head and Neck Cancer *Precancerous /Nonmalignant Condition*	Phase II ongoing
	Rosiglitazone in Preventing Oral Cancer in Patients With Oral Leukoplakia/NCT00369174	Rosiglitazone	Head and Neck Cancer *Precancerous /Nonmalignant Condition*	Phase II completed
RAR	Chemoprevention Study of Oral Cavity Squamous Cell Carcinoma/NCT00201279	13-cis Retinoic acid	Oral Cavity Squamous Cell Carcinoma	Phase III completed
	Isotretinoin Plus Interferon in Treating Patients With Recurrent Cancer/NCT00002506	Isotretinoin (combined with Interferon a)	Head and Neck Cancer Esophageal Cancer	Phase II ongoing
	Isotretinoin, Interferon Alpha, and Vitamin E in Treating Patients With Stage III or Stage IV Head and Neck Cancer/NCT00054561	Isotretinoin (combined with Interferon a and Vitamin E)	Head and Neck Cancer	Phase III completed
ER	Combination Chemotherapy and Tamoxifen in Treating Patients With Solid Tumors/NCT00002608	Tamoxifen (combined with Cisplatin and Doxorubicin)	Head and Neck Cancer	Phase II completed

precancerous lesions (see [26, 60]). Also, certain retinoids inhibited the development of second primary tumors in patients who had been previously treated for an early-stage cancer but remained at high risk to relapse ([26, 60] and references within). However, other studies using isotretinoin or other retinoids (e.g., retinyl palmitate) did not observe any benefit in second primary tumor development, recurrence, or mortality of HNSCC or lung cancer [26, 62]. Current trials (Table 2) are therefore aiming to resolve these controversies by recruiting appropriate study populations as well as by the use of novel drugs and improved treatment protocols.

Reduced RARβ mRNA levels have been observed not only in several malignant tumors ([26, 56] and references therein) but also in premalignant oral lesions ([63] and references within). Unfortunately, until recently no antibodies convincingly detecting RARβ were available. Thus, most of the studies demonstrating RARβ downregulation were based on in situ hybridization and could therefore only show a decrease in the amount of mRNA. Ralhan et al. were the first to demonstrate decreased expression of the RXRα and RAR$\alpha/\beta/\gamma$ on protein level correlating with different stages of OSCC development and progression [64]. The molecular mechanism leading to downregulation or loss of RARβ is poorly understood, but it was suggested that expression of RARβ could depend on the intracellular level of retinoids [26]. Several studies demonstrated a decrease in the amount of RARβ during vitamin A deficiency as well as its upregulation by RA. Additionally, there is evidence that retinoic acid induces the expression of RARβ mRNA in certain cell lines, but not in the malignant counterparts of these cells. Thus, transformed cells may have developed an aberrant response to retinoic acid due to the deregulated expression of coactivator/repressor proteins [26]. Ralhan et al. found a significant association between the increase in RARβ mRNA levels and clinical responses of premalignant oral lesions to isotretinoin [65, 66]. Hence, RARβ indeed seems to contribute to the suppression of the premalignant phenotype and malignancy and may be causally linked to the clinical outcome in chemoprevention trials with retinoids [26, 67]. If so, RARβ may indeed serve as a useful diagnostic marker in retinoid trials (Table 2) for the prevention of oral carcinogenesis [68]. RAR modulation by its agonist ligand all-*trans* retinoic acid (ATRA) represents a successful example of how targeting of an NR contributes to an impressive clinical benefit in liquid tumors ([26] and references therein). Lessons learned from these studies clearly show that the therapeutic benefit could be further enhanced by combining ATRA with chromatin modulating agents, such as histone deacetylase inhibitors [69]. Nevertheless, the design of receptor specific drugs as well as an in depth understanding of the molecular regulation of RAR biology is required in order to fully exploit its therapeutic benefit and minimize potential side-effects in the area of head and neck cancer [7, 26].

3. Estrogen Receptor-Like Receptors

This subfamily is composed of the estrogen receptors (ERα and ERβ), the estrogen-related receptor, and the 3-ketosteroid receptors [10].

Table 3: Nuclear receptor target genes playing pivotal roles in diverse biological processes and cellular homeostasis were described to be differentially expressed in head and neck cancer.

NR	Target gene	Function	Reference - target gene	Reference - head and neck
PPAR	**G0S2**	Cell cycle	Zandbergen et al. *Biochem J* 2005	Tokumaru et al. *Cancer Res* 2004
	PDK1	Energy homeostasis	Degenhardt et al. *J Mol Biol* 2007	Wigfield et al. *Br J Cancer* 2008
RAR	**p21WAF1/CIP1**	Cell cycle	Liu et al. *J Biol Chem* 1996	Kapranos et al. *Anticancer Res* 2001
	BIRC5/surviving	Apoptosis	Pratt et al. *J Cell Biochem* 2003	Engels et al. *J Pathol* 2007
	C/EBPε	Transcription factor	Schwarz et al. *Mol Cell Biol* 1997	Bennett et al. *Cancer Res* 2007
	CRABP	Carrier protein	Nezzar et al. *Mol Vis* 2007	Won et al. *Metabolism* 2004
	cyclins, **CDK**	Cell cycle	Bour et al. *Trends Cell Biol* 2007	Jeannon et al. *Clin Otolaryngol Allied Sci* 1998
ER	**c-Myc**	Transcription factor	Markaverich et al. *Steroids* 2006	Pries et al. *Int J Mol Med* 2008
	Cyclins	Cell cycle	Eeckhoute et al. *Genes Dev* 2006	Nakashima et al. *Eur Arch Otorhinolaryngol* 2005 Lotayef et al. *Br J Cancer* 2000
	CRABP	Carrier protein	Li et al. *J Biol Chem* 2003	Vo et al. *Anticancer Res* 1998
	CXCl12/SDF-1	Chemokine/ligand	Hall et al. *Mol Endocrinol* 2003	Rehman et al. *J Biol Chem* 2008
	cathepsin D	Protease	Bretschneider et al. *Mol Oncol* 2008	Strojan et al. *Anticancer Res* 2000

Besides the estrogen receptors themselves, many of the genes regulated by the ER/estrogen-axis are critical for cell proliferation, inhibition of apoptosis, stimulation of invasion and metastasis, as well as for the promotion of angiogenesis (see [10, 11] and references within). Since these processes clearly state hallmarks of cancer cells, it is well accepted that ERs are implicated in various cancer types [9, 21]. Sex hormone receptors are expressed not only in sexual organs but, amongst others, also in the vascular epithelium [70], the lung epithelium [71], and the larynx [72]. The expression of sex hormone receptors could also be demonstrated in HNSCC by several studies [12, 13]. Both ER isoforms as well as the progesterone receptor (PR) were detectable in cancer cells of the oral cavity, the salivary gland, and in laryngeal/hypopharyngeal cancers, whereas the tumor stroma was mostly negative [12, 13]. Expression of ERα inversely correlated with that of ERβ in esophageal carcinoma, and a correlation of ERβ levels with tumor dedifferentiation and staging was suggested [73, 74].

Clinical Aspects of Estrogen Receptors in HNSCC. Considering the impressive benefit of endocrine therapy in breast cancer, targeting sex steroid hormone receptor as a potential therapeutic strategy is also discussed for HNSCC [12, 75]. Currently, two main strategies are pursued in endocrine therapy of ER-positive tumors. One is based on steroidal antiestrogens like tamoxifen, which bind to the ER, block its function, and ultimately induce receptor degradation [8, 11]. The other is based on aromatase inhibitors and luteinizing hormone-releasing hormone agonists, which reduce the level of circulating estrogen, thereby inhibiting ER activation by depriving the receptor of its ligand [11]. Tamoxifen was already shown to inhibit proliferation and invasion of HNSCC cell lines, resulting in apoptosis, which could be further enhanced upon combination with cisplatin [76–78]. Thus, a therapeutic role of antiestrogens or aromatase inhibitors in the clinical management of HNSCC is currently under investigation, and the results of just completed clinical trials (Table 2) are eagerly awaited.

However, the precise molecular roles and impact of estrogen receptor-like receptors for the onset and/or progression of head and neck cancer remain to be clarified. This knowledge will be required, in order to rationally decide whether to further investigate the potential of modern endocrine therapy also for this tumor entity.

4. Conclusion

NRs are associated with head and neck cancer and hence seem to be at least partially amenable for prevention and/or treatment strategies. So far, three NR groups have mainly been linked with HNSCC, the retinoic acid and the peroxisome proliferator-activated and the estrogen receptors. Also, target genes activated by these NR subfamilies (Table 3) have been implicated as key elements in the molecular circuits involved in head and neck cancer development and progression. Reports on other members of the NR superfamiliy are rather scarce for this tumor entity, suggesting that they have not been investigated so far. Taking the thyroid hormone receptor as an example, many studies on its relevance for various malignancies have been conducted, whereas its role in head and neck cancer, including even thyroid carcinomas, has not been analyzed in detail [79]. Likewise, data on the cancer-related biological functions of orphan NRs are still missing for this tumor entity [7, 8]. As now cancer cell metabolism is beginning to be considered as "cancer's Achilles' heel", it may be conceivable to speculate that molecules present in diet, tobacco, or beetle nut might

deregulate the cell's metabolism by affecting NRs and as such contribute to head and neck carcinogenesis [27, 80]. Of note, the development of novel NR ligands with improved specificity and activity is currently intensively pursued in the area of metabolic diseases (see [7, 8, 81]). Hence, an interdisciplinary exploitation of the existing knowledge of NR pharmacobiology may result in novel HNSCC treatment approaches.

In sum, keeping in mind the enormous success of NR targeting therapeutics in several malignancies, a systematic investigation of NR biology as well as of its clinical relevance is highly desirable also for head and neck cancer. Together with the outcomes of current clinical trials (Table 2), such improved knowledge will hopefully result in strategies with improved benefit for the patient.

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Review Article

The Cancer Stem Cell Concept in Progression of Head and Neck Cancer

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Human head and neck cancer (HNC) is a highly heterogeneous disease. Understanding the biology of HNC progression is necessary for the development of novel approaches to its prevention, early detection, and treatment. A current evolutional progression model has limitations in explaining the heterogeneity observed in a single tumor nest. Accumulating evidence supports the existence of cancer stem cells (CSCs) as small subpopulations in solid tumors, including HNC. These CSCs can be selected by appropriate cell surface markers, which are cancer type specific and have been confirmed by unique *in vitro* and *in vivo* assays. Selected CSC populations maintain a self-renewal capability and show aggressive behaviors, such as chemoresistance and metastasis. In addition to introducing the CSC concept in solid tumors, this short review summarizes current publications in HNC CSC and the prospective development and application of the CSC concept to HNC in the clinic.

1. Introduction

Head and neck cancer is the sixth most common cancer and is responsible for almost 200,000 deaths around the world each year [1–3]. In the United States, head and neck squamous cell carcinoma (HNSCC) accounts for more deaths annually than cervical cancer, melanoma, or lymphoma. Although recent molecular studies have advanced our understanding of the disease and provided a rationale for the development of novel therapeutic strategies, HNSCC is still associated with severe mortality. Its 5-year survival rate has not been improved in more than 30 years [4]. In addition, the 5-year survival rate is even lower for HNSCC patients with a single homolateral lymph node metastasis (LNM) and is less than 25% for patients with bilateral LNM. Understanding the biology of HNSCC, progression will greatly assist in treatment decisions and in the development of new strategies for prevention and control of this disease.

Human neoplastic tumors, particularly HNSCC, are highly heterogeneous [5–7]. Currently, the progression of HNSCC is considered to result from evolution through stepwise alterations in multiple molecular and cellular pathways [8, 9]. However, this evolution concept has limitations in explaining the heterogeneity observed in a single tumor nest. It has been known for a long time that there are subpopulations of cells within solid tumors that contain different biological behaviors, such as metastatic potential [10, 11].

Accumulating evidence supports the subpopulation observation, particularly, the existence of so-called cancer stem cells (CSCs) [12–17]. Although CSCs in solid tumors including HNSCC have not been precisely identified, the CSC hypothesis opens a new era in understanding the initiation and progression of cancers. This short review will briefly introduce the CSC concept, summarize the current progress of CSC studies in HNSCC, and discuss the potential application of the CSC concept to the clinical management of HNSCC.

2. Cancer Stem Cell Concept

CSCs are defined as a small subset of cancer cells that constitute a pool of self-sustaining cells with the exclusive ability to maintain the tumor. Currently, there are two

hypothetical explanations for the existence of CSCs. CSCs may arise from normal stem cells by mutation of genes that render the stem cells cancerous. Or, they may come from differentiated tumor cells that experience further genetic alterations and, therefore, become dedifferentiated and acquire CSC-like features.

The CSC concept is "an old idea reemerging at an important time" [12]. If the CSC hypothesis is true, many aggressive behaviors of cancer cells, such as chemoresistance and metastasis, may be better understood. Current CSC research is focusing on the identification of CSC in solid tumors, since stem cells in hematopoietic malignancies such as leukemia have been well characterized [12–16]. However, many difficulties are encountered when exploring the existence of CSCs in solid tumors, due to the inaccessibility of tumor cells and the lack of appropriate functional assays [17]. An important breakthrough in the study of solid tumor CSCs was the identification of breast cancer CSCs and their biomarkers by Clarke and his colleagues in 2003 [18]. Since then, CSCs have been reported in neoplasms of brain, prostate, lung, colon, pancreas, liver, melanoma, and skin [19–33]. Among them, the breast CSC model with well-defined biomarkers is more advanced than in other types of cancers [34–36]. Using this model, molecular signatures and signaling pathways have been further explored [34, 37].

There are three main characteristics that define CSCs: (1) differentiation, which provides the ability to give rise to a heterogeneous progeny, (2) self-renewal capability that maintains an intact stem cell pool for expansion, and (3) homeostatic control that ensures an appropriate regulation between differentiation and self renewal according to the environmental stimuli and genetic constraints of each organ tissue, which accounts for the tissue specificity of CSCs. Currently, xenograft assays for different organ sites have been established for testing CSCs. As suggested by the AACR Workshop on Cancer Stem Cells in 2006, the orthotopic xenograft assay is considered the golden standard for the identification of CSCs [12]. This type of assay allows reliable testing for all three characteristics of CSCs. In current studies, cancer cells from either tumor tissues or cell lines are initially sorted by specific cell surface markers. The selected cell population is then injected into experimental animals for tumorigenesis testing. If as few as 100–500 cells of the selected cell population are tumorigenic, the featured cell surface markers can serve as CSC-specific biomarkers. In a breast cancer study by Al-Hajj et al. [38], human breast cancer tissues or cells with or without expression of CD44 and CD24 were injected into the mammary fat pad of immune-deficient nonobese diabetic/severe combined immune-deficient (NOD/SCID) mice, which have greater immune deficiency than nude mice. Using this model, the breast CSC-specific biomarkers $CD44^+/CD24^-$ were determined. Similar xenograft assays in NOD/SCID mice were used to identify CSCs of brain, colon, and lung with a $CD133^+$ profile [19, 21, 39–41]. Not only the NOD/SCID mouse models but also nude mice are choices for an orthotopic xenograft assay. Visvader and Lindeman have recently summarized mouse models and CSC markers used for isolation of CSC, including CD133, CD44, ALDH1A1, and epithelial cell adhesion molecule (EpCAM) [17]. As shown in Table 1, there is no universal CSC marker for all types of cancer. CSC markers may be tumor type specific, depending on the niche of each type of CSC. In addition to in vivo assays for CSC identification, many in vitro experiments have also provided evidence for the existence of CSCs. For example, studies by Collins et al. focused on a cell population in patients' tumor tissues featuring $CD44^+$/integrin$\alpha 2\beta 1^{high}/CD133^+$ [22]. These cells were examined by colony-formation and long-term serial culture assays and showed self renewal and regeneration of phenotypically mixed populations.

3. CSC-Related Cancer Progression Models

Accumulating evidence suggests that CSCs contribute not only to tumor initiation, but also to aggressive tumor behaviors such as chemoresistance and metastasis.

3.1. CSC-Like Cells Constitute Part of a Chemoresistant Population. It has been noted that although chemotherapy kills the majority of cancer cells in tumor tissues, it may leave a population of cells behind. These cells overexpress the ATP-binding casstte (ABC) drug transporters which protect cancer cells from damage by cytotoxic agents. Coincidently, a side population (SP) of tumor cells which are defined by their inability to accumulate the fluorescent dye Hoechst 33342 due to overexpression of the ABC transporter ABCG2 has been confirmed to hold CSC features in several types of cancers including hematopoietic, prostate, and glioma CSCs [42–44]. ABCG2 and other ABC transporter proteins, therefore, have served as CSC markers [45] (Table 1). Chemoresistant activity has been identified in some CSC-like cell populations. For example, a study of a colorectal cancer cell line that is resistant to 5-fluorouracil (5FU) and oxaliplatin by Dallas et al. showed 5- to 22-fold enrichment of a double CSC marker $CD133^+/CD44^+$ population [46]. Another study by Hermann et al. showed that human pancreatic cells that survived prolonged treatment with gemcitabine had a 50-fold increase in a $CD133^+$ population [32].

Considering CSCs a target population for the treatment of human cancer has opened new directions for research efforts in the field. The development of inhibitors against the ABC transporter ABCG2 has been explored in clinical studies [47]. On the other hand, targeting specifically activated signaling pathways in CSCs may provide an effective strategy to eliminate this cell population. Dallas et al. reported that chemoresistant colorectal cancer CSC-like cells showed increased expression of insulin-like growth factor-1 receptor (IGF-1R). This cell population responded to inhibition by an IGF-1R monoclonal antibody more effectively than its nonresistant counterpart [46]. Several signaling pathways, including the Wnt, TGF-β, and CXCR4 pathways, have been suggested to be activated in CSCs [17, 48, 49]. Therapeutically targeting these pathways deserves further investigation.

3.2. Migrating or Metastatic Cancer Stem Cells (mCSCs). The existence of mCSCs was first hypothesized in 2005 by Brabletz et al., based on their observations in colorectal

Table 1: Putative CSC makers in solid tumors.

CSC markers	Tumor types	% CSC markers in tumor cells	Minimal cell no. for tumor formation	Refs
$CD44^+/CD24^{-/low}$	Breast	11–35	200	[18]
$CD44^+$	Head and neck	0.1–42	5000	[57]
	Prostate	0.3–38	100	[26]
$CD44^+/EpCAM^{hi}$	Colon	0.03–38	200	[31]
$CD44^+/CD24^-/ESA^+$	Pancreas	0.2–0.8	100	[27]
$ALDH1^+$	Breast	3–10	500	[71]
$CD133^+$	Brain	6–29	100	[21]
	Brain	2-3	500	[39]
	Colon	1.8–25	200	[40]
	Colon	0.7–6	3000	[27]
	Head and neck	0.8–4.2	1000	[60]
	Pancreas	1–3	500	[32]
	Lung	0.32–22	10^4	[19]
Side population	Prostate	0.05–0.2	100	[33]
$ABCG5^+$	Melanoma	1.6–20	10^6	[30]

cancer [50, 51]. They proposed that there are two forms of CSCs in tumor progression—stationary CSC (sCSC) and mobile or migrating CSC (mCSC). They proposed that sCSCs are embedded in epithelial tissues or epithelial-based tumors and cannot disseminate. In contrast, mCSCs, which are derived from sCSC by acquiring a transient epithelial-mesenchymal transition (EMT), are located at the tumor-host interface and mediate tumor cell metastasis. In a colorectal cancer model, Brabletz et al. observed that not only the expression levels of EMT-related biomarkers but also their locations in the tumor nest were significantly associated with metastasis. They found that loss of E-cadherin (E-cad) usually resulted in nuclear localization of β-catenin, which is a typical feature of EMT, and nuclear β-catenin was accumulated in dedifferentiated tumor cells at the tumor-host interface. The authors then interpreted these observations in the context of the sCSC and mCSC hypotheses, suggesting that sCSC and mCSC are responsible for formation of the primary tumor and metastasis, respectively. Both sCSC and mCSC can lead to differentiation and tumor heterogeneity. Particularly, metastatic tumors generated from mCSC may experience a mesenchymal-epithelial transition (MET) in the metastatic organ site, which may explain why EMT can not be clearly observed pathologically in many metastatic lesions. In fact, the mCSC hypotheses can be used to explain the "heterogeneous morphology of the primary tumor and how metastases can recapitulate the heterogeneity in differentiation" and "tumor-cell dormancy and disease recurrence" [50]. Two recent publications support the mCSC hypotheses. Mani et al. reported that the stem-like cells identified in breast cancer were associated with EMT markers [49, 52]. A $CD133^+/CXCR4^+$ stem-like population isolated by Hermann et al. was suggested to be essential for metastasis of pancreatic cancer [32, 53].

3.3. Hierarchical and Stochastic Models of CSCs in Solid Tumors. Although the concept of developmental hierarchy of solid tumors has been discussed in several papers, the hypothetical hierarchical model of CSC/progenitors was clearly proposed in 2007 by Tang et al. based on their studies in prostate CSCs [43, 54]. This model described a hierarchical organization of phenotypically and functionally distinct cells at different stages of prostate tumor maturation. Their study demonstrated that a highly purified $CD44^+$ population was still heterogeneous and enriched in tumorigenic and metastatic progenitors. That is, not only CSC but also progenitors can be tumorigenic in the NOD/SCID mouse model. These two types of tumor cells share the common marker $CD44^+$, but they can be distinguished by other well-defined markers including $ABCG2^+$ and $\alpha2\beta1^+$, which are specific for tumor progenitors. Recently, Odoux et al. identified chromosomal instability that usually supports a stochastic model in the mCSC population isolated from liver metastasis of colon cancer [55]. They, therefore, proposed a new model which suggested that both stochastic and hierarchical models can be used to explain the mCSC population (Figure 1).

4. CSC Studies in HNSCC

To date, only a few studies of HNSCC CSC have been reported [56]. Using both NOD/SCID mice and Rag2/cytokine receptor common γ-chain double knockout (Rag2γDKO) mice, Prince et al., the same group that identified breast CSCs, reported that as few as 5×10^3 $CD44^+$ HNSCC cells could generate tumors in the mice and demonstrated tumor heterogeneity [57]. Examining samples from human HNSCC tissues revealed that the $CD44^+$ population varied from 0.1% to 41.7%. This cell

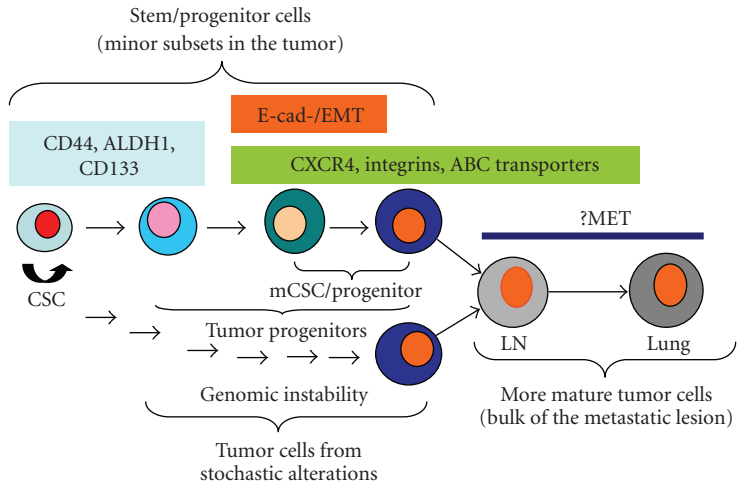


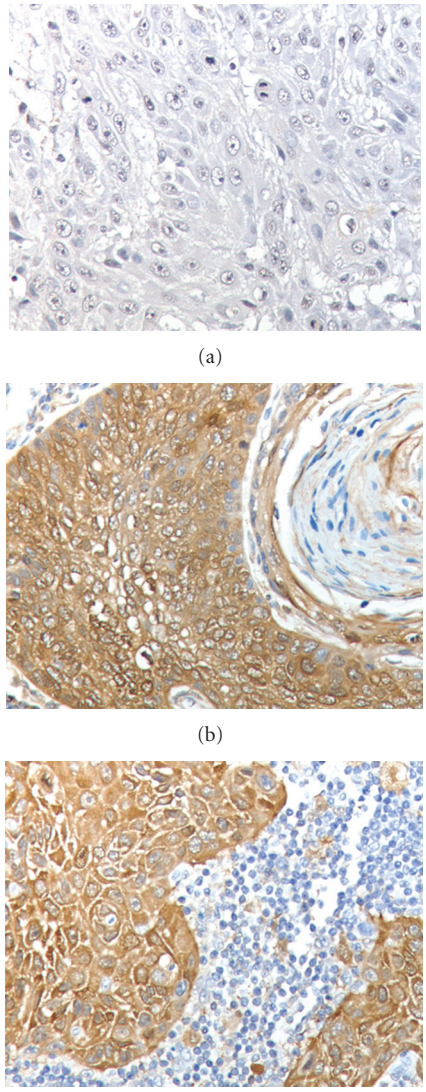
Figure 1: Hierarchical and stochastic models of CSC in progression of solid tumors.

population also inclusively expressed BMI1, a nuclear protein that also plays a role in self renewal in other CSCs, while exclusively expressed the differentiation marker involucrin. Unlike breast CSCs, this group found that epithelial-specific antigen (ESA) expression was not enriched in the tumorigenic cells, suggesting that HNSCC has CSC biomarkers distinct from those in breast cancer. A $CD44^+$ population was also reported by Okamoto et al. to characterize HNSCC CSC-like cells [58]. It was found that $CD44^+$ cells possessed not only a capacity for forming tumor spheres, proliferation, migration, and invasion in vitro, but also a resistance to chemotherapeutic agents. Supporting this observation, four relevant chemoresistant genes, *ABCB1*, *ABCG2*, *CYP2C8*, and *TERT*, were upregulated in the $CD44^+$ population. Recently, an SP was identified by Zhang et al., and proved to enhance the capability of tumor formation in nude mice as compared with non-SP [59]. In another study, oral cancer stem-like cells were enriched through sphere formation and found to express Oct-4, Nanog, CD133, and ABCG2 [60]. Nanog/Oct-4/CD133 triple-positive status predicted a poor prognosis for patients with oral cancer. CD133 is also reported as an HNSCC stem-like cell marker by studies using a head and neck cancer cell line [61]. These data can be supported by many observations showing that a small population of HNSCC tumor cells exists and demonstrates strong self-renewal and proliferation capabilities, even in the early stage of tumor development [62–64]. In tumor cells of epithelial origin, this subpopulation shows a dedifferentiation phenotype and plasticity, which facilitates metastasis of HNSCC. In fact, this tumor subpopulation is also responsible for more aggressive phenotypes, such as resistance to cancer therapeutic drugs and metastasis [50, 51].

Whether putative CSCs play a role in metastasis of HNSCC or not the existence of mCSC has not been reported. But our previous study provides indirect evidence supporting the existence of such a population. We found that a highly metastatic subpopulation selected from a xenograft mouse model expressed high levels of CSC markers, including CXCR4 and integrin $\beta 1$, and altered levels of EMT markers such as E-cadherin and vimentin [65–67]. CXCR4 has been investigated as a putative CSC marker and is also an ideal target for the treatment of metastatic HNSCC. Integrin $\beta 1$ is mainly expressed in the basal layer of the normal epithelium as an epithelial stem cell marker [64, 68]. In abnormal epithelium (hyperplasia and dysplasia), integrin $\beta 1$ is found to be expressed in the upper layers of the epithelial tissues. It is also expressed in a variety of tumor tissues. Integrin $\beta 1$ overexpression has been suggested to expand the CSC compartment by inhibiting differentiation and apoptosis, therefore contributing to tumor progression and metastasis [68]. A recent study by Kirkland and Ying showed that $\alpha 2\beta 1$ integrin regulated lineage commitment in multipotent human colorectal cancer cells [69]. Whether the metastatic populations contain CSC-like features or not is currently under investigation.

5. Implications of CSC in the Development of Biomarkers and Therapy for HNSCC

From a clinical perspective, if the CSC or CSC-like population represents the more aggressive HNSCC population, the early detection and targeted treatment of these cells become an urgent need in order to better manage this disease. CSC-specific markers provide unique tools for identifying these putative aggressive cell populations. An immunohistochemistry study of primary HNSCC reported by Prince and Ailles showed that CD44 staining was associated with more basal-appearing cells [56]. $CD44^+$ cells were costained with markers for the basal normal squamous epithelium, CK5/14, while $CD44^-$ cells were associated with the differentiation marker involucrin, supporting the organization of HNSCC by developmental hierarchy, as predicted by the CSC theory of carcinogenesis. However, some studies of CD44 as a CSC marker in human HNSCC tissues contradict these in vitro and in vivo studies. A recent study by Mack and Gires reported CD44s and CD44v6 expressions in head and neck epithelial tissues [70]. They found a similarly high



(a)

(b)

(c)

Figure 2: ALDH1 expression in HNSCC tissues: (a) nonmetastatic primary tumor with negative ALDH1 expression, (b) metastatic primary tumor with positive ALDH1 expression, and (c) corresponding lymph node metastases. (Magnification: 400x).

level of CD44s and CD44v6 expression in normal, benign, and malignant epithelia of the head and neck. A similar observation was also obtained in our laboratory (data not shown). Therefore, the value of CD44s as a marker for a small CSC population in HNSCC needs to be reconsidered. We believe that there is a necessity to precisely define more HNSCC CSC markers with an aim of further improving our ability to isolate HNSCC CSCs.

Another possible CSC marker expressed in HNSCC is ALDH1. ALDH1 has been considered a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome [71]. Expression of ALDH1 in HNSCC and dysplastic mucosa tissue samples was examined by Visus et al. [72]. They found that 12 of 17 HNSCC and 30 of 40 dysplastic mucosa tissues expressed this protein. However, this study did not correlate ALDH1 expression status with aggressiveness or prognostic features of the disease, such as metastasis, chemoresistance, or survival. Our recent study of HNSCC tissues demonstrated a statistically significant increase in ALDH1 expression in tumors with LNM compared to tumors without LNM ($P < .0003$, Figure 2). Although ALDH1 has not been reported as a marker for HNSCC CSC, our study suggests that ALDH1 may be a potential marker for tumor progression and metastasis in HNSCC.

In addition to their predictive and prognostic value, the identification of CSCs in HNSCC will also provide target populations that require more aggressive treatment than can be achieved with conventional therapies, such as a combination treatment with chemotherapy and an agent targeting CSC-specific signaling pathways. As discussed in Section 3.1., a combination of chemotherapy with inhibitors of the ABC transporters overexpressed by CSCs may have potential clinical application. Furthermore, recent progress in nanotherapeutics has shown the ability of nanoparticles to bypass ABC transporters when delivering anticancer drugs to tumor cells, providing a new strategy to overcome chemoresistance of CSCs [73].

6. Conclusions

Recent progress in the study of CSCs in solid tumors has provided researchers and clinicians in head and neck cancer new concepts to better understand the heterogeneity of this disease with. Once CSC or CSC-like populations are defined with appropriate biomarkers, these biomarkers can be used for accurately detecting tumor-initiating cells or metastatic cells in primary tumor biopsies, which will aid clinicians in their treatment decisions and in the accurate prognosis of HNSCC.

Currently, there are no consistently well-defined biomarkers or matured technologies to identify CSC or CSC-like populations in HNSCC. Efforts are being made to improve this situation by developing in vitro models and appropriate HNSCC CSC culture systems and refining techniques for the selection of well-defined cell populations from clinical samples. Furthermore, major signaling pathways in CSC or CSC-like populations of HNSCC are under investigation. The major cellular signaling mediators should be ideal targets for the development of new therapeutic agents to specifically eradicate high-risk HNSCC cells, which may also hold drug-resistant phenotypes. These studies are part of a growing interest toward personalized treatment for HNSCC.

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Review Article

Understanding and Targeting the Eukaryotic Translation Initiation Factor eIF4E in Head and Neck Cancer

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The eukaryotic translation initiation factor eIF4E is elevated in about 30% of human malignancies including HNSCC where its levels correlate with poor prognosis. Here, we discuss the biochemical and molecular underpinnings of the oncogenic potential of eIF4E. Studies in human leukemia specimens, and later in a mouse model of prostate cancer, strongly suggest that cells with elevated eIF4E develop an oncogene dependency to it, making them more sensitive to targeting eIF4E than normal cells. We describe several strategies that have been suggested for eIF4E targeting in the clinic: the use of a small molecule antagonist of eIF4E (ribavirin), siRNA or antisense oligonucleotide strategies, suicide gene therapy, and the use of a tissue-targeting 4EBP fusion peptide. The first clinical trial targeting eIF4E indicates that ribavirin effectively targets eIF4E in poor prognosis leukemia patients and more importantly leads to striking clinical responses including complete and partial remissions. Finally, we discuss the relevance of these findings to HNSCC.

1. Generalized Role for eIF4E in Cancer

The eukaryotic translation initiation factor 4E (eIF4E) is a protein that plays a central role in the regulation of gene expression at the posttranscriptional level. eIF4E binds the 7-methyl guanosine "m^7G cap" structure found on the 5′ end of mRNAs. In the cytoplasm, eIF4E catalyses cap-dependent protein synthesis [1, 2]. Importantly, eIF4E effects the translation of some mRNAs, known as eIF4E sensitive, more than other transcripts. For instance, elevated eIF4E levels selectively increase translation of mRNAs coding for a variety of potent growth stimulatory proteins such as VEGF, Pim-1, and ornithine decarboxylase (ODC) [3–5]. In the nucleus, eIF4E mediates in the export of another subset of mRNAs (such as cyclin D1 and ODC mRNAs) to the cytoplasm [5–7]. Thus eIF4E can modulate gene expression at two levels: by exporting mRNAs to the cytoplasm increasing their concentration therein and by enhancing the translational efficiency of transcripts that are already in the cytoplasm. Not all transcripts are affected at both levels. Importantly, eIF4E requires its m^7G cap binding function in order to act in either of these functions. Clearly, dysregulation of eIF4E will profoundly affect the cellular proteome.

The process of malignant transformation requires multiple molecular events involving activation of proto-oncogene products that stimulate growth and inactivation of suppressor genes that inhibit cellular proliferation. Together, these events result in selective dysregulation of cellular metabolism and growth. Critical control points in the cell cycle, DNA replication, and protein synthesis are just a few of many potential sites where alterations of normal functions may result in tumorigenesis. Because the overexpression of eIF4E results in the upregulation of multiple gene products that play critical roles in cycle progression and survival, it is not surprising that the elevation of eIF4E has been detected in various malignancies [3].

eIF4E is overexpressed in many epithelial cell cancers, including breast [8–12], colon [13, 14], bladder [15–19], cervix [20, 21], lung [22–24], and squamous cell carcinoma of the head and neck [25–32]. Some studies report that eIF4E is overexpressed in almost 100% of tumors of the breast, head and neck, and colon [8, 27, 32]. Several retrospective

studies indicate that eIF4E elevation is correlated with poor prognosis. As discussed below, high eIF4E levels in the HNSCC correlated with higher incidence of relapse [26–29, 32]. eIF4E overexpression was detected at a range of 3–30 fold in breast carcinomas, compared to normal breast tissue [8, 10], and eIF4E levels were significantly increased in vascularized malignant ductules of invasive carcinomas [33]. Breast cancer patients with high eIF4E expression (>7-fold to normal) experienced a statistically significant poorer clinical outcome with a higher risk for recurrence and cancer related death [11]. Further, increased levels of eIF4E are observed in non-Hodgkin's lymphomas but not in benign lesions [34, 35]. Here, eIF4E levels correlated with the aggressiveness of these lesions [34, 35]. In prostate cancer, eIF4E levels were also correlated with worse prognosis [36]. In acute myeloid leukemia (AML), elevated eIF4E levels are characteristic of the poor prognosis M4 and M5 AML subtypes [37].

Given that both normal and cancer cells express eIF4E, it is important to develop therapeutic strategies that target cancer cells without harming normal cells. There is evidence that cancer cells have developed an oncogene addiction to, or dependency on, eIF4E. In studies in primary human leukemia specimens, subtypes of leukemias with elevated levels of eIF4E are sensitive to inhibition of eIF4E by antagonists at levels 100-fold less than those that effect normal bone marrow or other leukemic subtypes [38]. More recent studies suggest a similar case in a prostate cancer mouse model [39].

In animal models, eIF4E overexpression is correlated with not only increased numbers of tumors but also increased invasion, metastases, and angiogenesis [3, 15, 40]. Mice with transgene overexpression of eIF4E developed a variety of cancers of distinct histological origin [41]. These cancers develop despite the fact that the level of eIF4E overexpression in these mice is much less than the corresponding levels of eIF4E overexpression found in patients [32, 33, 37]. Further, a lymphoma mouse model showed that eIF4E overexpressing mice developed more lymphomas [42].

2. Dysregulation of eIF4E in HNSCC

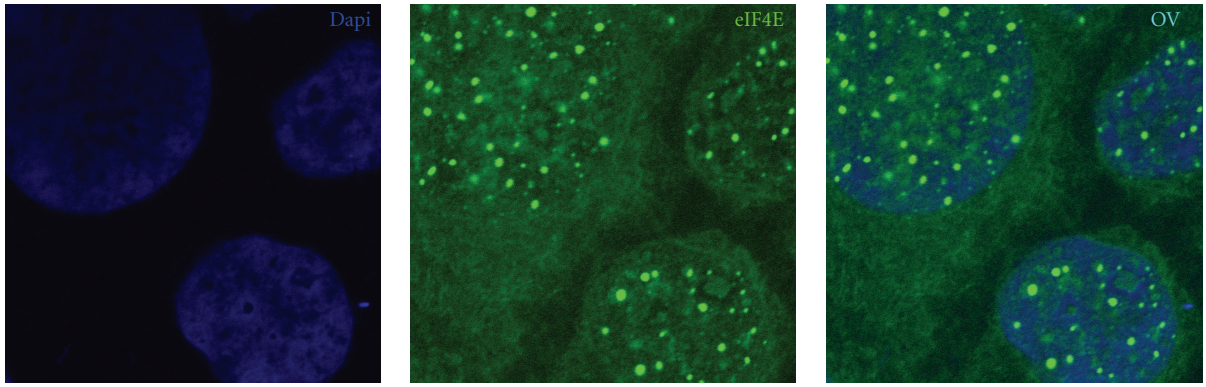
eIF4E is found to be elevated in the vast majority (in some studies even 100% of cases) of HNSCC specimens, with levels being 3 to 24 fold elevated relative to normal controls [26–30, 32]. High eIF4E levels in surgical margins are predictive of increased risk of recurrence in HNSCC [26–29]. Overexpression of eIF4E in >5% of the basal layer of histologically tumor-free surgical margins of HNSCC patients predicted a significantly increased risk of recurrence [27]. This prediction is important for patient outcome as most HNSCC patients will succumb due to local recurrence [26, 28, 29]. It has been demonstrated that eIF4E overexpression is associated with eIF4E gene amplification in both HNSCC and in breast carcinomas [30, 43–45]. An increased level of eIF4E gene amplification was observed when benign tumors and invasive carcinomas of the head and neck were compared. Benign tumors only had moderate evidence for gene amplification, while malignant tumors had a 4–15 fold level of amplification [43]. eIF4E protein levels were elevated in premalignant lesions in the larynx, but to a lesser extent than observed in HNSCC [25]. These studies suggest that progression to the malignant phenotype paralleled eIF4E gene amplification and overexpression [43]. Also, there was a progressive increase in the degree of eIF4E gene amplification and protein expression when comparisons were made among samples from tumor free margins of resected carcinoma specimens, tumor free regions adjacent to tumor core and tumor core samples [44]. This suggests that molecular events such as eIF4E gene amplification may precede cellular morphological changes, and that surgical margins which appear tumor free microscopically, may have elevated eIF4E protein levels. Thus, eIF4E levels could be used as a marker for prediction of early recurrence. It has been postulated that somewhere in the multistep pathway of carcinogenesis, elevation of eIF4E is a necessary event in progression of most solid tumors, and that eIF4E does not only reflect the proliferative status of cells but also their malignant properties [28, 46].

Consistent with their derivation from hypopharyngeal squamous carcinoma, FaDu cells [48] have elevated eIF4E [49], and as seen in many cell types, eIF4E is found in both the nucleus and cytoplasm (Figure 1). Further, eIF4E levels are elevated in FaDu cells due to both gene amplification, and increased mRNA stability [50]. Thus, there appears to be multiple ways to elevate eIF4E levels (see below).

3. Biochemical Underpinnings of eIF4E's Biological Effects

eIF4E overexpression profoundly alters the cellular proteome. However, experiments as early as 1980 [51] and more recent studies using knockdown strategies indicate that alterations in eIF4E expression do not uniformly alter the proteome [3, 5, 52–57]. In other words, the expression of some genes is more affected by modulation of eIF4E levels. These genes are referred to as eIF4E sensitive. In the cytoplasm, eIF4E recruits the transcript to the ribosome thereby increasing its translational efficiency. When eIF4E is overexpressed, sensitive transcripts have a higher ribosomes/mRNA ratio enabling more efficient translation without modulating mRNA levels in the cytoplasm. Notably, sensitive mRNAs have more highly structured 5′ UTRs versus insensitive housekeeping mRNAs such as GAPDH or β-actin which contain short, unstructured 5′ UTRs [3, 52, 58]. Transcripts controlled at this levels often code for proteins involved in proliferation such as c-Myc, Pim 1, VEGF, and ODC [4, 5, 55, 59].

Up to 68% of eIF4E is found in the nucleus of cells from a wide variety of species ranging from yeast to humans [7, 60–63]. These include FaDu cells, which have high eIF4E levels relative to normal cells and have eIF4E in both the nucleus and cytoplasm (Figure 1). As in the cytoplasm, only a subset of transcripts is sensitive to eIF4E dependant mRNA export [5]. These mRNAs contain a discrete 50 nucleotide element in their 3′UTR known as the eIF4E sensitivity element (4E-SE) [6, 64, 65]. Removal of the 4E-SE



(a) (b) (c)

Figure 1: FaDu cells immunostained for eIF4E showing cytoplasmic and nuclear localization. Cells were stained using eIF4E mAb conjugated directly to FITC (green) and nuclear marker DAPI (blue) as described [37, 47]. Micrographs were collected on laser scanning confocal microscope using 100X objective and 2x digital zoom.

ablates eIF4E sensitivity [65]. Many mRNAs sensitive to eIF4E at the export level code for proteins that promote proliferation and survival. Increased export of the transcripts leads to increased levels of the mRNA available to the translation machinery, without altering translation efficiency [5]. In the nucleus, eIF4E is found in the nucleoplasm, in nuclear bodies co-localising with 4E-SE containing mRNAs or colocalising with promyelocytic leukaemia (PML) nuclear bodies (with no RNA). PML is a potent inhibitor of its mRNA export function and a potent inhibitor of eIF4E mediated transformation [47, 60, 66, 67].

Regulation of transcripts by eIF4E can occur at the mRNA export level, the translation level, or both (Figure 2). For instance, cyclin D1 transcripts are only sensitive to eIF4E at the mRNA export level [5, 60, 61, 65]. VEGF transcripts are only sensitive to eIF4E at the level of translation [5, 58]. In contrast, ODC transcripts are sensitive to eIF4E at both the mRNA export and translation levels [5]. ODC is regulated at both levels because it contains both the complex 5′UTR sensitising it to translation and the 4E-SE in its 3′UTR sensitising it to eIF4E dependent mRNA export. Importantly, 4E-SE containing mRNAs is exported through a pathway that is distinct from bulk mRNA export [64]. Unlike bulk mRNA export which is TAP/NXF1 dependent, eIF4E dependent mRNA export is CRM1 dependent and requires the 4E-SE and the mRNA export factor LRPPRC [64, 68].

The combinatorial effects that eIF4E have on gene expression position it as a central node in an RNA regulon governing proliferation and cell survival [64, 65]. The RNA regulon is a theoretical construct that outlines a means by which posttranscriptional gene expression can be coordinated [69, 70]. In this model, elements in the UTRs of transcripts sensitise groups of transcripts to the same level of regulation. Transcripts with the same combination of elements, known as USER codes, will be coregulated. In this way, transcripts coding for proteins acting in the same biochemical pathway can have their production coordinated and thus the biochemical output of the pathway optimised. In the case of eIF4E, the complex 5′UTR and the 4E-SE in the 3′UTR can be considered to be USER codes for translation and export, respectively [6].

An example of the RNA regulon is the ability of eIF4E to modulate Akt signalling. eIF4E overexpression, via its mRNA export function, upregulates the expression of an activator of Akt, NBS1 [71, 72]. Furthermore, it enhances the expression of several downstream effectors of Akt including c-myc, cyclin D1, and cyclin E1 [5, 64]. eIF4E rescues serum-starved fibroblasts from serum-induced apoptosis. However, eIF4E loses this activity in $Akt1^{-/-}$ cells whereas reintroduction of Akt1 enables eIF4E to rescue the cells again [71]. Thus, through the coordinated regulation of genes involved in the Akt pathway, eIF4E can promote cellular survival. These observations are particularly interesting in the context of HNSCC progression. In a study of HNSCC tumors and surgical margins, elevated levels of eIF4E correlated with elevated Akt activation [73].

In summary, eIF4E modulates gene expression at two levels: mRNA export and translation. These functions are coordinated through the RNA regulon. In many cases, eIF4E sensitive mRNAs act in the same biochemical pathways such as cell cycle progression or survival pathways. This coordination potently drives the oncogenic potential of eIF4E [6].

4. Molecular Basis for eIF4E Mediated Transformation

eIF4E overexpression leads to transformation in cell culture, as well as in animal models, as described above. Specifically, eIF4E overexpression leads to loss of contact inhibition of fibroblasts, growth in soft agar and increased proliferation [47, 58, 61, 74–76]. eIF4E overexpression rescues cells from certain types of apoptotic stimuli [77–81]. In fibroblasts, serum deprivation induced apoptotic rescue of eIF4E is Akt1 dependent [82]. Both the nuclear and cytoplasmic functions of eIF4E contribute to its oncogenic potential [37, 47, 58, 60, 61]. For instance, a mutant of eIF4E, W73A, which acts in

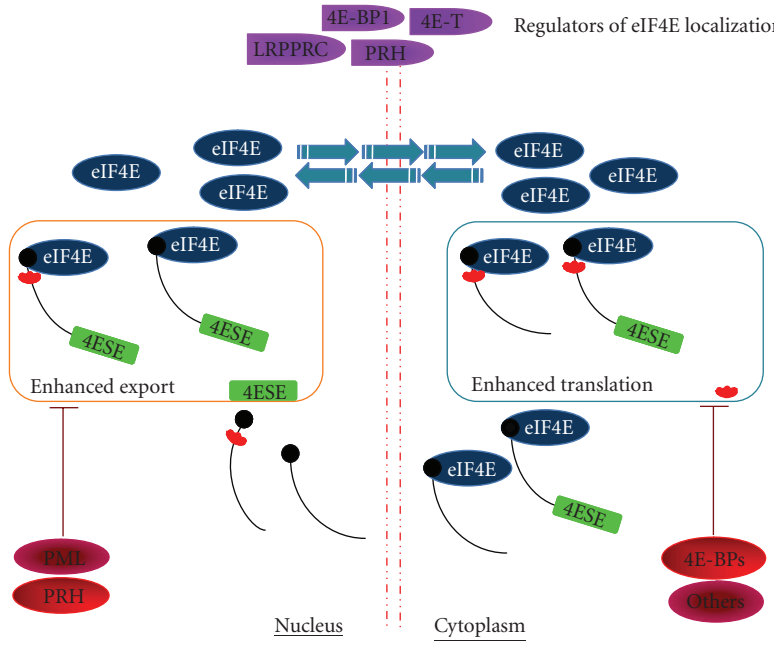


FIGURE 2: A diagram summarizing the nuclear and cytoplasmic functions of eIF4E. Some factors that directly regulate eIF4E functions and proteins involved in regulation of eIF4E subcellular distribution are shown. Not all regulators are shown for the sake of clarity. mRNAs are depicted as black lines with black balls denoting the 5′ m^7cap and with/without complex 5′UTRs shown in red and 4ESE element shown in green.

mRNA export but is deficient in promotion of translation, acts in both transformation and survival to the same extent as wild-type eIF4E [47, 60, 76].

5. Redundant Regulation of eIF4E

Regulators of eIF4E functions are positioned to modulate the eIF4E regulon, co-ordinately modulating cell cycle progression, and cell survival (Figure 2). One of the best-characterized regulators of eIF4E is eIF4E binding protein 1 (BP1) [58, 83]. This protein uses a conserved eIF4E binding site to associate with eIF4E, and thereby precludes access of eIF4E to eIF4G and the rest of the translation machinery [58]. This binding site is defined as follows: YXXXXLϕ (where X is any residue and ϕ is a hydrophobic residue). Studies suggest that BP1 increases cap affinity and thereby sequesters both eIF4E and the RNA in question from the translational machinery [1]. The association of BP1 with eIF4E is modulated by phosphorylation of BP1 [58, 83]. Phosphorylation of BP1 leads to a reduction in its interaction with eIF4E and thereby and results in increased translational activity of eIF4E. Phosphorylation is mTOR dependent and thus rapamycin treatment leads to reduced phosphorylation of BP1, increasing its association with eIF4E and thereby reducing translation of eIF4E sensitive mRNAs [84]. In contrast to eIF4E, BP1 overexpression sensitizes Ras transformed cells to apoptosis when treated with certain cytostatic drugs [85]. In addition, BP1 overexpression represses eIF4E mediated transformation of NIH 3T3 cells [58]. However, $BP1^{-/-}$ and $BP1^{-/-}$ $BP2^{-/-}$ mice do not develop cancers more readily than controls [86–89], highlighting the importance of redundancy of regulators in the control of eIF4E. Studies on BP1 in the literature focus on BP1 as a regulator of the cytoplasmic functions of eIF4E [58, 84]. However, endogenous BP1 associates with eIF4E in both the nuclear and cytoplasmic compartments and thus likely modulates eIF4E activity at both the level of translation and mRNA export (see [90] and our unpublished observations).

Counter-intuitively, BP1 levels are elevated in prostate and breast cancer and these levels correlate with a more advanced stage [91]. In esophageal cancers, there are more BP1-eIF4E complexes than in normal tissues, further complicating the accepted model of BP1 regulation of eIF4E [92]. Clearly, there is much more to be understood about BP1 and its implications for eIF4E activity.

There are many other regulators of eIF4E. The vast majority of these regulators contain the YXXXXLϕ motif like eIF4G and the BPs. These regulators include a set of over 200 homeodomain proteins that contain this motif. Some of these members are negative regulators of eIF4E, such as PRH/Hex. PRH is a nuclear protein that impedes eIF4E's mRNA export function [76]. PRH overexpression leads to the cytoplasmic redistribution of eIF4E [37, 76]. Other members of this group of homeodomain containing regulators include Emx2, Otx, Engrailed 2, Hox11, Bicoid, and HoxA9 [93]. HoxA9 can stimulate both the nuclear and cytoplasmic functions of eIF4E [94]. Emx2 travels from one neuron to another through the synapse enabling localized translational control of eIF4E via signals to the adjacent neuron. In this way, Emx2 controls eIF4E activity remotely

[93]. Thus, eIF4E function can be regulated in a tissue and context dependent manner.

There is also a discrete class of eIF4E regulators that utilize a RING domain to impede eIF4E function. These regulators include the promyelocytic leukemia protein PML, HHARI, and arenaviral Z proteins from LCMV and Lassa viruses [47, 60, 95]. PML and the Z proteins use their RING motifs to associate with eIF4E and inhibit eIF4E function by reducing the affinity of eIF4E for the m^7G cap by up to 100-fold [28, 37, 96]. These were the first proteins reported to reduce the affinity of eIF4E for the m^7G cap. PML is a key cellular inhibitor of the oncogenic activities of eIF4E. The ability to inhibit eIF4E function is closely tied with the ability of PML to impair cap binding, and thus the mRNA export activity of eIF4E [47, 64, 65, 71, 97]. Similarly, Z also impairs eIF4E cap binding and function [97]. Notably, PML and Z do not alter eIF4E levels, and therefore do not appear to act directly or indirectly in its protein stability, unlike other RINGs [97].

Clearly, the regulation of eIF4E activity is redundant and multifactorial. There are tissue specific regulators such as the homeodomain proteins and more ubiquitous regulators such as PML and BP1 (Figure 2). Redundancy of regulators is seen for both the nuclear and cytoplasmic arms of eIF4E activity.

6. Controlling eIF4E Localization—a Key Step in the Regulation of eIF4E

Clearly, modulating the subcellular distribution of eIF4E will have profound impacts on the sets of genes it regulates and thus on its biological effects. For instance, eIF4E localization is substantially altered during Xenopus gastrulation [7]. Furthermore, eIF4E nuclear-cytoplasmic localization changes dramatically during differentiation of mouse embryonic stem cells to macrophages (KLBB, unpublished observation).

As discussed above, eIF4E is found in both the nuclear and cytoplasmic compartments. Recent studies indicate that within the cytoplasm, eIF4E is found not only associated with actively translating transcripts, but also with cytoplasmic structures known as processing bodies (P-bodies) [98, 99]. These structures contain a variety of factors including many associated with RNA degradation as well as eIF4E [100]. RNAs associated with these structures are sequestered from the translational machinery. It is thought that P-bodies are a temporary storage depot for these RNAs while their fate (in terms of degradation, sequestration, translation, etc.) is being decided [100]. Thus, in the cytoplasm, eIF4E is associated with both the translation machinery and in some cases with mRNAs that are being sequestered from this machinery (e.g., in P bodies), perhaps left there until the time is right for these mRNAs to be translated.

Ultimately, the biochemical pathways in which eIF4E functions (mRNA export, mRNA translation, or mRNA sequestration) depend on the subcellular distribution of eIF4E. Little is known about what regulates nuclear entry of eIF4E and what determines which cytoplasmic compartments in which eIF4E will be found. To date, the only factor known to directly modulate the subcellular distribution of eIF4E is the eIF4E transporter protein (4E-T) [98, 101]. 4E-T uses its conserved eIF4E binding site to interact directly with the dorsal surface of eIF4E. The original study suggested that 4E-T transported eIF4E protein into the nucleus [101]. However, several other studies, including subsequent studies by the Sonenberg group [98], indicate that overexpression of 4E-T leads to relocalization of the majority of nuclear eIF4E to the cytoplasm, where a subset is found in P-bodies. The molecular mechanism for this redistribution is not yet known.

Other factors also modulate the subcellular distribution of eIF4E, including BP1 [90], the proline rich homeodomain protein PRH [37, 76], and the leucine rich protein LRPPRC [68] (Figure 2). PRH is a potent inhibitor of the mRNA export function of eIF4E [76]. PRH overexpression leads to redistribution of nuclear eIF4E to the cytoplasm [37, 76]. LRPPRC overexpression leads to re-distribution of eIF4E within the nucleus. Here, upon LRPPRC overexpression, LRPPRC competes for PML leading to reduced PML-eIF4E co-localization. This redistribution correlates with increased eIF4E dependent mRNA export [68]. In summary, these factors are positioned to impact the nuclear and cytoplasmic arms of eIF4E activity and thus alter the effects of eIF4E on the proteome.

There are other means to modulate the subcellular distribution of eIF4E. Interestingly, transduction of primary leukemia specimens (M4/M5 AML) with the inhibitor of NFκB activity, IκB-SR, leads to a substantial re-organization of eIF4E, reducing the amount of eIF4E found in the nuclear fraction and increasing the amount in the cytoplasm, and reorganization of the remaining eIF4E nuclear bodies into structures which are morphologically indistinguishable from normal cells [37, 93]. Thus, the subcellular distribution of eIF4E appears linked to NFκB activity. As expected, transduction of IκB-SR leads to reduced eIF4E dependent mRNA export in these specimens [37, 93]. In this way, eIF4E localization is linked to NFkB activity.

In addition, the subcellular distribution of eIF4E can be modulated by small molecules [38, 47, 102]. Treatment of cells with the m^7G cap analogue (m^7GpppG) leads to disruption of eIF4E nuclear bodies and re-distribution of eIF4E to the cytoplasm [47, 102]. Treatment with a physical mimic of the m^7G cap, ribavirin, has a similar effect where it leads to an increased fraction of eIF4E in the cytoplasm [38]. Consistently, ribavirin treatment leads to reduction in eIF4E dependent mRNA export. Note that ribavirin or m^7GpppG, under these conditions, does not alter the levels of eIF4E [38, 47, 102].

In summary, factors such as 4E-T that so drastically affect the subcellular localization of eIF4E, are positioned to affect eIF4E's physiological activities in proliferation and oncogenic transformation (Figure 2). In addition, given that eIF4E modulates the expression of some transcripts only at one level (such as cyclin D1 at the export level or VEGF at the translation level), modulation of its subcellular distribution is likely to lead to differential effects on eIF4E sensitive transcripts. In this way, these eIF4E traffickers could modulate gene expression differentially favouring/disfavouring subsets of genes (e.g., export versus translation) and thereby

modulate the biological effects of eIF4E. This level of modulation would allow a more tailored response to cellular stresses and stimuli.

7. How Does eIF4E Become Elevated in Cancer?

Given that elevated eIF4E levels are found in many human cancers and are associated with poor prognosis [28, 30, 43, 103], it is critical to understand how eIF4E levels become elevated. There are likely multiple mechanisms that could account for elevated eIF4E mRNA levels in these primary patient specimens, for example, gene amplification, transcriptional dysregulation, and alterations in mRNA stability. In fact, elevated eIF4E levels may result from any combination of these. For instance, eIF4E levels are elevated, at least in part, in breast cancer and head and neck squamous cell carcinomas due to amplification of the eIF4E gene [13, 104, 105]. Studies in cell culture indicate that the eIF4E promoter contains an E-box, and that its expression is regulated by c-myc [5, 6, 64]. Interestingly, c-myc is a downstream mRNA export and mRNA translation target of eIF4E which suggests existence of a potential feedback loop [106, 107]. As eIF4E is made in c-myc null mice, there must be other means by which it is induced [37]. Further, eIF4E mRNA levels are substantially reduced in primary leukemia specimens transduced with the IκB-SR [3, 108]. In addition, some studies have found increased eIF4E expression during hypoxic conditions by IHC analysis of confined breast cancer biopsies [50]. In this way, the hypoxia that accompanies tumor growth may stimulate eIF4E expression.

Another mechanism that appears to be involved in the elevation of eIF4E in HNSCC is HuR dependent stabilization of eIF4E transcripts. Specifically, in FaDu cells, both HuR and eIF4E levels are elevated relative to control cells. Here, the mRNA stability factor, HuR, associates with eIF4E mRNA and enhances its stability [109]. HuR is a member of a family of proteins which modulate the stability of mRNAs by associating with U or AU rich elements (denoted AREs) typically in the 3′UTR of these messages [109]. Hu/ELAV family members are primarily neuronal with the exception of HuR, which is ubiquitously expressed. HuR modulates the expression of many proliferative mRNAs which contain AREs including (but not limited to): cyclin D1, cyclin B1, c-myc, VEGF, and so forth [109, 110]. Interestingly, many of these target mRNAs are also export and /or translational targets of eIF4E (e.g., all of the ones listed above). HuR has been implicated in oncogenesis. Its overexpression is correlated with the formation of tumors in mouse xenograft models [111, 112]. Microarray data of normal and cancer tissues indicated that HuR is elevated in human breast and lung cancer [96, 113, 114]. Further, HuR promotes angiogenesis, as does eIF4E [37]. The overlap in mRNA targets coupled to the fact that both eIF4E and HuR are involved in transformation and elevated in human cancers, suggests that eIF4E could be a downstream effector of HuR activity. Thus HuR is positioned to modulate the eIF4E regulon by both altering its expression and the expression of eIF4E's downstream effectors. Future studies that monitor HuR levels in HNSCC could be very interesting and may suggest HuR as another prognostic marker.

8. Targeting eIF4E in HNSCC- from Cells to Patients

To date, targeting of eIF4E in HNSCC remains in the preclinical stage. Three main pre-clinical strategies have been described: knockdown of eIF4E levels through the use of antisense oligonucleotides or RNA interference, suicide gene therapy, hormone analog—4EBP fusion peptide, and targeting eIF4E activity with ribavirin.

Inhibition of eIF4E using antisense oligonucleotides to eIF4E was first performed in HeLa cells and Ras-transformed mouse fibroblasts, and resulted in the reversal of the malignant phenotype [115–117]. Decreasing levels of eIF4E in the human breast cancer cell line MDA-MB-435 and human prostate cancer cell line PC-3 diminished their angiogenic and tumorogenic properties [33, 39]. The first laboratory to find eIF4E levels elevated in both breast and HNSCC, the De Benedetti lab, was also the first to target eIF4E in HNSCC cells [33, 49]. Using antisense RNA to eIF4E, they demonstrated that they lowered both eIF4E levels and the levels of its downstream targets, VEGF and FGF-2. FaDu cells treated with antisense oligonucleotides also show reduced oncogenic properties of these cells including displaying increased contact inhibition, reduced growth in soft agar, and reduced tumorigenicity in xenograft mouse models [49]. A related strategy used small interfering RNAs targeting eIF4E either alone or in combination with cis-platin in the UMSCC22B HNSCC cell line [118]. As expected, siRNA to eIF4E lowered eIF4E levels and reduced the oncogenicity of this cell line. The addition of cis-platin increased the effects of the knockdown of eIF4E alone. This same strategy, combining siRNA to eIF4E with cis-platin, was also used in breast carcinoma cells with success [119].

Antisense oligonucleotides (ASOs) were also used by the Graff lab in a human prostate cancer xenograft mouse model [39]. Here, mice that intravenously received anti-sense oligonucleotides showed significant reduction of eIF4E expression and suppressed tumor growth. No toxicity was observed. The ASOs used also target murine eIF4E, leading to an 80% reduction of eIF4E in mouse liver; however there was no affect on body weight, organ weight, or liver transaminase levels. Eli Lilly is currently pursuing clinical trials using this strategy.

Suicide gene therapy is a method of introducing a gene, the expression of which will make a tumor cell uniquely susceptible to attack and destruction [120]. This strategy utilizes delivery of herpes simplex virus-thymidine kinase (HSV-Tk) by nonreplicative adenovirus vectors to the cells and subsequent ganciclovir (GCV) treatment [121, 122]. The HSV-Tk has the ability to phosphorylate and activate prodrug GCV to its cytotoxic triphosphate form with 1000-fold higher efficiency than its mammalian homologues. As a consequence, cells transfected with HSV-Tk can be

targeted for death by treatment with ganciclovir, while normal cells would remain mainly unaffected [121, 122]. Although this strategy gained wide popularity as potential treatment for HNSCC, this strategy had two principal challenges: acceptable cytotoxic specificity to tumor cell targets and adequate delivery of the suicide gene. In order to specifically target eIF4E overexpressing cells, a long 5′UTR (from FGF-2) was fused to the thymidine kinase gene (5′UTR-Tk) to preferentially sensitize expression of this gene to eIF4E levels [123, 124]. This system was reported highly efficient in a broad spectrum of breast cancer cell lines [124]. Using a mouse minimal residual disease soft-tissue metastasis model for HNSCC, the Li group examined the efficacy of this strategy to target solid tumors cells that are overexpressing eIF4E [123]. In this study, mice that received Ad-HSV- 5′UTR-Tk fusion and GCV treatment showed longer disease free survival than the control group [123].

In order to inhibit eIF4E in ovarian cancers, Ko et al. [125] designed 4EBP-based peptide fused to an analog of gonadotropin-releasing hormone (GnRH) to specifically target ovarian and other endocrine cancer cells, that are widely overexpressing the GnRH receptor. This fusion peptide inhibited growth of the GnRH receptor expressing tumor cells and showed potent antitumor effect in a mouse xenograft model of epithelial ovarian cancer, without significant cytotoxic effects in other tissue.

9. Successful Targeting of eIF4E in the Clinic

To date, eIF4E has been successfully targeted only in a particularly aggressive form of acute myeloid leukemia French American British (FAB) subtype M4/M5 AML. These poor-prognosis leukemias are characterized by elevated eIF4E levels [37]. In these studies, ribavirin, a competitive inhibitor of the natural ligand of eIF4E the m^7G cap, was used to target its biochemical and oncogenic activities [38]. In a phase II proof-of-principle clinical trial of refractory, relapsed or patients who cannot undergo induction chemotherapy were treated with ribavirin [126]. eIF4E inhibition led to striking clinical improvement including complete remission, partial remission, and blast response. Ribavirin was originally used as an antiviral drug and was well tolerated with no therapy related toxicities observed. Note that in these studies, ribavirin was the only cytotoxic chemotherapy permitted.

Clinical response correlated with inhibition of eIF4E activity and redistribution of the eIF4E protein from the nucleus to the cytoplasm [126]. In these AML patients, eIF4E was both highly upregulated and found mainly in the nucleus [38, 82]. The mRNA export activity of eIF4E is also upregulated in these specimens [37, 126]. After 28 days of treatment with ribavirin, eIF4E was markedly re-distributed from the nucleus to the cytoplasm [126]. Surprisingly, eIF4E protein levels were also downregulated, which is the first time this downregulation has been reported postribavirin treatment (note that in the previous experiments, ribavirin treatment was followed in cell culture for up to 48 hours, not 28 days as used for patients [127–130]). It is possible that this downregulation occurs via a negative feedback loop due to prolonged inhibition of eIF4E. Alternatively, decreased eIF4E levels could be a result of differential sensitivity within a heterogeneous cell population to ribavirin. At the same time, the production of eIF4E mRNA export targets such as cyclin D1 and NBS1 mRNA is repressed in patients. Further, eIF4E dependent Akt activation is reduced, which is consistent with its requirement for NBS1.

Durability of clinical response is key to success. In the treatment of M4 and M5 AML, a regimen of chemotherapy typically combines Ara-C with danurubicin or idarubicin [127–130]. This regimen, named 7+3, induces remission in most patients. However, in the absence of consolidation therapy (typically with Ara-C), remissions only last 2–4 months [127–130]. Like many targeted monotherapies such as ATRA in APL or flt3 inhibitors in AML [131–134], after 2–4 months of ribavirin treatment, development of drug resistance was observed [126]. In these cases, although eIF4E levels remain low, eIF4E relocalizes to the nucleus, which generally correlated with relapse of the disease. The molecular events underpinning the return of eIF4E to the nucleus are not known, but clearly these events play a critical role in the response of these cells to ribavirin. To try to overcome resistance, ribavirin will be combined with other chemotherapy regimens. Although these studies are in AML patients, the ability to target eIF4E has clear implications for the development of treatments for HNSCC and other cancers with elevated eIF4E.

10. From Leukemia to HNSCC

Several previous studies indicate that ribavirin is an effective inhibitor of growth in FaDu cells [38, 82]. In xenograft mouse models, studies had shown that genetically reducing the levels of eIF4E protein by antisense RNA substantially impaired tumour growth [39, 119]. Similarly, addition of oral ribavirin to mice after FaDu xenograft led to significantly smaller tumours than for control animals [38]. Further, ribavirin inhibited anchorage dependent growth in FaDu cells in culture and significantly reduced levels of cyclin D1 and NBS1 proteins, and decreased Akt activation [82]. These findings in cell culture as well as results from treatment of AML patients suggest that targeting eIF4E with ribavirin in HNSCC may yield promising clinical results.

11. Conclusions

In this paper, we have described the biochemical function and biological effects of eIF4E. We summarize the roles and regulation of eIF4E in gene expression (Figure 2). We discussed the dysregulation of eIF4E in multiple cancers and the current strategies being considered to target its activity. eIF4E is an indicator of poor prognosis in HNSCC and hopefully, therapeutic approaches targeting eIF4E will benefit these patients.

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Review Article

Immunotherapy of Head and Neck Cancer: Current and Future Considerations

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Recommended by Amanda Psyrri

Patients with head and neck squamous cell carcinoma (HNSCC) are at considerable risk for death, with 5-year relative survival rates of approximately 60%. The profound multifaceted deficiencies in cell-mediated immunity that persist in most patients after treatment may be related to the high rates of treatment failure and second primary malignancies. Radiotherapy and chemoradiotherapy commonly have severe acute and long-term side effects on immune responses. The development of immunotherapies reflects growing awareness that certain immune system deficiencies specific to HNSCC and some other cancers may contribute to the poor long-term outcomes. Systemic cell-mediated immunotherapy is intended to activate the entire immune system and mount a systemic and/or locoregional antitumor response. The delivery of cytokines, either by single cytokines, for example, interleukin-2, interleukin-12, interferon-γ, interferon-α, or by a biologic mix of multiple cytokines, such as IRX-2, may result in tumor rejection and durable immune responses. Targeted immunotherapy makes use of monoclonal antibodies or vaccines. All immunotherapies for HNSCC except cetuximab remain investigational, but a number of agents whose efficacy and tolerability are promising have entered phase 2 or phase 3 development.

1. Introduction

Head and neck cancer is a prevalent condition in the United States and the eighth leading site of new cancer cases among men. It is estimated that 35,310 new cancers of the oral cavity and pharynx will have been diagnosed in 2008 in the United States, and that 7,590 Americans will have died due to such cancers [1]. More than 80% of head and neck cancers (excluding cancers of the thyroid, salivary glands, and nasopharynx; and nonmelanoma skin cancer) are head and neck squamous cell carcinomas (HNSCCs) [2].

Death rates from cancers of the oral cavity and pharynx declined from 1979 to 2000 in the United States, but they have since then remained stable. The overall 5-year relative survival rate at diagnosis is 59.1%, with a range from 81.8% for early disease at diagnosis to 26.5% for advanced disease. For diagnoses at all stages combined, the 10-year relative survival rate for cancers of the oral cavity and pharynx is 48%. For cancer of the larynx, the 5-year relative survival rate at all stages of diagnosis is 62.9%, ranging from 81.1% for early cancers to a dismal 23.9% for cancers with distant metastases at the time of diagnosis [1].

Mortality in head and neck cancers in the United States is higher in blacks than in whites: for cancer of the larynx, the 5-year survival rate in 2000 was 67% for whites and 40% for blacks; for cancer of oral cavity and pharynx, the rate was 65% for whites and 46% for blacks [3].

In light of these discouraging data, the development of novel therapies for HNSCC has become a priority. One of the most exciting research avenues is immunotherapy, thanks to advances in the understanding of the relationships between tumors and the host immune system, as well as to developments in the technology for identifying molecular therapeutic targets. This article reviews the rationale for

immunotherapy in HNSCC and the principal approaches under investigation.

2. Etiology, Diagnosis, and Staging of HNSCC

The development and progression of HNSCC are considered to result from stepwise alterations of cellular, genetic, biochemical, and molecular pathways at multiple epithelial sites within the aerodigestive tract [4]. This progression probably explains, in part, the high incidence of second primary tumors, the tendency for patients to present with premalignant lesions at multiple sites in the aerodigestive tract, and the high rate of progression of these premalignancies [5].

Tumor carcinogenesis in HNSCC involves dynamic interactions among many factors. Exposure of the upper aerodigestive tract to alcohol or tobacco is one of the chief risk factors for many HNSCCs, and exposure to both increases the risk beyond what would be expected if the agents simply had additive effects [2]. Another common risk factor is alteration of the function of the p53 tumor suppressor gene, which may be caused by either gene mutation or infection with an oncogenic type of human papillomavirus (HPV) [6, 7]. In some patients, particularly those with oropharyngeal cancer not associated with p53 mutation or the molecular impacts of alcohol and tobacco, HPV infection can cause head and neck cancer even in the absence of other molecular alterations [4, 8]. All of these risk factors are likely to result from and contribute to suppression of the patient's immune system, as is the tumor itself [9].

Diagnosis of HNSCC is based on a history and physical examination and computed tomography and/or magnetic resonance imaging as needed, chest imaging, pathology review, and biopsy [10]. In advanced HNSCC, positron emission tomography is an increasingly useful new modality for assessing lymph node involvement, distant metastases, and synchronous second primary tumors [11].

Relatively small primary HNSCCs with no nodal involvement are usually classified as stage I or II, and large primary tumors that may have invaded nearby structures or spread to regional lymph nodes are classified as stage III or IV [10]. Generally, stage I or II disease is discussed as "early stage" and stage III or IV disease is termed "advanced stage" [12].

3. Current Therapeutic Options

Approximately 40% of patients with HNSCC present with early-stage disease, and either surgical resection or radiotherapy is recommended as a single treatment modality [10]. Most patients (60%) present with locally advanced disease [10] and require a multidisciplinary approach using some combination of surgery, radiotherapy, and chemotherapy [4, 13].

In addition to considering the stage of cancer, oncologists must equally consider the site of disease. Lesions in the oral cavity are often treated with surgery followed by radiotherapy or chemoradiotherapy (CRT). Tumors located in the oropharynx, hypopharynx, nasopharynx, or larynx are usually treated with CRT firs [14].

As it is in many other kinds of cancer, immunotherapy is emerging as an important new option in treating HNSCC. The monoclonal antibody (MAb) cetuximab, which binds to the EGF receptor, is approved by the US Food and Drug Administration as first-line treatment of locally or regionally advanced HNSCC in combination with radiotherapy. As a single agent, cetuximab is indicated for the treatment of patients with recurrent or metastatic HNSCC for whom prior platinum-based therapy has failed [15]. Although technically considered a molecular targeted agent that inhibits the EGFR, cetuximab is a chimeric MAb. Its administration is often associated with a generalized allergic skin rash that correlates directly with tumor responses. Whether the benefit of adding this agent is due to EGFR inhibition and downstream molecular effects on pathways of cell proliferation and apoptosis or due to antibody-mediated immune responses is unclear. It is less likely due to a direct allergic response since nonneutralizing antibodies to cetuximab are only detected in 5% of treated patients. Recent evidence has shown that MAbs mediate antibody-dependent cellular cytotoxicity, and induce activation of cellular immunity, including natural killer and T cells [16]. Other immunotherapies being explored for treatment of HNSCC are discussed later in this paper.

In recent years, there have been many improvements in the modalities used to treat HNSCC. Minimally invasive surgical techniques followed by improved reconstruction procedures frequently result in better functional and esthetic outcomes. Improved microvascular reconstructions have enhanced functional results of major tumor resections. Intensity modulation in the use of radiation therapy may be reducing toxicity, and altered fractionation schedules may be improving local disease control and late toxicity [4]. Multidrug chemotherapy regimens incorporating the newest agents and molecular targeted therapies have shown some efficacy and tolerable toxicity in both recurrent and previously untreated patients.

In addition to efficacy considerations, impact on quality of life remains a major consideration in selecting appropriate treatment for HNSCC. The tumors themselves commonly jeopardize physiologic functions, such as the patient's ability to chew, breath, and swallow; the senses of taste, smell, and/or hearing; as well as personal characteristics such as voice and appearance [10]. Common side effects of radiotherapy include fibrosis of normal tissue, scarring, and long-term dry mouth or dysphagia [17]. CRT involves the substantial risks for severe acute and long-term side effects associated with both chemotherapy and radiotherapy, including mucositis, dermatitis, pain, dysphagia, dry mouth, local, or systemic infections, dental problems, depression, speech difficulties, and occasionally breathing difficulties, as well as immune suppression [14]. Combination with altered fractionation or intensified radiation increases the burden. Cisplatin, the preferred chemotherapeutic agent in CRT [10], is severely toxic when used in combination with other drugs and radiation, and patients unable to tolerate cisplatin have an especially high cumulative risk of death: 20% to 25% at 2 years [14].

4. Novel Therapeutic Directions: Engaging the Immune System and Antitumor Immunity

The HNSCC patient's immune system is an important element in the development of the disease and, in many cases, in the response to treatment. The microenvironment in which HNSCC arises is populated with numerous immune cells and soluble factors produced by these cells. Both cutaneous skin and aerodigestive tract mucosa are highly immunoreactive organs. In this environment, it is likely that many newly appearing tumor cells will be rapidly eliminated, leaving those which survive particularly resistant to the body's innate and adaptive immune mechanisms [18].

As with other cancers, there are numerous methods by which HNSCC may avoid recognition and destruction by the immune system. One strategy is to escape immune system recognition via downregulation of human leukocyte antigens (HLAs), which are necessary to present antigens on malignant cells to T cells [19, 20], or via apoptosis of circulating T cells, which seems to be mediated at least in part by tumor-derived Fas ligand [21]. Another potential mechanism is secretion of immunosuppressive factors such as prostaglandin E_2 [22], vascular endothelial growth factor (VEGF) [23], interleukin (IL)-10, or transforming growth factor-β [24]. Additionally, immune defenses can be directly inhibited by "suppressor T cells," now known as regulatory T cells (T_{reg}) [9]. Immune reactivity is not simply turned on or off, rather, HNSCC and certain other cancers avoid the immune response by modulating responses that are more effective against tumors, for example, T_H1 responses, and enhancing those which are less effective, for example, T_H2 responses. T_H1 responses are classically defined by the production of interferon (IFN)-α, granulocyte macrophage colony-stimulating factor (GM-CSF), and IL-2, whereas T_H2 responses are defined by expression of cytokines such as IL-4, IL-6, and IL-10 [24].

In most types of cancer, these processes are thought to take place concurrently [24]. Some immune system deficiencies, however, are specific to HNSCC and a few other cancers [25], and they are thought to contribute to the poor long-term survival rate in HNSCC. Patients with HNSCC have been shown to have lymph nodes that are reduced in size and have diminished T-cell content. Reduced T-cell function has been linked to shorter disease-specific survival [26]. Defects in dendritic cell (DC) function are also a hallmark of immune system dysfunction in HNSCC [27]. For example, the accumulation of histiocytes/DCs in the distended sinuses of lymph nodes, known as sinus histiocytosis, is a reflection of DC defects, and is present in the lymph nodes of HSNCC patients. The buildup of these cells in the nodal sinuses prevents their entry into the node parenchyma, and maturation is, therefore, impaired, preventing optimal T-cell stimulation [28]. Low infiltration of DCs in tumor environments (linked to abnormalities in the TcR-associated zeta chain in TILs) was correlated with poor prognosis for disease survival [29].

Specific defects in cell-mediated immunity may also include progressive decreases in dermal delayed-type hypersensitivity responses, T-cell counts in blood, proliferative responses of blood T cells to mitogens or antigen stimulation, and blood monocyte functions such as chemotaxis and cytotoxicity [25]. One example is the production by HNSCC and some other cancers of chemoattractive factors (e.g., VEGF) to attract immunosuppressive CD34(+) progenitor cells that inhibit the capacity of intratumoral lymphoid cells to become activated [23, 30]. Intriguingly, cell-mediated immunity may decline even before the tumor develops, whereas levels of B cells in blood, immunoglobulin, and complement are usually normal. Therefore, alterations in humoral immunity seem modest in HNSCC patients [25]. These findings reflect the fact that HNSCC is intrinsically characterized by deficits in the cellular immune system. These cancers arise within the oral, nasal, or laryngeal mucosa, and interact with the local, regional, and systemic immune cells likely to affect the initiation and promotion of tumors in these environments [18].

5. Immunotherapy: Future Directions for HNSCC Treatment

Immunotherapy is an attractive option for cancer treatment because both humoral immunity and cell-mediated immunity involve cells with a variety of clonally distributed antigen receptors that can distinguish normal cells from cancerous cells. Another advantage is that the immune system can adapt to the evolution of cancer cells and can respond in a systemic fashion [31]. Signs of an immune response have been shown to correlate with positive outcomes for cancer patients. For example, the presence of tumor-infiltrating T cells has been correlated with progression-free survival and/or overall survival in various cancers, including advanced ovarian cancer [32], advanced melanoma [33], and head and neck cancer [34]. Because the immunobiology of HNSCC is so intimately associated with the host immune system, the reversal of immunosuppression is a particularly attractive therapeutic goal in this tumor type [18]. The remainder of this article describes immunotherapies now in development for treatment of HNSCC.

5.1. Systemic Cell-Mediated Immunotherapy in HNSCC. Systemic cell-mediated immunotherapies are nonspecific, and attempt to replace the entire immune system by mounting either a systemic and/or locoregional antitumor response. For example, adoptive transfer therapy is a form of passive therapy that entails ex vivo expansion and modification of the patient's own immune cells, followed by their reinfusion. The initial use of this approach was based on evidence from murine studies in which regression of established tumors was demonstrated [18]. An example of its clinical application was shown in patients with stage IV nasopharyngeal carcinoma, which expresses Epstein Barr virus (EBV) antigens. EBV-specific autologous T cells were reactivated and expanded exogenously from peripheral blood lymphocytes by stimulating them with EBV-transformed autologous B cells. Aside from mild inflammatory reactions in 2 patients, treatment

was well tolerated, and 6 of 10 patients demonstrated control of disease progression [35]. Other groups have reported the feasibility of generating tumor-reactive T cells and the low toxicity of this approach in advanced HNSCC [36, 37].

In transfected dendritic cell therapy, autologous dendritic cells are transfected with patient tumor DNA, then reinfused. A proof-of-concept study in HNSCC showed that this approach yielded effective antigen-presenting cells, without signs of tumor-induced suppression of dendritic cells [38]. Another novel approach is the use of intratumoral dendritic cells in combination with immunosuppressive chemoradiation. Augmentation of immune responses, long-term tumor regressions, and increased apoptosis associated with decreases in intratumoral regulatory T cells have recently been shown in an animal model of head and neck cancer [39].

Cytokine-based immunotherapy works by delivering proinflammatory cytokines either locoregionally and/or systemically to elicit an antitumor response. A number of cytokines are being explored for treatment of HNSCC, including GM-CSF, IL-2, IFN-γ, IL-12, and an investigational multicytokine biologic known as IRX-2. Table 1 lists the approaches to systemic cell-mediated immunotherapy for HNSCC that are currently in clinical trials [40], of which some are discussed in more detail in what follows.

OncoVEX$^{GM\text{-}CSF}$. OncoVEX$^{GM\text{-}CSF}$ is a second-generation oncolytic herpes simplex virus that delivers GM-CSF. In a phase 1 trial, multiple doses of OncoVEX$^{GM\text{-}CSF}$ were safe and well tolerated in patients with a range of solid tumor types, GM-CSF was expressed, and there was evidence of antitumor activity [41]. According to preliminary data from a phase 1/2 study specific to node-positive advanced head and neck cancer, the combination of CRT and OncoVEX$^{GM\text{-}CSF}$ produced pathologic complete response in 6 of 8 patients, and non-CRT-related toxicities were mild [42].

Interleukin-2. The main function of IL-2, one of the major proinflammatory cytokines produced by T cells, is to enhance the growth and cytotoxic response of activated T cells [43]. Multiple studies have shown that IL-2 enhances cellular immune responses to tumors by stimulating the proliferation and activation of several types of leukocytes with antitumor activity, including natural killer cells, lymphokine-activated killer cells, antigen-specific T-helper cells, cytotoxic lymphocytes, macrophages, and B cells [44]. The nonspecific immune reaction first causes tumor shrinkage, followed by tumor-specific, delayed-type hypersensitivity, and long-lasting immune memory [45]. Complete or partial responses have been reported after IL-2 or IL-2-based immunotherapy in head and neck cancer patients [43]. IL-2 has been administered to HNSCC patients using a variety of delivery methods, including intralesional injection (recombinant IL-2) and synthetic gene delivery systems.In addition to the benefits of IL-2 itself, the attributes of some delivery methods may have immunologically beneficial effects, whereas other methods, such as viral-based vectors, can increase toxicity. In a murine model,giving IL-2 in a plasmid/cationic lipid formulation resulted not only in expression of the IL-2 transgene but also in induction of endogenous IFN-γ and IL-12 [44].Several novel methods of administering IL-2 have been investigated, including direct administration of low-dose recombinant IL-2 around the chin and neck lymph nodes in HNSCC patients [45].

Interferon-γ. Interferon-γ has not been well studied in HNSCC, but systemic administration of the recombinant form in a phase 1/2 study in 8 patients produced clinically measurable immunologic responses in 4 of 9 HNSCC tumors evaluated, resulting in clinically measurable response in 3 patients and stable disease in 4 (1 patient progressed). During 22 days of treatment, a carcinoma in situ in the piriform sinus disappeared, and the other 3 tumors were reduced in bulk by 40%, 40%, and 18% [46].

Interferon-α. Interferon-α has been added to other drugs in the treatment of HNSCC. The combination of IFN-α, cisplatin, and 5-fluorouracil was associated with an overall response rate of 55% in patients with advanced esophageal cancer, accompanied by considerable toxicity [47]. In a phase 2 study of interferon-α plus isotretinoin and vitamin E in patients with locally advanced HNSCC, the 5-year progression-free survival rate was 80% and the 5-year overall survival rate was 81.3% [48]. Combination treatment with low dose recombinant IL-2 and interferon alpha-2a has also produced significant clinical tumor regressions in 2 of 11 (18%) heavily pretreated patients with recurrent disease [49].

Interleukin-12. Interleukin-12 has effects on both the innate and adaptive immune systems. It is important in inducing cellular immunity because it fuels the production and activation of cytolytic T cells and natural killer cells and induces the production of cytokines. In a study of 30 patients with previously untreated HNSCC, injection of recombinant IL-12 into the primary tumor was shown to increase the number of natural killer cells and alter the distribution of B cells in the lymph nodes of the 10 treated patients. These effects included redistribution of lymphocytes from the peripheral blood to the lymph nodes in the neck; a significant increase in natural killer cells and a lower percentage of $T_H$cells in the lymph nodes and the primary tumor; and a 128-fold increase in IFN-γ mRNA in the lymph nodes. Finally, the T_H2 profile in the lymph nodes of IL-12-treated patients switched to a T_H1 profile [50].

IRX-2. IRX-2 is a promising systemic cell-based strategy for HNSCC immunotherapy that employs a multifaceted approach to stimulating immune response. A primary cell-derived biologic IRX-2 contains multiple cytokines: IL-1, -2, -6, and -8, tumor necrosis factor-α, IFN-γ, G-CSF, and GM-CSF.It is sterile, endotoxin-free, and serum-free, and is produced from purified human mononuclear cells that are stimulated by phytohemagglutinin (PHA) under GMP conditions [51]. Additionally, in the regimen, cyclophosphamide is used to inhibit suppressor T-cell function, indomethacin is

Table 1: Systemic cell-mediated immunotherapies in clinical development in head and neck cancer [40].

Agent	Phase	Status	Study Type	Description
IFN-α	2 (NCT00004897)	Active, not recruiting ($N \sim 15$–45)	Open-label trial	Patients with stage I–III esophageal cancer receive combination chemotherapy and recombinant IFN-α followed by surgery and/or RT
	3 (NCT00054561)	Completed ($N = 376$)	Multicenter randomized controlled trial	To compare the combination of isotretinoin, recombinant IFN-α, and vitamin E with observation only in patients with stage III or IV HNSCC
Pegylated IFN-α2b	2 (NCT00276523)	Completed ($N = 72$)	Randomized controlled trial	Pegylated IFN-α2b at 3 different dose levels is compared with no treatment prior to resection of stage II–IV HNSCC
IL-2	2 (NCT00006033)	Completed ($N = 80$)	Multicenter open-label	To compare IL-2 gene with methotrexate in the treatment of recurrent or refractory stage III/IV HNSCC
	3 (NCT00002702)	Recruiting ($N \sim 260$)	Multicenter randomized, controlled trial	To compare surgery and RT with and without rIL-2 in patients with SCC of the mouth or oropharynx
IL-12	1/2 (NCT00004070)	Active, not recruiting ($N \sim 28$–34)	Multicenter rising-dose study	Patients with unresectable, recurrent, or refractory HNSCC receive IL-12 gene twice during week 1 and once weekly during weeks 2–7
ALT-801 (a recombinant fusion protein with an IL-2 component)	1 (NCT00496860)	Recruiting ($N \sim 46$)	Multicenter dose-escalation study	To determine the MTD of ALT-801 in previously treated patients with progressive metastatic malignancies, including HNC
IRX-2	2 (NCT00210470)	Closed ($N = 27$)	Multicenter open-label trial	Study of IRX-2 with cyclophosphamide, indomethacin, and zinc in patients with newly diagnosed, resectable stage II–IV HNSCC. The study is being conducted to confirm the safety and biological effect of the IRX-2 regimen in the same population to be studied in a planned randomized phase 3 trial. The primary focus will be on observations made from the start of treatment through the planned surgical resection of the primary tumor.

HNC: head and neck cancer; HNSCC: head and neck squamous cell carcinoma; IFN: interferon; IL: interleukin; MTD: maximum tolerated dose; RT: radiotherapy.

used to block immunosuppression due to the prostaglandins synthesized by the tumor and by suppressor macrophages, and zinc is used to reverse cellular immunodeficiency [52].

It has been shown that ex vivo treatment with IRX-2 leads to dose- and time-dependent apoptosis suppression of T cells ($P < .001$ to $P < .005$). IRX-2 also potentiated antitumor effects of immune cells, such as upregulation of key signaling molecules' expression on dendritic cells to increase their functions. Local delivery of IRX-2 induced systemic changes in both peripheral blood memory and naive T cell subsets [53].

The results of a multicenter phase 2 trial of IRX-2 have recently been reported. In this trial, 27 previously untreated, resectable patients with stage II-IV oral cavity (15), oropharynx (8), larynx (3), or hypopharynx (1) HNSCC received the IRX-2 regimen prior to surgery. The regimen consisted of intravenous cyclophosphamide on day 1, followed by bilateral perilymphatic injections of IRX-2 (115 U bilateral daily) from day 4 to 15, and daily oral indomethacin, zinc, and omeprazole from day 1 to 21. The IRX regimen was well tolerated, with minimal acute toxicity (grade <2). Tumor responses (>12% decrease on blinded CT review) were seen in 16% of patients, and 74% patients had either reduction or stable tumor size. Significant changes in tumor and lymph node lymphocytic infiltration were observed in the IRX-treated patients. Data on estimated 2-year overall survival (72%) and disease-free survival (67%) were favorable compared to those reported for 81 concurrent treatment matched controls [54, 55].

5.2. Targeted Immunotherapy in HNSCC. Technological advances have allowed researchers to identify several kinds of tumor-associated antigens that are now under investigation as therapeutic targets in HNSCC [56]. One category is

Table 2: Monoclonal antibodies (excluding anti-EGFR agents) in clinical development in head and neck cancer [40].

Agent	Phase	Status	Study Type	Description
Bevacizumab	Clinicaltrials.gov search retrieves records for 3 phase 1 trials 2 phase 1/2 trials 11 phase 2 trials 1 phase 3 trial	The phase 1, 1/2, and 2 trials are completed or ongoing The phase 3 trial is recruiting	Several	The early-phase trials are exploring several different regimens The phase 3 trial is a multicenter, randomized, controlled trial in which patients with recurrent or metastatic HNSCC receive chemotherapy ± bevacizumab. Chemotherapy consists of cisplatin, docetaxel, and fluorouracil.
anti-CD45 MAb	1 (NCT00608257)	Completed ($N = 18$)	Dose-escalation study	Patients with EBV-positive nasopharyngeal cancer receive autologous EBV-specific cytotoxic T cells in combination with anti-CD45 MAb
MN-14 (anti-CEA MAb)	1/2 (NCT00004048)	Active, not recruiting ($N \sim 30$)	Dose-escalation study	Patients with medullary thyroid cancer undergo radioimmunotherapy with MN-14 alone or combined with doxorubicin and peripheral blood stem cell rescue

CEA: carcinoembryonic antigen; EBV: Epstein-Barr virus; HNSCC: head and neck squamous cell carcinoma; MAb: monoclonal antibody.

tumor-specific antigens (also called germ cell antigens or cancer testes antigens), which are silenced in normal tissues but are reactivated in certain tumors [31]. For example, up to 71% of HNSCCs express antigens from at least 1 of 6 melanoma antigen genes (MAGEs) [57], notably MAGE-1 and MAGE-3 [58]. Antigen from NY-ESO-1, a gene expressed in normal ovary and testis, is highly expressed in a variety of tumor types [59], including HNSCC [60].

Another category of tumor-associated antigen is tumor-specific mutated proteins that are unique to the tumor and may contribute to the malignant phenotype, for example, tumor suppressor gene p53 [31]. Preclinical work suggests not only that p53 is mutated in many more cases of HNSCC than originally thought but also that wild-type p53 is often associated with highly oncogenic strains of HPV (types 16 and 18) [61].

Antigens overexpressed in tumors are a third category of targets under investigation. Notable examples are carcinoembryonic antigen (CEA), HER-2/neu, VEGF, and EGFR [31]. Antigens derived from oncogenic viruses, such as the HPV E6 and E7 oncoproteins, are also important targets in HNSCC [62–64].

5.2.1. Monoclonal Antibody (MAb) Immunotherapy. Advancements in technology have allowed identification and large-scale production of monoclonal antibodies, which are highly specific to their target, are better tolerated than cytotoxic drugs, and can induce tumor cell apoptosis [31]. These advantages have made MAb immunotherapy a compelling field of research (Table 2) [40].

EGFR is overexpressed in more than 90% of HNSCCs [10], and overexpression is often associated with poor clinical prognosis and outcome, including reduced disease-free and overall survival. A variety of EGFR inhibitors have been developed that function either by binding to the extracellular ligand binding domain of the EGF receptor (e.g., MAbs such as cetuximab), or by inhibiting the intracellular tyrosine kinase activity of the receptor [65, 66]. While the exact mechanisms of action of these inhibitors are unclear, cetuximab has been shown to activate antibody-dependant cellular cytotoxicity (ADCC). The in vivo success of cetuximab in combination with radiation has inspired exploration of other anti-EGFR agents. These include matuzumab [67], panitumumab (also called ABX-EGF) [68], ICR62 [69], nimotuzumab (also called h-R3) [70], MAb 806 [71], and zalutumumab [66]. Anti-EGFR agents have recently been reviewed elsewhere [66].

VEGF is highly expressed in most human cancers [72, 73], and in HNSCC its expression may be a significant factor in survival [74]. Therefore, recent studies have combined the antiangiogenic agent bevacizumab with chemotherapy. For example, an ongoing phase 2 trial ($N = 14$) pairs bevacizumab with pemetrexed in first-line treatment of recurrent and/or metastatic HNSCC; interim results show an overall response rate of 45% among the 11 evaluable patients, but also a high rate of bleeding complications in susceptible patients [75]. Bevacizumab is also being investigated in head and neck cancer in combination with erlotinib, a small-molecular-weight tyrosine kinase inhibitor [76].

There is evidence that VEGF and VEGF receptor-2 are coexpressed in HNSCC and that coexpression is associated with a higher proliferation rate and worse survival [74]. Adjuvant therapy with VEGFR-2 inhibitors might disrupt both the paracrine and autocrine actions of VEGF and be beneficial in HNSCC patients [77].

An investigational anti-VEGF antibody, 2C3, appears to control tumor metastasis by a mechanism somewhat different from that of bevacizumab: in a preclinical study of breast cancer, it inhibited lymphangiogenesis and decreased intratumoral lymph vessel development [78].

Table 3: Vaccines in clinical development in head and neck cancer [40].

Agent	Phase	Status	Study Type	Description
ALVAC-CEA vaccine	2 (NCT00003125)	Active, not recruiting ($N \sim 24$)	Partially randomized pilot study	For patients with CEA-expressing advanced tumors, including HNC. In stage I, patients receive vaccinia-CEA vaccine and then ALVAC-CEA (CEA-avipox) vaccine, or the reverse sequence. In stage 2, patients receive whichever vaccine was superior, plus GM-CSF ± IL-2.
Anti-CEA RNA-pulsed DC vaccine	1 (NCT00004604)	Active, not recruiting ($N \sim 18$)	Dose-escalation study	To determine the MTD of the vaccine in patients who have refractory metastatic cancer, including HNC, that expresses CEA
EBV LMP-2 peptide vaccine	1 (NCT00078494)	Completed ($N = 99$)	Randomized study	Patients with nasopharyngeal cancer that has been controlled with standard therapy receive 1 of 2 LMP-2 vaccines to determine which better prevents cancer recurrence. LMP-2 is a protein produced by EBV.
HPV-16 E7/E6 peptide vaccine	1 (NCT00019110)	Completed ($N = 40$–46)	Multicenter open-label study	Patients with advanced or recurrent cancers, including HNC, receive a vaccine that contains the HPV-16 E7 and E6 peptides
JAX-594 (thymidine kinase-deleted vaccinia virus plus GM-CSF)	1 (NCT00625456)	Recruiting ($N \sim 24$)	Dose-escalation study	To find the MTD of JAX 594 in patients with refractory solid tumors, including HNSCC
MAGE-A3/HPV-16 vaccine	1 (NCT00257738)	Recruiting ($N \sim 90$)	Dose-escalation study	Patients with HNSCC receive a vaccine comprised of MAGE-A3 and HPV-16 peptides
	1 (NCT00704041)	Recruiting ($N \sim 48$)	Dose-escalation study	To evaluate 4 doses of the MAGE-A3/HPV-16 vaccine in 2 cohorts of HNSCC patients those with MAGE-A3-positive tumors and those with HPV-16-positive tumors
Multiple-peptide vaccine (LY6K, VEGFR1, VEGFR2)	1 (NCT00561275)	Completed ($N = 6$)	Open-label trial	Patients with esophageal cancer receive a vaccine containing multiple peptides and GM-CSF
p53-pulsed DC vaccine	1 (NCT00404339)	Recruiting ($N \sim 50$)	Randomized safety trial	Patients with HNSCC receive autologous DCs loaded with wild-type p53 peptides, ± T-helper peptide epitope
Ras peptide vaccine	2 (NCT00019331)	Completed ($N = 60$)	Single-center trial	To compare 3 regimens of vaccine therapy with tumor-specific mutated Ras peptides plus IL-2 or GM-CSF in patients with metastatic solid tumors, including HNC, that potentially express mutant Ras.
Fowlpox-CEA-TRICOM vaccine (fCEA-TRI)	1 (NCT00028496)	Completed ($N = 48$)	Dose-escalation study	To evaluate fCEA-TRI ± GM-CSF in patients with advanced or metastatic cancer, including HNC.
	1 (NCT00021424)	Completed ($N = 20$)	Dose-escalation study	To find the MTD of fCEA-TRI in patients with advanced SCC of the oral cavity or oropharynx or nodal or dermal metastases
	1 (NCT00027534)	Completed ($N = 6$–18)	Dose-escalation study	Immunotherapy comprises autologous DCs treated with fCEA-TRI in patients with CEA-expressing advanced or metastatic cancer, including HNC.

CEA: carcinoembryonic antigen; DC: dendritic cell; EBV: Epstein-Barr virus; HNC: head and neck cancer; HNSCC: head and neck squamous cell carcinoma; IL: interleukin; GM-CSF: granulocyte macrophage colony-stimulating factor; HPV: human papillomavirus; LY6K: lymphocyte antigen 6 complex, locus K; MAGE: melanoma antigene gene; MTD: maximum tolerated dose; TRICOM: TRIad of COstimulatory Molecules (aimed at stimulating a cytotoxic T-cell response); VEGFR: vascular endothelial growth factor receptor.

Another new treatment strategy is to target CEA, an antigen present on the surface of a majority of HNSCC tumors [31, 79], via MAb immunotherapy plus radiotherapy. A phase 1 trial combined high-dose labetuzumab, a ^{90}Y-labeled humanized anti-CEA MAb, with doxorubicin and peripheral blood stem cell rescue for patients with advanced thyroid cancer. Objective responses were rare, but the therapy was well tolerated and there was evidence of antitumor activity [80]. Another study in advanced thyroid cancer evaluated bispecific MAb (BsMAb), which targets both CEA and diethylenetriamine penta-acetic acid. Combination therapy with BsMAB and a ^{131}I-labeled bivalent hapten was associated with a median survival time of 110 months, significantly longer than the 61 months seen in untreated patients ($P < .03$) [81].

5.2.2. Cancer Vaccines. Two common types of therapeutic cancer vaccines are peptide/protein-based or dendritic cell-based. To produce the first type, an adjuvant is combined with 1 or more peptides/proteins commonly expressed on HNSCC such as p53, MAGE, or HPV. It is expected that the immune system, in response to the adjuvant, will also respond to tumor cells that express the antigen(s). For the second type, dendritic cells are removed from cancer patients through leucopheresis and stimulated with an appropriate tumor antigen, then reinjected so that they will activate T cells specific to the patient's tumor. The strategies can be combined, as when dendritic cells are pulsed with mutant p53 peptides [82, 83]. A phase 1 trial of this approach is under way [40]. Dendritic cells can also be pulsed with MAGE peptides. In one recent study, a vaccine that combines MAGE-1 and MAGE-3 peptides was administered following surgery and chemotherapy for 2 patients with primary malignant melanoma of the esophagus, which has an extremely poor prognosis. One patient had stable disease for 5 months and survived for 12; the second was without tumor recurrence for 16 months after treatment, and, following esophagectomy, had survived for 49 months at the time of trial report publication [84]. In a phase 1/2 study, an MAGE-3 peptide + ASO2B adjuvant vaccine produced clinical responses in 6 of 12 patients with metastatic tumors (mainly melanoma), but the response could not be clearly correlated with cytokine profile, levels of anti-MAGE-3 antibody, or IgG subclass [85]. A phase 2 pilot study has recently been completed that made use of vaccines constructed of HPV 16 peptides E6 and E7 alone or in combination with MAGE-3 peptides [40]. A common issue challenging the further development of clinically useful vaccines is the need to develop new and more effective vaccine adjuvants.

Cancer vaccines for HNSCC can also be based on DNA or RNA. The nucleic acid containing the gene for the antigen is manipulated exogenously so it will be taken up, expressed, and processed by antigen-presenting cells, in the hope that the immune system will target tumor cells containing the same antigen. Vaccines of this type have shown potential for targeting CEA when recombinant fowlpox or ALVAC (canarypox) viruses, which do not replicate in human cells, are used as vectors, with and without GM-CSF [86, 87]. Nucleotide-based vaccines targeting HPV are also being studied in HNSCC. Research in China showed that in a mouse model of esophageal SCC, a fusion protein vaccine combining the HPV-16 oncoproteins E6 and E7 significantly inhibited tumor growth and size ($P < .01$), and 25% of vaccinated animals remained tumor-free at 2 days [88]. In another study, Chen et al. constructed a vaccine which linked *Mycobacterium tuberculosis* heat-shock protein 70 to HPV-16 E7; the E7-specific T-cell response to murine tumors that expressed HPV-16 E7 was at least 30-fold higher with the fusion vaccine than with a vaccine based on unmodified E7 [59]. A list of cancer vaccines in clinical trials for treatment of head and neck cancer is provided in Table 3 [40].

6. Conclusions

Given the well-established role of immune system dysfunction in HNSCC, immunotherapy is an attractive treatment option, potentially associated with more tolerable side effects and improved efficacy. Recent advances in identifying HNSCC tumor antigens have provided targets for monoclonal antibodies and other modes of immunotherapy. In particular, advances in the understanding of cell-mediated immunity have led to several promising approaches to HNSCC treatment that involve systemic cell-mediated immunotherapy, such as the delivery of cytokines that can stimulate a durable immune response and tumor rejection. These novel treatment modalities, either as monotherapy or combined with other forms (e.g., MAb therapy), represent future directions in the treatment of HNSCC.

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Review Article

Recent Advances in Image-Guided Radiotherapy for Head and Neck Carcinoma

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Radiotherapy has a well-established role in the management of head and neck cancers. Over the past decade, a variety of new imaging modalities have been incorporated into the radiotherapy planning and delivery process. These technologies are collectively referred to as image-guided radiotherapy and may lead to significant gains in tumor control and radiation side effect profiles. In the following review, these techniques as they are applied to head and neck cancer patients are described, and clinical studies analyzing their use in target delineation, patient positioning, and adaptive radiotherapy are highlighted. Finally, we conclude with a brief discussion of potential areas of further radiotherapy advancement.

1. Introduction

Recent technological advances in the field of radiation oncology are revolutionizing the management of cancer with ionizing radiation. Through the use of highly conformal techniques, the ability to deliver curative doses to sub-millimeter accuracy is unprecedented to now. In particular, intensity-modulated radiotherapy (IMRT) has had a substantial impact on the management of head and neck carcinoma (HNC), and its use is highly prevalent among radiation oncologists [1]. IMRT allows for the delivery of high doses to target volumes while simultaneously limiting the dose to organs at risk, so that once common toxicities, such as xerostomia, can be limited. However, for this to be achieved, sharp gradients in dose are produced, and therefore small changes in patient or tumor position may have large dosimetric implications. In particular, several studies have demonstrated that patient/tumor motion during IMRT specifically for HNC is clinically significant [2–4].

Image-guided radiotherapy (IGRT) is a novel array of techniques to help minimize the discrepancies due to variations in patient/tumor position. A strict definition of IGRT is the use of images to monitor or modify treatment delivery. However, IGRT can also be divided into three broad categories of image-based innovations: (1) the integration of functional and biological imaging into the treatment planning process to improve tumor contouring (or *target delineation*), (2) the use of various imaging modalities to adjust for tumor motion and positional uncertainty, and finally (3) the adaptation of treatment planning based on tumor response and changes in normal tissue anatomy [5]. The latter form of IGRT, known as *adaptive radiotherapy*, has the potential benefit of avoiding unintended normal tissue toxicity by altering the original treatment plan according to changes that may have occurred during the course of radiotherapy.

Treating HNC is often complex, owing to the importance of preserving critical organ functions, such as salivation, speech, and swallowing, that are key factors in determining quality of life after treatment. Since radiotherapy continues to play a central role in the definitive [6–10], adjuvant [11, 12], and recurrent disease [13] settings of HNC, it is likely that these innovations will continue to improve outcomes by minimizing toxicity and maximizing organ preservation. In addition, dose escalation with IMRT may lead to improved local control, which may ultimately extend survival if augmented by improvements in systemic therapies for metastatic disease. Although many of these sophisticated imaging and treatment modalities that employ IGRT are still yet to be proven beneficial in randomized controlled

trials, the theoretical benefits of improved disease-control and normal tissue sparing are currently being demonstrated in a variety of peer-reviewed publications, which is the focus of the following review.

2. Improved Target Delineation

The first type of IGRT involves the incorporation of new diagnostic imaging modalities into the initial tumor contouring stage of radiotherapy planning in order to more precisely identify areas that should be treated with radiation. Currently, most centers employ CT-based planning, where the patient is simulated in the treatment position and then the targeting of macroscopic and microscopic disease sites is performed on CT-acquired images alone. Although CT-based planning is common for HNC, recent studies have suggested that a large degree of interobserver variability exists in the contouring of the gross-target volume (GTV). Cooper et al. asked eight "expert" physicians to contour the same GTV in 20 patients with supraglottic carcinomas and found that the overlap in contoured volumes was only 53% with CT-alone [14]. As precise tumor localization is of growing importance with increasingly conformal radiotherapies, attention has now shifted to novel forms of imaging that provide additional biological and tumor information that can be included in the planning process in order to clarify areas of tumor burden.

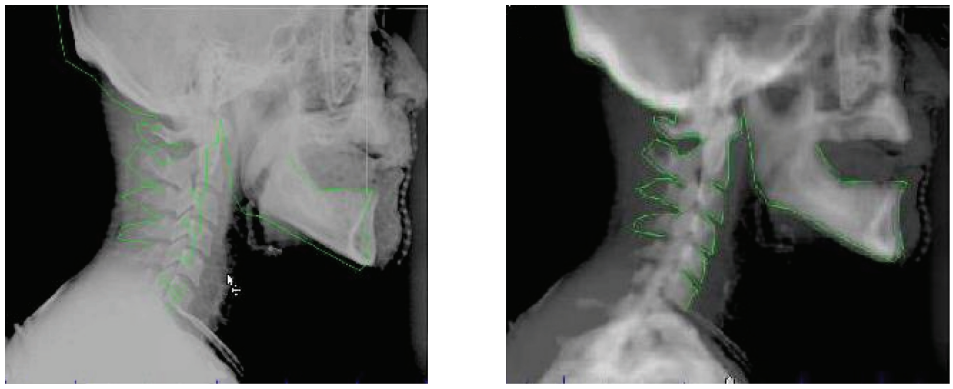
A key innovation in this form of IGRT is the use of 18-F-Flurodeoxyglucose (FDG)-PET. FDG is a radiolabeled analog of glucose that is selectively absorbed in tumor cells more than normal tissues, and thus it is useful in distinguishing neoplastic growth in tissues that otherwise appear radiographically normal. As such, FDG-PET has a well-established role in oncology and is commonly used in tumor staging for several cancers, including HNC [15–17]. However, increased interest has now focused on the use of FDG-PET in target delineation for radiation therapy in order to guide the contouring of tumor margins and extended fields. Since most tumor contouring is performed on CT-based images, this is accomplished by using sophisticated software to perform an accurate overlay (or *registration*) of PET and CT images. In this fashion, target delineation can be performed on the fused PET-CT image. Alternatively, some centers are now equipped with hybrid PET-CT scanners that are capable of acquiring both PET and CT scans during a single session [18]. This has the added benefit of imaging the patient while in the treatment position.

Research on FDG-PET in HNC has shown that PET-based planning can significantly influence the size of the gross-tumor volume (GTV) that is outlined [19–24], the size of the nodal volume [23, 24], and assist in the detection of nodal metastases not visualized or enlarged by CT criteria [23, 24]. Most studies have found that PET-based planning tends to reduce the GTV, however some studies have shown that PET-based planning can also increase the size of volumes contoured [5, 19]. Furthermore, new clinical evidence from patients treated with PET-CT planning is appearing in literature. Research has shown that PET-CT based planning can lead to excellent local control [18, 25], significant alterations in staging [22], and decreased normal tissue toxicity [18]. In particular, Vernon et al. reported on 42 patients with HNC who underwent PET-CT during planning and were followed for a median of 32 months [18]. A high level of disease control was obtained, and acute toxicities were relatively mild and improved with time.

Although the initial results of improved tumor localization through PET-CT planning are optimistic, several areas of concern exist. Guido et al. raise an issue regarding PET-CT planning in a recent study of 38 patients who were planned using PET-CT and CT-alone [26]. These researchers found that although the GTV was reduced in 92% of patients with the addition PET-CT from CT-only-based plans, the planning target volume (which includes areas of microscopic disease and additional margins for error) was not significantly different between the two planning modalities. As such, no clinical advantage would be expected from the combined PET-CT planning. Further research on technical issues such as this will have to be carefully addressed in the future before widespread implementation of these technologies. As of now, FDG-PET has a well-established role for tumor staging, monitoring tumor response, and follow-up of HNC patients. However, the routine use of PET-CT for planning is not yet recommended.

FDG-PET is a commonly used radioactive tracer; however several novel tracers are being employed in HNC imaging. Tumor hypoxia is a common occurrence in the tumor microenvironment and has a well-known role in the resistance of tumors to radiotherapy. Furthermore, it is thought that many hypoxia-induced treatment failures can be prevented in part by escalating the dose to hypoxic subvolumes of the GTV. However, this process depends on our ability to accurately identify hypoxic areas and deliver a targeted radiation boost to those localities. Recent advances in PET-based imaging combined with IGRT are now making "hypoxia-directed radiotherapy" possible [27]. [^{18}F]-misonidazole (FMISO) is a novel tracer that has been shown to accurately identify hypoxic areas in head and neck tumors [28–30]. In particular, Lee et al. have used FMISO-PET to identify hypoxic subvolumes in 10 HNC patients and subsequently escalated the dose to those areas with a local boost [31]. No outcomes were reported, but the feasibility of the technique has been established.

A recent study describes the treatment of 20 HNC patients who received routine pre- and mid-treatment FMISO scans in order to determine the effect of tumor hypoxia on patient prognosis [32]. Surprisingly, these results showed that neither the presence nor absence of tumor hypoxia as defined by FMISO was correlated with patient outcome. Although this may suggest that tumor hypoxia is not correlated with patient outcomes, the authors suggest several alternative explanations to this idea, including the notion of tumor reoxygenation during fractionated radiotherapy. Furthermore, a wealth of preclinical and clinical data support the worsening prognosis associated with hypoxia in HNC [27, 33–36]. In any case, further investigation is necessary to ascertain whether the outcomes of HNC can be improved by specifically targeting hypoxic zones.



(a) (b)

Figure 1: Example of 2D kV image used for verification of patient positioning. A 2D projection was created from the planning CT, and the bony anatomy was contoured (solid line). This image was then overlapped with a kV image taken immediately prior to treatment delivery. The overlay is shown before (a) and after (b) adjustments are made.

Other non-FDG tracers have also been investigated for their role in HNC patients. In particular, 1-(^{11}C)-acetate PET (ACE-PET) has been shown to be a promising tracer for HNC staging and target delineation and may be used to complement FDG-PET [37]. The molecule, 3′-deoxy-3′-^{18}F-fluorothymidine (^{18}F-FLT), is also of growing interest to HNC management. FLT is phosphorylated by the cytosolic enzyme thymidine kinase-1 (TK1) and is subsequently trapped intracellularly [38]. TK1 activity is increased during DNA synthesis, and thus ^{18}F-FLT trapping is a marker of proliferation. Research specifically in HNC has shown that FLT uptake is correlated with decreased survival [39], has good reproducibility [40] and may potentially be useful in determining tumor response to radiotherapy [41]. Finally, similar to FMISO, Cu(II)-diacetyl-bis(N(4)-methylthiosemicarbazone) (Cu-ATSM) is a marker of hypoxia but through an entirely different mechanism [27]. This tracer has also been evaluated in HNC and was shown to provide another feasible approach for hypoxia-directed radiotherapy [42]. However, further research is necessary before the routine implementation of this or other novel PET tracers into daily clinical use.

3. Improved Treatment Delivery

The second type of IGRT involves the use of modern imaging modalities to assist in daily patient positioning. Most radiotherapy protocols involve several weeks of sequential daily treatment, and each day the patient needs to be repositioned into the exact position obtained during the initial planning CT. However, often small positioning errors occur, which introduces the possibility for considerable *interfraction* motion. In addition, if the patient is not properly immobilized during a radiotherapy session, there is also the potential for *intrafraction* motion. As six potential degrees of freedom are prone to changing between and during fractions, accurate positioning is an exceedingly complex challenge.

Over the years, several unique methods have been devised to address and minimize interfraction and intrafraction motion. Traditionally in HNC, thermoplastic masks composed of a mesh-like grating are placed over a patient's face and secured to the treatment couch in order to immobilize the patient's head during CT-simulation and treatment. The masks have markings on them that allow the radiation therapists to then re-position the patient prior to each fraction with the aid of optical arrays. Various types of masks [3, 43–47] and bite blocks [48] have been employed for HNC patients. However, due to the flexibility of the head and neck region, these immobilization techniques have a potential for considerable setup variability [45, 49–51].

Another common way to verify patient positioning is through the use of two-dimensional (2D) portal film imaging (see Figure 1). This is done using devices attached to the treatment machine that are capable of taking two-dimensional megavoltage (MV) [48, 52, 53] or kilovoltage (kV) [54] radiographs. Typically, this is performed at the beginning of each week of radiotherapy; however newer schemes have been devised for daily kV imaging that are more sensitive to day-to-day interfractional changes [55]. Although these 2D-radiographs are adequate for detecting large positioning errors, they are problematic for a number of reasons. First, they tend to have poor image quality, making it difficult to identify set up inaccuracies [56–58]. Second, they can only visualize bony structures, so changes in soft tissue are not detected using this method. Third, 2D-radiographs are not adequate for detecting rotational movement of the head [49, 59, 60].

As such, recent advances in *three-dimensional* (3D) (or *volumetric*) in-room imaging have offered new solutions to the limitations of conventional patient positioning. One solution that has been proposed involves the use of a conventional CT scanner mounted on a rail system, which is placed in the treatment room and shares a couch with the linear accelerator. This system is capable of taking high-quality, three-dimensional images after patient immobilization in order to verify setup between day-to-day treatments [51, 61, 62]. These images are of higher quality than traditional portal images, and they provide adequate resolution for soft tissue

identification. However, the CT-on-rails system does have some distinct disadvantages. First, the addition of a full-size CT gantry into the treatment room can be cumbersome. Second, these systems are incapable of detecting intrafractional motion. Finally, this system introduces the need for movement of the couch between the CT scanner and the linear accelerator, which increases the time of the procedure [63].

Cone-beam CT (CBCT) is another novel form of 3D in-room imaging that can minimize patient positioning inaccuracies. CBCT is a scaled-down version of a CT scanner that is built into the treatment machine. Images taken from a CBCT at the time of treatment can be overlaid on the original planning CT, and specialized software can be used to detect positioning errors with millimeter accuracy [59]. Similar to 2D portal imaging, two types of CBCT exist: MV and kV. CBCT with kV imaging is reported to have better image contrast and smaller signal-to-noise ratios than MV CBCTs [64]. CBCT imaging has been used to correct for interfractional motion in a clinically feasible amount of time [63]. In addition, this technology is being studied for the detection of intrafractional motion, which could potentially be used for improved accuracy as well [65, 66]. Finally, CBCT-based correction has also been used to increase treatment accuracy in the setting of IMRT, thus allowing for larger target doses, while simultaneously sparing healthy tissues [67, 68]. There are concerns; however, about the additional radiation dose delivered with frequent CBCT imaging [69, 70]. In particular, studies have estimated that daily cone-beam CT imaging can lead to an increase of 5.3–6.7 cGy to skin per scan [71] and a total of 300 cGy over an entire treatment course [72]. This may correspond to a 2%–4% increase in secondary malignancies [71]. No long term data on the actual incidence of secondary malignancies is currently available, and continued investigation will have to be performed to address this question.

In the past few years, helical tomotheraphy (HT) has become an increasingly popular technique that employs daily volumetric imaging to visualize both patient setup errors and tumor/organ variations [73, 74]. HT combines a 6 MV CT with a therapeutic linear accelerator that is mounted onto a ring gantry. During treatment, the patient is translated through the ring while the gantry continuously rotates, resulting in helical fan beam radiation delivery. The radiation beam is dynamically modifed using a binary multileaf collimator, which allows for IMRT and the creation of highly conformal dose distributions. In addition, using the on-board 6 MV CT scanner, daily image guidance can be performed with the patient in the actual treatment position [75]. Thus, direct target position verification can be achieved prior to radiation delivery [73].

Research on HT in HNC patients has been promising. A prospective evaluation comparing HT to 3D-conformal radiotherapy (3D-CRT) in 60 patients with disease at various anatomic sites found that HT was subjectively equivalent or superior to 3D-CRT in 95% of plans [76]. Furthermore, studies have shown that HT can achieve sharper dose gradients, improve dose homogeneity, and provide better sparing of the parotids than conventional IMRT [77–79] or stereotactic radiosurgery [80]. HT with daily position corrections using MV CT is also safe and easy to implement into a daily clinical routine [74]. Clinical outcomes using HT in HNC patients have also been encouraging and have shown decreased dose, as well as toxicity, to the parotids without compromising survival, locoregional control, and disease-free survival in comparison to conventional and non-HT IMRT approaches [81, 82].

Digital tomosynthesis (DTS) offers another method of 3D in-room imaging for patient setup verification. Similar to CBCT and HT, DTS provides volumetric tomographic imaging; however it works by reconstructing 3D slices from a limited number of 2D cone-beam projections. These images may be of a lower resolution; however advocates of this technology argue that it is comparable to CBCT in terms of imaging quality. Furthermore, since DTS constructs images from a limited number of arcs, it may result in lower cumulative doses, as well as reduced treatment times in comparison to other modalities [83, 84]. These advantages may be of added benefit to pediatric patients, where reduced dose and decreased treatment times are a high priority.

Optical methods have also been studied for daily image-guidance [85]. Several groups have reported on systems utilizing in-room cameras for imaging 3D surface reconstructions in real time [86–88]. Others have used specialized cameras with infrared markers for determining target position [3, 89–93]. These systems are reported to detect setup errors with high precision, as well as little setup time. This technology has also been used in combination with in-room radiographic imaging with promising results [44, 94–96]. Unlike other radiographic modalities, optical modalities are noninvasive and do not expose the patient to added radiation dose. In addition, these techniques account for intrafraction motion and can be done in a relatively short amount of time [85].

In conclusion, volumetric (3D) imaging in the HNC setting is superior to conventional 2D portal imaging in many ways. However, the extent to which this technology should be applied is unclear. In particular, the frequency with which 3D imaging for setup verification should be performed is unknown and is the subject of current debate. Some have argued that weekly or biweekly scans are adequate [59], while others have suggested that daily scanning is necessary [97]. Additional investigation will be necessary to clarify these questions.

4. Adaptive IGRT

The third broad category of IGRT is called adaptive IGRT (ART). ART is a new, and still evolving, concept with the potential to greatly improve the delivery of radiotherapy. The current standard of treatment planning in radiotherapy involves obtaining an image at the start of treatment. The plan is then generated on that image and delivered to the patient over the course of his/her therapy. We know in head and neck cancer; however, that over the course of the 6–7 weeks of radiotherapy, there can be significant changes in the patient's anatomy based on shrinkage of the primary tumor or involved lymph nodes and loss of overall body

weight [98–100]. Applying the original plan to the now altered patient anatomy can lead to increasing the dose delivered to the surrounding normal tissues, including the parotid glands and spinal cord [101–104]. Sparing these normal tissues is an important consideration, because post-radiation xerostomia has a significant impact on quality of life [105–107] and dose constraints regarding the spinal cord and brain stem are always of concern due to potentially devastating consequences. ART allows us to "adapt" the treatment plan in response to the changes that occur so that we can maximally spare these normal tissues while maintaining complete coverage of the tumor volume.

A study by Barker et al. examined the rate of tumor regression and the total overall tumor regression by obtaining CT images during treatment 3 times per week over the course of radiotherapy and quantifying the volumetric and geometric changes that occurred [99]. They estimated that the GTV decreased by a median rate of approximately 1.8% per day. The median total GTV decrease was approximately 70% (range 10%–92%) over the course of treatment, and this shrinkage tended to be asymmetric. The parotid volume also decreased by a median of 28.1% and moved medially with a median translation of 3.1 mm which correlated with patient weight loss. Vakilha et al. demonstrated a median reduction of parotid volume of 49.8% and a median medial translation of the parotids of 8.1 mm over the course of treatment [108].

Medial translation of the parotid glands from tumor regression and patient weight loss tend to bring the parotids into higher dose regions and therefore increase the dose to the parotids [101]. In addition, shrinkage of the parotids can result in a much larger percentage of the parotid receiving high doses than anticipated. O'Daniel et al. estimated that the median dose increase to the ipsilateral parotid was 3 Gy, and 45% of patients experienced increases between 5–7 Gy [103]. Though these doses seem small, the parotid is a very radiosensitive tissue and even small changes in dose can have a large impact. Blanco et al. estimated that salivary function decreased at a rate of 5% per 1 Gy increase in mean dose [107]. They also noted that 70% of patients that received a mean dose of greater than 26 Gy to both parotids experienced grade 4 xerostomia.

In order to avoid the unintentional overdosing of the surrounding tissues, some investigators have studied re-planning the radiation treatments in response to changes in patient anatomy. Kuo et al. performed a prospective trial in which 10 patients with enlarged lymph nodes were re-planned after delivery of 45 Gy [101]. Twenty-one Gy was then delivered according to the new plan to complete the radiation treatment. The patients were then analyzed to compare the differences between the dose that was delivered after re-planning to the dose that would have been delivered without re-planning. Their results show that the dose to the parotid glands was reduced by approximately 2–4 Gy by re-planning.

Hansen et al. performed a retrospective analysis on patients that were re-planned for weight loss or tumor regression [102]. Comparison of the two plans showed that not re-planning led to decreases in target coverage and increases in dose delivered to the surrounding tissues. They found that the dose to 95% of the planned target volume was reduced in 92% of patients in the old plan compared to the new plan (range, 0.2–7.4 Gy). In addition, the maximum dose to the spinal cord was higher in the original plan compared to the new plan in all patients (range 0.2 to 15.4 Gy). The brainstem maximum dose was also increased in 85% of patients (range 0.6–8.1 Gy).

Though research in the field of ART is mostly preliminary, it does show promising evidence of an improvement in the delivery of radiotherapy. Though the theoretical benefits of ART are highly desirable, there are still many barriers to overcome before widespread adoption will be feasible. First, it is unclear when and how often re-planning should be done. Would re-planning once be sufficient or would it need to be done more frequently, such as weekly or even daily? Alternately would it be more appropriate to develop defined thresholds that, if met, would necessitate re-planning? Attempts are underway to identify the optimal re-planning schedule, but for now, this schedule must take into account the technical difficulties and the time required to create a new plan. Currently, occasional re-planning can be done, but frequent re-planning would overwhelm the available resources. New technologies such as deformable image registration and automated target delineation in conjunction with higher computational power will be required before widespread adoption of this new strategy can occur.

5. Future Technologies

In the future, IGRT will likely continue to expand by incorporating newer and more sophisticated imaging modalities. In this section, we briefly discuss several cutting-edge technologies that are in the early stages of investigation in HNC, including molecular-based CT, high-resolution ultrasound, magnetic resonance imaging (MRI), and proton therapy.

Molecular-based CT imaging is a promising modality that may offer several advantages for tumor delineation. As CT is one of the most commonly employed diagnostic imaging modalities in hospitals today, it has widespread availability and convenience of use. However, CT is generally not thought of as a molecular/cellular imaging modality owing to the lack of targeted contrast agents. A recent report by Popovtzer et al. at the University of Michigan at Ann Arbor has described the use of gold nanoparticles that selectively and sensitively target tumor antigens [109]. Using in vitro models of HNC, these researchers demonstrated that the attenuation coefficient for molecularly targeted cells is over 5 times greater than for normal cells. As such, nanotechnology-based CT may improve target delineation by providing more accurate microtumor identification during planning. Furthermore, since CT is easily accessible to most physicians, this technique could be rapidly introduced if proven to be both feasible and efficacious.

Aside from CT and PET, several other imaging modalities have also been investigated for their potential role in radiotherapy planning for HNC. High-resolution ultrasound was studied by Wein et al. who demonstrated a feasible

method for incorporating ultrasound-based information of the architecture of cervical lymph nodes into the planning CT for target delineation [110]. MRI has also been examined in HNC. A recent study by Gardner et al. has found that MRI fused to the planning CT can decrease the amount of interobserver variation in critical organ and target volume delineation for patients who have intracranial tumor extension, heavy dental work, or contraindication for contrast-enhanced CT [111]. To these authors knowledge, no clinical data has yet been reported. However, based on these preclinical studies, MRI and high-resolution ultrasound may contribute to improved outcomes in HNC patients.

Proton therapy is another appealing form of radiotherapy owing to its superior dose distribution properties, which allow smaller volumes of normal tissue to be irradiated than is possible for any photon beam technique. Accordingly, initial clinical experiences of proton therapy in HNC have been encouraging and have shown reduced normal tissue toxicity in sinonasal, nasopharyngeal, and oropharyngeal malignancies [112]. Although long-term efficacy studies are still immature, the preliminary data is encouraging. Furthermore, recent interest in combining proton therapy with modern improvements in image-guidance and dose-localization has arisen. In particular, just as the intensity of photon beams can be modulated in IMRT, the intensity of proton beams can also be modified to produce intensity-modulated proton therapy (IMPT) [113]. Although a mature technique is still unavailable, an offline study in HNC patients has shown that IMPT has a better ability to spare organs at risk and is associated with a significantly reduced risk of secondary malignancy induction in comparison to IMRT with photon beams [114]. The feasibility of combining proton therapy with various forms of IGRT, such as MRI- and kV-based modalities, has also been demonstrated and may lead to a further reduction in normal tissue toxicity when clinical data becomes available [115, 116]. Based on preliminary reports such as this, future proton-therapy research is eagerly anticipated.

6. Conclusion

With the advent of highly precise conformal therapies, such as IMRT, the accurate localization and delivery of radiotherapy will be increasingly important in the decades to come. Recent advances in image-guided radiotherapy provide increased tumor localization by improving the identification of areas of tumor burden, by minimizing the effects of patient setup errors caused by intra-/interfraction motion, and by allowing for adaptive replanning to changes that occur in the tumor or patient during long courses of radiotherapy. In doing so, these changes are leading to improvements in the therapeutic ratio, where doses are increased at diseased-sites and minimized at normal tissues.

Although the promise of IGRT is great, it is not without its hurdles. Importantly, there are large financial and educational barriers in the initial setup and implementation of new imaging modalities. Furthermore, there is still no existing level I evidence demonstrating the benefit of IGRT over standard radiotherapeutic modalities. Evidence from existing retrospective and nonrandomized studies; however, strongly supports the beneficial role of IGRT. Further research is currently under way, and the results are expected to continue to validate the use of IGRT in the management of HNC patients.

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Review Article

Clinical Applications of FDG PET and PET/CT in Head and Neck Cancer

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18F-FDG PET plays an increasing role in diagnosis and management planning of head and neck cancer. Hybrid PET/CT has promoted the field of molecular imaging in head and neck cancer. This modality is particular relevant in the head and neck region, given the complex anatomy and variable physiologic FDG uptake patterns. The vast majority of 18F-FDG PET and PET/CT applications in head and neck cancer related to head and neck squamous cell carcinoma. Clinical applications of 18F-FDG PET and PET/CT in head and neck cancer include diagnosis of distant metastases, identification of synchronous 2nd primaries, detection of carcinoma of unknown primary and detection of residual or recurrent disease. Emerging applications are precise delineation of the tumor volume for radiation treatment planning, monitoring treatment, and providing prognostic information. The clinical role of 18F-FDG PET/CT in N0 disease is limited which is in line with findings of other imaging modalities. MRI is usually used for T staging with an intense discussion concerning the preferable imaging modality for regional lymph node staging as PET/CT, MRI, and multi-slice spiral CT are all improving rapidly. Is this review, we summarize recent literature on 18F-FDG PET and PET/CT imaging of head and neck cancer.

1. Introduction

In 2008, head and neck cancers accounted for approximately 4% to 5% of all the malignant disease in the United States [1]. Head and neck squamous cell carcinoma (HNSCC) comprises the vast majority of head and neck cancer (HNC). Oncologic imaging plays an important role in head and neck cancers as imaging findings can aid significantly detection, staging, restaging, and therapy response assessment of these tumors. Accurate staging at the time of diagnosis is critical for selection of the appropriate treatment strategy. Unfortunately, at the time of initial diagnosis more than 50% of patients already present with regional nodal metastases or even distant metastases.

Diagnosis of a head and neck cancer is usually achieved by a combination of patient history, physical examination, and either nasopharyngoscopy and/or laryngoscopy with directed biopsies. Panendoscopy may be necessary to reveal the extent of a tumor. Morphologic imaging with computed tomography (CT) and/or magnetic resonance imaging (MRI) with intravenous contrast are often performed either prior to panendoscopy to noninvasively assess the aerodigestive tract or afterwards to provide information about primary tumor size, infiltration, involvement of surrounding structures, and regional nodal involvement. There is growing evidence, however, that these modalities have limitations in their diagnostic accuracy. CT and MR imaging rely on criteria of contrast-enhancement patterns and nodal size for detection of lymph node metastases which are not specific and may escape detection of metastases within normal size lymph nodes. There is also growing evidence that 18F-FDG PET imaging is a very sensitive and valuable imaging tool in evaluation head and neck cancer. The main drawback of 18F-FDG PET alone is the limitation with respect to lesion localization. However, the advent of PET/CT now overcomes this limitation and permits the evaluation of both metabolic and anatomic characteristics of disease, which has proven to be a major advance for staging, detection carcinoma of

Table 1: Studies comparing accuracy of FDG PET and PET/CT with CT and MRI for detection of lymph nodes metastases.

Author year	Number of patients	Tumor Subtypes	Results	Notes
Beak et al. [2], 2009	15	Periorbital	PET/CT accuracy (98%) > CT 84%	- CT: 16 slice. - PET modified Tx in 39%.
Roh et al. [3], 2007	167	HNSCC	PET or PET/CT accuracy (92%-93%) > CT/MR 85%-86%	- PET/CT significantly better for detection of primary tumor
Gordin et al. [4], 2007	35	Nasopharyngeal	PET/CT accuracy 91% > PET 80% > CT 60%	- Retrospective - PET/CT modified TX in 57%
Kim et al. [5], 2007	32	Oropharyngeal	PET sensitivity 21% higher than CT/MR ($P < .05$)	- PET/CT significantly better for detection of primary tumor
Dammann et al. [6], 2005	79	Oral cavity and oropharynx	PET accuracy 96% > MRI 94% > CT 92%	- Nonhyprid PET/CT used
Ng et al. [7], 2005	124	Oral cavity SCC	PET accuracy 98.4% > CT/MR 87.1%	- Prospective

unknown primary, treatment monitoring, and evaluation of residual or recurrent disease.

2. Staging

Accurate staging at the time of diagnosis is the most important factor for treatment planning and determination of prognosis [8]. One attractive feature of 18F-FDG PET as a modality for initial TNM staging is that it covers most of the body within a single study. PET therefore provides information on the primary tumor, nodal metastases, distant metastases, and potential 2nd primary carcinomas. A literature survey on the use of 18F-FDG PET in head and neck cancer (HNC) compared to CT indicates that PET has a higher sensitivity (87% versus 62%) and specificity (89% versus 73%) for staging cancer [9]. Addition of PET/CT to initial staging of patients with HNC has also been shown to have a measurable impact on the treatment selection [10, 11].

2.1. Primary Tumor. Numerous reports on initial staging have shown that 18F-FDG PET is at least as sensitive as MRI or CT in detecting the primary tumor [3, 7, 10–17]. This is related to the fact that smaller or submucosal malignancies may be difficult to distinguish from adjacent tissues on anatomical imaging. A better sensitivity of 18F-FDG PET for detecting primary tumor comparing to CT/MRI imaging has been shown in oral cavity cancer [18, 19]. However, the current practice is not in favor of utilizing 18F-FDG PET for local staging of all newly diagnosed head and neck squamous cell carcinoma (HNSCC). This is due to the higher anatomic resolution of MRI and contrast enhanced multislice CT compared to 18F-FDG PET. Nevertheless, in a recent study by Baek et al. including 40 patients with oral cavity cancer and dental artifacts on CT or MRI, it was demonstrated that 18F-FDG PET/CT can provide more useful clinical information and higher sensitivity, particularly in deep tumors, compared to CT and MR. The diagnostic performance for the detection of the primary tumors in the oral cavity was 96.3% for PET/CT, 77.8% for CT, and 85.2% for MRI [20].

2.2. Nodal Metastases. Nodal staging has a significant impact on outcome in terms of disease free survival and overall survival after therapy [21]. Metastatic lymph node disease is found in approximately 50% of the patients at the time of primary diagnosis [6, 22]. Several reports have verified that 18F-FDG PET has a higher sensitivity and specificity than CT or MR imaging for detection of lymph node metastases in head and neck cancer [23, 24]. In a review by Schöder and Yeung, an average sensitivity of 87%–90% and a specificity of 80%–93% were reported for 18F-FDG PET/CT; a sensitivity of 61%–97% and specificity of 21%–100% were reported for morphologic imaging modalities including MRI and CT [25]. Several recent studies comparing 18F-FDG PET, 18F-FDG PET/CT, and CT/MR are summarized in Table 1. Results showed that integrated 18F-FDG PET/CT may play an important role in identifying lymph node metastases in head and neck squamous cell carcinoma (HNSCC). However, MRI is usually used for local staging as it provides almost comparable accuracy to 18F-FDG PET in locoregional metastases in addition to best primary tumor delineation [26].

Occult lymph nodes (clinical N0 disease) still represent a dilemma for both imaging modalities and surgeons. Although earlier reports have favored PET over other anatomic imaging modalities as PET has been shown to have a sensitivity of 78% and an accuracy of 92% (compared with a sensitivity of 57% and an accuracy of 76% for CT) for the detection of nodal metastases in clinical N0 disease [27]. Two recent reports by Nahmias et al. and Schoder et al. comprising 47 and 37 patients, respectively, demonstrated that 18F-FDG PET/CT is not accurate enough for detection

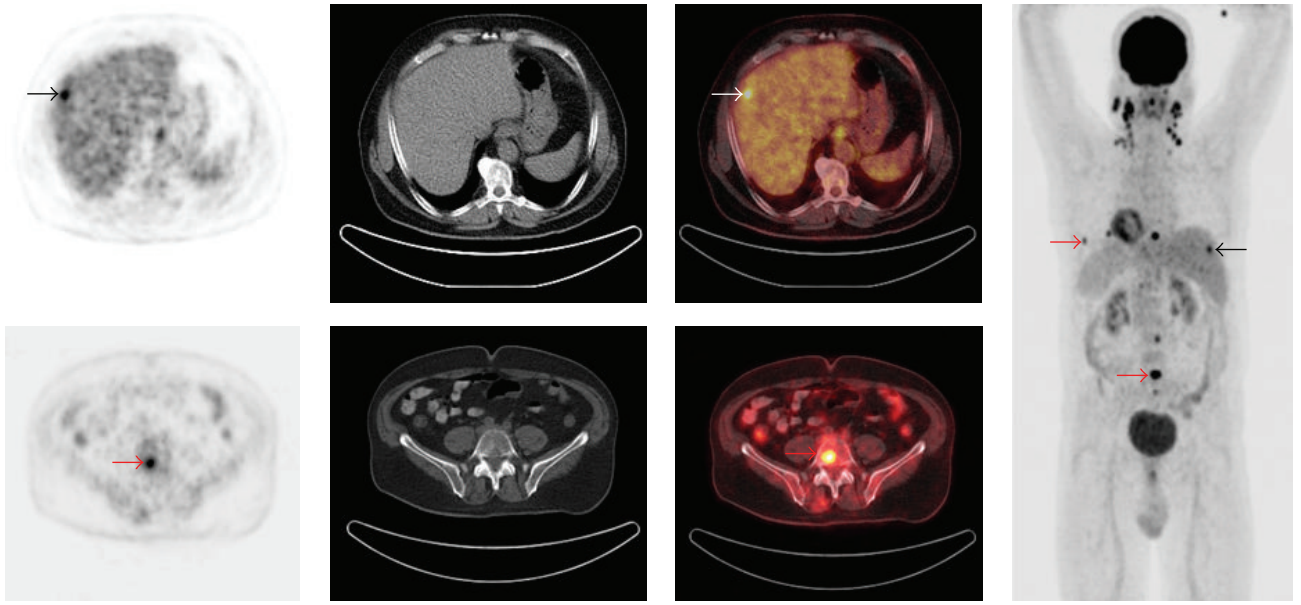


Figure 1: A 61-year-old man with nasopharyngeal SCC and bilateral cervical lymph node metastases underwent PET/CT for staging. Axial PET, CT, PET/CT, and maximum intensity projection (MIP) images are shown. PET/CT revealed focal FDG uptake in the right liver lobe indicating liver metastasis (black, white arrows). PET/CT also revealed multiple focal FDG uptakes in the lumbar spine, sternum, and ribs indicating multiple bone metastases (red arrows). PET/CT was valuable for detection distant metastases.

of occult nodal disease in previously untreated patient and would not help the surgeon in the management strategy of the patient, particularly if the study is negative. They reported sensitivity and a specificity ranging from 67% to 79% and 82% to 95%, respectively. False negative findings were likely related to either the presence of microscopic metastases not detected by PET/CT, or by the proximity of nodal metastases to the primary tumor which might have obscured their detection [28, 29]. Schroeder et al. verified these results and suggested that elective neck dissection in patients with clinical N0 head and neck cancer squamous cell carcinoma (HNSCC) should not be based upon cross-sectional imaging (CT, MR, PET/CT) at the resolutions currently available [30]. However, Kovacs et al. examined the potential role of 18F-FDG PET and sentinel node biopsy in 62 patients for the purpose of neck dissection. Their results suggest that patients showing positive lymph node on PET scan undergo a neck dissection due to the high specificity, whereas a sentinel node biopsy should be performed in patients with a negative PET scan. This strategy avoided 12 patients futile neck dissections with false-positive CT findings and a negative sentinel node biopsy [31].

2.3. Distant Staging. The role of 18F-FDG PET for staging of distant metastases in HNC is acknowledged as one of the most powerful indication in HNC (Figure 1). There is a general agreement that 18F-FDG PET is indicated for initial staging of HNC when there is suspicious of distant metastases and synchronous 2nd tumor. The incidence distant metastases increases with locally advanced disease (T3-T4), N2, or N3 disease, extracapsular extension of lymph node involvement, and perineural invasion [32, 33]. A synchronous 2nd tumor, particularly in aerodigestive tract, is often associated with a history of heavy nicotine or alcohol consumption and patients with hypopharyngeal tumors. Recent studies on the use of 18F-FDG PET for the detection of distant metastases and synchronous 2nd tumor in HNC are summarized in Table 2. These studies showed that PET detected distant metastases or 2nd primaries in up to 15.6% of the patients. The true positive findings were 82%. Moreover, PET showed a better accuracy once it was compared to conventional imaging as demonstrated by Ng et al., Chua et al., and Liu et al. [34–36].

3. Carcinoma of Unknown Primary

Cervical lymph node metastases from an unknown primary tumor account for approximately 1-2% of newly diagnosed head and neck cancers [40]. In 5% to 80%, depending on the patient selection, the primary tumor could not be identified by physical examination, panendoscopy, and conventional imaging, including CT and/or MRI [41, 42]. Treatment of these patients often includes extensive fields of irradiation to include the entire pharyngeal mucosa, larynx, and bilateral neck. The wide-field irradiation reduces the risk of tumor recurrence. However, it also causes significant morbidity, particularly in terms of xerostomia [43]. Therefore, the accurate identification of occult primary sites is important because the therapy can then be focused to the known site of origin, decreasing treatment-related morbidity, and improving therapeutic efficacy [44].

The utility of 18F-FDG PET to identify carcinomas of unknown primary has been examined. A comprehensive review by Rusthoven summarized the impact of 18F-FDG

Table 2: Studies evaluating the performance of FDG PET for the detection of distant metastases and synchronous 2nd tumor in HNC.

Author year	Number of patients	Positive PET	True positive (distant mets + 2nd primary)	False positive	Notes
Ng et al. [34], 2009	111	16	13/16	3/16	CT/MR detect 4/16
Chua et al. [35], 2009	68	6	5/6	1/6	CT + BS detect 4/6
Liu et al. [36], 2007	300	61	50/61	11/61	
Yen et al. [37], 2005	118	32	24/32	8/32	
Goerres et al. [12], 2003	34	8	7/8	1/8	PET modified Treatment in 15%
Sigg et al. [38], 2003	58	8	7/8	1/8	PET modified Treatment in 5%
Schwartz et al. [39], 2003	33	7	7/7	0/7	
Total	722	138	113/138	25/138	

PET for the situation of carcinoma of unknown primary. A total of 16 studies comprising 302 patients, published between 1994 and 2003, were included. In all of these studies, patients underwent physical examination and CT or MRI, with the majority undergoing panendoscopy as well. The gold standard for primary tumor verification was tissue biopsy. Of the 302 patients, 18F-FDG PET detected 24.5% of tumors that were not apparent after conventional workup. 18F-FDG PET imaging also led to the detection of previously unknown metastases in 27.1% of the patients (regional, 15.9%; distant, 11.2%). The overall of sensitivity of PET for the primary tumor detection was 88%, with a specificity of 75%, and an accuracy of 79%. When detection efficacy was evaluated with respect to localization, a lower sensitivity for cancers in base of tongue, and a lower specificity for cancers in the tonsil were noted [45]. In this review, we performed a meta-analysis including studies published between 2000 and 2009 that specifically addressed the performance of 18F-FDG PET or PET/CT in detecting carcinoma of unknown primary in patients presented with cervical lymph node metastases and negative or inconclusive standard workup. For this group, 18F-FDG PET and PET/CT detected the primary tumor in 51 of 180 patients (28%) (Table 3).

Two recent reports in the era of advanced morphologic imaging technology also verified the vital utility of 18F-FDG PET and PET/CT in cancer of unknown origin. Johansen et al. showed in a prospective study comprising 67 patients with cancer of unknown primary that a therapeutic change of treatment was made in 25% as a consequence of 18F-FDG PET findings [54]. Roh et al. compared the performance of combined 18F-FDG PET/CT and CT alone in 44 patients with cervical metastases from unknown primary tumors. They reported that 18F-FDG PET/CT was significantly more sensitive than CT (94.0% versus 71.6%, $P < .001$), but the two methods had similar specificities (94.8% versus 96.5%, resp.). 18F-FDG PET/CT correctly detected distant metastases in 6 out of 6 patients [55]. Based on these results, 18F-FDG PET and PET/CT can be recommended as early diagnostic modality in the workup for carcinoma of unknown primary and neck metastases (Figure 2).

4. Treatment Response Assessment

In recent years, the use of combined chemoradiotherapy (CRT) has been shown to have a significant impact on the treatment of head and neck cancer [56]. 18F-FDG PET is a valuable modality for monitoring response to treatment as it can assess metabolic activity rendering malignant process. Martin et al. demonstrated in a recent study including 78 patients that PET was significantly superior to clinical examination or conventional imaging with respect to the assessment of patients after chemoradiotherapy. Accuracy of PET in therapy response assessment was significantly better than clinical assessment and conventional imaging (CT/MR) ($P < .002$ and $P < .001$, resp.). The authors also suggested that patients with a complete response on posttreatment PET have a significant survival advantage [57].

Monitoring response to radiation therapy can be complex due to posttreatment changes like inflammation and edema. 18F-FDG PET has been investigated in the assessment of early response to chemotherapy regimen and if modification or discontinuation are needed or not (Figure 3). Several reports have illustrated that patients with favorable response to therapy demonstrate a continued reduction in metabolic activity and hence decreased FDG uptake over multiple PET studies compared to baseline values. Prognostic value of 18F-FDG PET regarding survival and response to therapy appears promising but needs more confirmation.

5. Residual and Recurrent Disease

The utility of anatomical imaging in the posttreatment situation is limited because of fibrosis, tissue edema, and anatomical distortion [58, 59]. The early detection of residual or recurrent head and neck cancer following radiation therapy and/or chemotherapy poses a diagnostic challenge. A survey of the literature showed that 18F-FDG PET is the most sensitive noninvasive modality presently available for differentiating posttreatment changes from residual or recurrent

Table 3: Studies evaluating performance of 18F-FDG PET or PET/CT for the detection of carcinoma of unknown primary in patients with negative workup.

Author year	Number of patients	All positive	True positive	False positive	Percent detected by PET
Padovani et al. [46], 2009	13	9	7	2	54%
Silva et al. [47], 2007	25	9	3	6	12%
Fakhry et al. [48], 2006	20	10	7	3	35%
Wong and Saunders [49], 2003	17	8	5	3	29%
Fogarty et al. [50], 2003	21	6	1	5	5%
Johansen et al. [51], 2002	42	20	10	10	24%
Kresnik et al. [52], 2001	15	12	11	1	73%
Jungehulsing et al. [53], 2000	27	7	7	0	26%
Total	180	81	51	30	28%

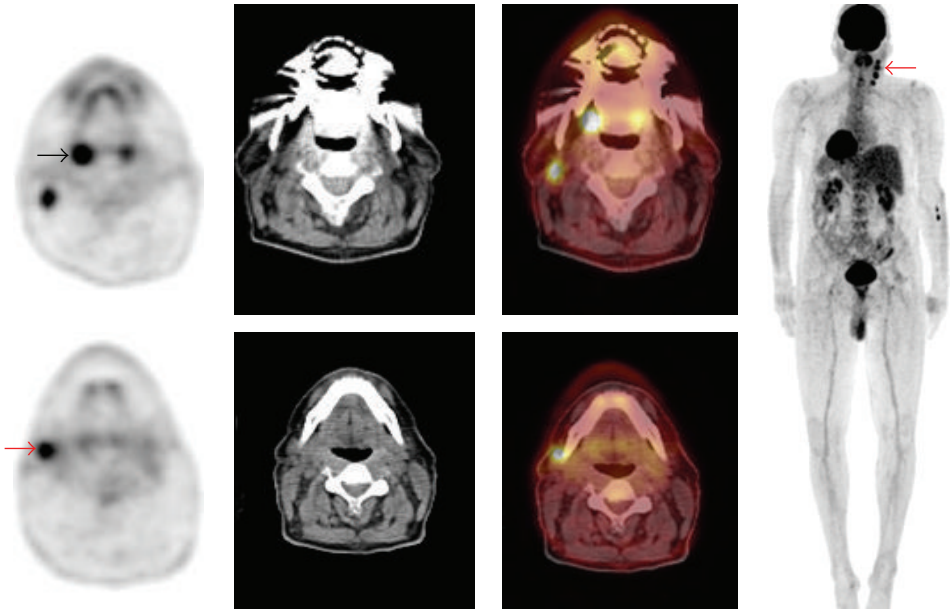


Figure 2: A 61-year-old man presented with right side cervical lymphadenopathy proved to be carcinoma of unknown primary. Patient underwent PET/CT to reveal primary tumor. Axial PET, CT, PET/CT, and maximum intensity projection (MIP) images are shown. PET/CT showed asymmetrical FDG uptake in the palatine tonsils with intense FDG uptake in the right tonsil (black arrow) as well as multiple hypermetabolic cervical lymph nodes in the right side (red arrows). This patient subsequently underwent surgical resection and histopathology revealed squamous cell carcinoma in the right tonsil. PET/CT was valuable in revealing the primary tumor in this case.

disease and that its performance is higher compared CT and MR for this purpose.

6. Residual Disease

A 3-4 months interval between the end of radiotherapy and evaluation of residual malignant tissue provides the best specificity and sensitivity for PET. This is due to reducing false positive findings associated with nonspecific inflammatory activity, and reducing false negative findings encountered during first 8 weeks postchemoradiotherapy which may increase the risk of seeding the dissection scar if viable tumor cell was left in the tumor bed [60–67]. The NPV of PET following therapy is very high (up to 97%) and associated with a very good prognosis, whereas positive 18F-FDG PET must be correlated with clinical status and a biopsy is needed to rule out nonspecific uptake [60, 61, 64–67]. Performance of 18F-FDG PET early after chemoradiotherapy has been evaluated to assess residual tumor as many surgeons prefer to perform salvage surgery within 6 to 8 weeks after radiation, before postradiation fibrotic changes develop. Kim et al. found in a prospective study in 97 patients that early imaging one month following completion of radiation therapy can be performed with high sensitivity (88%) and specificity (95%) [68]. Delbeke and Martin and Kostakoglu and Goldsmith agreed in two reviews that persistent tumor uptake one month after radiation therapy is strongly suggestive of residual disease and that

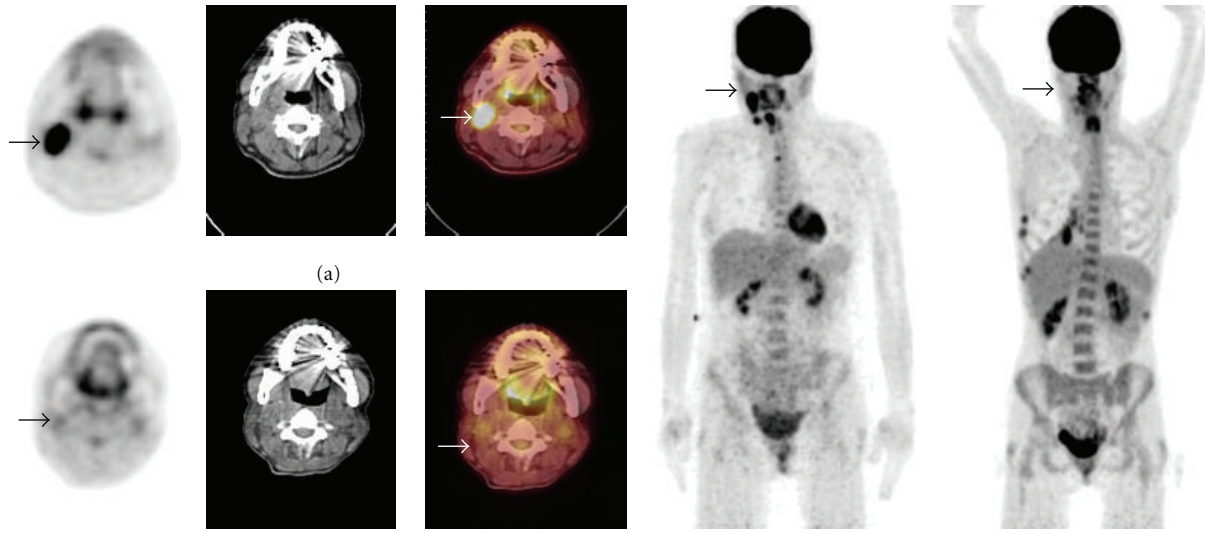


Figure 3: 40-year-old women with right side larynx squamous cell carcinoma and a right side cervical lymph node metastases underwent PET/CT imaging before and 1 month after completion of chemoradiotherapy. (a) Pretherapy axial PET, CT, PET/CT, and MIP images reveal intense FDG uptake in the right cervical lymph node (arrows). (b) After treatment axial PET, CT, PET/CT, and MIP images reveal decrease FDG uptake in the corresponding locations (arrows). Appearance was consistent with early response to chemoradiotherapy. FDG PET/CT was valuable in monitoring early response to treatment.

a positive PET scan can result in immediate initiation of secondary treatment strategies due to early detection of resistance to chemotherapy [69, 70]. On the other hand, Rogers et al. found in a prospective study with a small number of patients (12 patients) a low sensitivity of 45% for a 1-month posttherapy 18F-FDG PET, compared to the 6–8 week posttreatment surgical histopathology [71]. The current role of 18F-FDG PET and PET/CT are indicated to exclude residual disease and to select patients who are candidates for salvage surgery after chemoradiotherapy. Although there is general consensus that waiting 3 months postradiation sustains best sensitivity and specificity, early imaging is justified in some scenarios, but with cautious interpretation considering time interval posttherapy and clinical findings.

The possible role of 18F-FDG PET in avoiding patients futile neck dissection after treatment by excluding residual locoregional disease has also been evaluated. Ong et al. demonstrated in a recent study comprising 65 patients that 18F-FDG PET/CT has a high negative predictive value (NPV) and specificity (97% and 89%, resp.) for excluding residual locoregional disease after chemoradiotherapy and that neck dissection may be omitted safely in patients without lymphadenopathy, while in patients with residual lymphadenopathy, a lack of abnormal ^{18}F-FDG uptake in these nodes also excludes viable tumor with high certainty but still further assessment is needed [72]. Yao et al. suggested that 18F-FDG PET can avoid patient neck dissection if the postradiotherapy 18F-FDG PET scan is negative since it has had a high predictive value for negative pathology in neck dissection or fine-needle aspiration even with large residual lymphadenopathy [73]. Nevertheless, Tan et al. found in a retrospective study comprising 48 patients that 18F-FDG PET was not a good predictor of residual disease [74]. Standardization of the role of 18F-FDG PET in avoiding neck dissection in a prospective study particularly when lymphadenopathy presents is necessary before negative 18F-FDG PET/CT may become the only, or at least most-decisive, criterion in the management of the neck after chemoradiotherapy.

7. Recurrence

Klabbers et al. reviewed studies published between 1994 and early 2003 regarding the utility of 18F-FDG PET for detection of residual and recurrent head and neck tumors after radiation and/or chemoradiotherapy. The results showed that 18F-FDG PET has a better sensitivity (86%) and specificity (73%) compared with CT and/or MRI (56% sensitivity and 59% specificity, resp.) [75]. Ryan et al. reported on 108 patients and found that 18F-FDG PET/CT detected locoregionally persisting or recurrent head and neck SCC with a sensitivity of 82%, a specificity of 92%, a positive predictive value (PPV) of 64%, a negative predictive value (NPV) of 97%, and an overall accuracy of 90% [76].

18F-FDG PET and PET/CT have a high sensitivity and moderate specificity for detecting recurrent disease at the primary tumor site, regional lymph node metastases, and distant metastases. Wong performed a meta-analysis on studies published between 1999 and 2002 that showed relatively high sensitivity (84–100%) with moderate specificities (61–93%) regarding 18F-FDG PET in recurrent tumor of HNCSC [84]. Several studies published in the last 4 years on the utility of PET or 18F-FDG PET/CT for the detection of recurrence

Table 4: Studies evaluating the performance of 18F-FDG PET and PET/CT for the detection of recurrent disease in head and neck cancers.

Authors year	Number of patients	Sensitivity	Specificity	Accuracy	Notes
Abgral et al. [77], 2009	91	100%	85%	90%	FDG PET/CT
Wang et al. [78],2009	44	100%	98%	98%	Prospecrive PET performance > CT
Cermik et al. [79], 2007	50	83%	93%		
Álvarez Pérez et al. [80], 2007	60	98%	90%		Prospective
Salaun et al. [81], 2007	30	100%	95%	97%	
Goerres et al. [82], 2004	26	91%	93%		Prospective
Kubota et al. [83], 2004	36	90%	78%	81%	Prospective Accuracy significantly higher than CT/MR

are summarized in Table 4. The performance of PET with respect to the identification of recurrent disease following treatment demonstrated a high sensitivity (83%–100%) and relatively high specificity (78%–95%). The higher specificity in these studies compared to an earlier report, published by Wong et al., may be related to more awareness of proper time point (2-3 months) for imaging after treatment.

8. PET/CT in Radiation Treatment Planning

New high-precision radiotherapy (RT) techniques, such as intensity-modulated radiation therapy (IMRT), 3-dimensional conformal radiotherapy (3D-CRT), and proton beam therapy allow conformal treatment of tumor and to avoid unacceptable damage to normal tissues leading to possible improvement of tumor control and decrease of treatment-related toxicity. These techniques depend on imaging modalities allowing accurate tumor volume delineation and response assessment during treatment. The potential application of 18F-FDG PET/CT for intensity modulated radiation therapy (IMRT) planning is an area of major interest. PET/CT may increase the gross target volume (GTV) because metabolically active tumor can be detected in normal-sized nodes. On the other hand, PET/CT-based target volume could be smaller than CT-based target volume alone in the case of patients in whom the tumor may be partially necrotic. The radiation treatment plan might be modified significantly if distant metastases are detected on the PET scan. The radiation target volumes may be significantly modified when 18F-FDG PET data are incorporated into radiation treatment planning. However, PET has been found to fail frequently to identify viable tumor in areas of bone marrow infiltration and perineural extension that are highly suspect on MRI (21).

Soto et al. suggested recently, based on a retrospective study comprising 61 patients, that 18F-FDG PET/CT should play an important but not exclusive role in defining the gross target volume (GTV) depending on the correlation between pretreatment 18F-FDG PET-defined biologic target volume (PET-BTV) and the anatomical sites of locoregional failure (LRF) after 3-D CRT or IMRT for head and neck cancer [85].

Rothschild et al. reported in a recent case control analysis of 45 patients with pharyngeal carcinoma that PET/CT and treatment with IMRT improved cure rates compared to patients undergoing IMRT without PET/CT. The event-free survival rate of the PET/CT-IMRT group was 90% and 80% at 1 and 2 years, respectively, compared to 72% and 56% in the control group without PET/CT ($P = .005$) [86].

Wang et al. investigated 28 patients with head and neck carcinoma treated with IMRT based on an 18F-FDG PET/CT defined gross tumor volume (GTV). They reported that tumor staging was significantly changed in 50% of cases (14/28 patients) as compared with CT-based staging, with 12 patients having higher T stages and 6 patients having higher N stages. Furthermore, a 17 months median follow-up period posttherapy did not reveal any locoregional recurrence indicating that PET-guided planning of the radiation field is accurate [87].

On the other hand, Breen et al. reported that GTV assessment in 10 patients with HNSCC was not significantly different between PET/CT and contrast CT scans, using 8 different observers. Furthermore, Breen et al. noted that there was greater consistency for the CT derived GTV's compared to the PET/CT derived volumes [88]. Table 5 summarizes recent studies on the use of 18F-FDG PET in radiotherapy planning. Ahn and Garg suggested in a review that the utility of a functional assay in defining target volume helps to determine areas to receive higher doses of radiation in cancers of the head and neck tumors [94].

One of the most controversial and challenging issues in applying PET/CT in radiation planning is contouring the outline of the tumor. Changing the PET window level can lead to a considerable overestimation or underestimation of the target volume. However, several techniques including threshold-based methods and gradient-based methods have been suggested and used, but still consensus needs to be met. Fifty percent of the tumor/image maximum intensity have been used for contouring by several groups [9, 95]. Others normalized volumes according to liver uptake [89, 91]. Wang et al. used an arbitrary SUV of 2.5 as a basis for contouring [87]. Berson et al. suggested in a recent report that developing an institutional contouring protocol

Table 5: Studies evaluating the role of FDG PET and PET/CT in radiation planning.

Author year	Number of patients	Study type	Results	Notes
Soto et al. [85], 2008	61 (9 LRF)	Retrospective	8/9 LRF within BTV-PET.	
Rothschild et al. [86], 2007	45	Case-control analysis	PET/CT with IMRT improved cure rates	Advanced pharyngeal carcinoma
Wang et al. [87], 2006	28	Prospective	PET/CT-based GTV significantly different from CT scans alone in 50% of cases	PET/CT upgraded T and N stage in 18 p.
Breen et al. [88], 2007	10		no significant differences in the GTVs between PET/CT and CT alone	CT volumes were larger than PET-CT
El-Bassiouni et al. [89], 2007	25		PET/CT-based volume significantly smaller than CT.	
Koshy et al. [90], 2005	36	Retrospective	TNM changed in 36%, RT volume and dose changed in 14%	
Heron et al. [91], 2004	21	Prospective	PET/CT improves delineation of normal tissues from tumor areas	PET/CT improves staging
Ciernik et al. [92], 2003	12HNC of 39	Retrospective	PET/CT changed GTV in 50% compared to CT	
Nishioka et al. [93], 2002	21		PET improves GTV, normal tissue sparing	PET alone

(IMRT) intensity-modulated radiation therapy, (GTV) gross target volume, (BTV) biological target volume, (LRF) locoregional failure

for PET/CT treatment planning is highly recommended to reduce interobserver variability [96]. Geets et al. compared gradient-based method and threshold-based method in patients with laryngeal cancer. They demonstrated that the gradient-based method is more accurate than the threshold-based method. The threshold-based method overestimated the true volume by 68% ($P = .014$) [97].

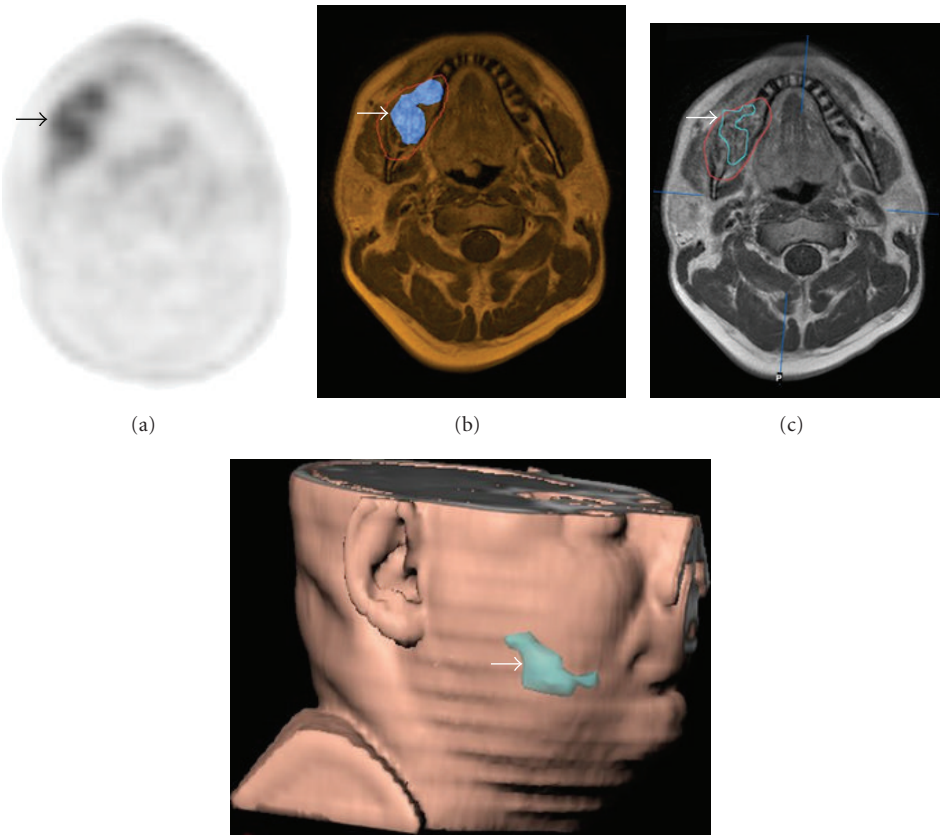
Although most authors demonstrated that PET/CT can change the gross tumor volume (GTV) and staging status for radiotherapy planning. Several issues are still to be addressed before the role of PET/CT for IMRT planning and gross tumor volume (GTV) delineation can be clearly defined. Is this change in GTV clinically significant? Can PET/CT provide prognostic information guiding the escalating of the radiation dose to area with higher metabolic activity? Furthermore, is the development of objective and reproducible methods for segmenting PET images achievable? Addressing these issues will help to identify the ultimate impact of this technology in radiation treatment planning which needs subsequent larger experimental studies with clinical outcome and cost-benefit analyses.

9. Non-FDG PET in Head and Neck Cancer

PET imaging has become a promising tool for detecting hypoxic subvolumes of tumors. Hypoxia represents a negative prognostic factor for radiation treatment of head and neck cancer where it is associated with a significant resistance to radiochemotherapy [98, 99]. However, mapping hypoxic region of tumor can positively impact on treatment outcome [100]. Several PET tracers have emerged for this purpose like 18F-fluoromisonidazole (18F-FMISO), 18F-labelled fluoroazomycin arabinoside (-[18F]FAZA and 2-(2-nitro-(1)H-imidazol-1-yl)-N-(2,2,3,3,3-pentafluoropropyl)-acetamide (EF5) [100–102]. Chao et al. demonstrated the feasibility of Cu(II)-diacetyl-bis(N(4)-methylthiosemicarbazone) (60Cu-ATSM PET) to create a hypoxia imaging-guided IMRT treatment plan through coregistering hypoxia 60Cu-ATSM PET to the corresponding CT images for radiation therapy of patients with head and neck cancer [103]. At our institution, Souvatzoglou et al. 18F-labelled fluoroazomycin arabinoside (18F-FAZA) as a feasible hypoxic agent in patients with head and neck cancer and demonstrated that FAZA can be potentially used for hypoxia-directed intensity-modulated IMRT dosing patients [104].

While 18-18F-FDG PET is very sensitive head and neck cancers, its specificity is not as high as its sensitivity due to false-positive results in inflammatory or infectious lesions. These lesions are frequent in this area, in particular after treatment by surgery and/or radiotherapy. For this purpose, O-(2-[18F]fluoroethyl)-L-tyrosine (18F-FET) has been introduced and investigated by several groups. Results suggested a possible role for FET in head and neck cancer to differentiate between inflammatory and malignancy in a selective cases. Nevertheless, it should not be used as alternative to FDG due to inferior sensitivity [105, 106].

The proliferation marker fluorodeoxythymidine 18F-3-deoxy-3-fluorothymidine (18F-FLT) has been investigated by de Langen et al. in 15 patients (including 6 patients with HNC) to evaluate the reproducibility of quantitative 18F-FLT measurements. The authors showed that quantitative



(a) (b) (c)

(d)

Figure 4: Patient with a squamous cell carcinoma of the right mandible (arrows). (a) The [18F]Galacto-RGD PET shows heterogeneous tracer uptake, which can also be clearly delineated in (b) the image fusion with the corresponding MRI scan. (c) shows the tumour volume in red as defined by MRI. By applying a threshold of SUV 3 and only using pixels with SUVs above this threshold, (d) a subvolume with more intense $\alpha v\beta 3$ expression can be defined which is shown in the 3D reconstruction (blue line in (c), blue area in (d)).

18F-FLT measurements are reproducible for predicting response to therapy in individual patients. However, authors recommended further studies correlating 18F-FLT response with pathological and clinical outcome [107].

Beer et al. investigated the application of [18F] Galacto-RGD-PET imaging of $\alpha v\beta 3$ expression, a receptor related to tumor angiogenesis and metastasis, in 11 patients with head and neck squamous cell carcinoma (HNSCC). Their results showed that use of 18F-RGD PET in a multi-modalities setting and definition of tumor subvolumes is feasible (Figure 4). The authors suggested that 18F-RGD PET imaging might be used for the assessment of angiogenesis and for planning and response evaluation of $\alpha v\beta 3$ targeted therapies [108].

In the preclinical settings, the role of molecular imaging with PET for monitoring the antiepidermal growth factor receptor (anti-EGFR) inhibitor therapy in solid tumors showing overexpression of EGFR like head and neck squamous cell carcinoma (HNSCC) has been investigated. Several radiopharmaceuticals including the proliferation marker fluorodeoxythymidine (18F-FLT) and the chimeric monoclonal antibody (64CU-DOTA-Cetuximab) have been considered promising for this purpose. However, further clinical and imaging studies are still needed.

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Review Article

Sentinel Node Detection in Head and Neck Malignancies: Innovations in Radioguided Surgery

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Sentinel node mapping is becoming a routine procedure for staging of various malignancies, because it can determine lymph node status more precisely. Due to anatomical problems, localizing sentinel nodes in the head and neck region on the basis of conventional images can be difficult. New diagnostic tools can provide better visualization of sentinel nodes. In an attempt to keep up with possible scientific progress, this article reviews new and innovative tools for sentinel node localization in this specific area. The overview comprises a short introduction of the sentinel node procedure as well as indications in the head and neck region. Then the results of SPECT/CT for sentinel node detection are described. Finally, a portable gamma camera to enable intraoperative real-time imaging with improved sentinel node detection is described.

1. Introduction

Sentinel lymph node biopsy is increasingly being used as a staging procedure for various malignancies. The sentinel node can be defined as the lymph node on the direct drainage pathway from the primary tumor [1]. Therefore, this particular node is likely to be the first node to harbor metastasis and can be used to provide information about the rest of the nodal basin. Based on the hypothesis of sequential tumor spread, sentinel node mapping can be used for nodal staging, being more precise than imaging procedures and less invasive than regional prophylactic nodal dissection. In melanoma, the sentinel node status has proven to provide relevant prognostic information and is widely performed to accurately stage melanoma patients [2]. The procedure has evolved to a routine staging method for patients with clinically localized breast cancer and is nowadays used to stage patients with other solid tumors as well. The role of sentinel node biopsy has not been clearly defined for head and neck squamous cell carcinoma. Positive sentinel nodes have shown to be a negative prognostic factor in oral cancer [3] and several authors have published good results regarding staging accuracy of the sentinel node biopsy in oral cancer patients [4–6]. It can be used to select patients for subsequent neck dissection and can reduce morbidity in many sentinel node negative patients who can be spared this operation.

Conventional planar lymphoscintigraphy is routinely used for preoperative sentinel node detection and localization. Dynamic planar images can show the draining lymph vessels directly after injection, while sequential static planar images show an overview of the number and localization of the sentinel nodes. Sentinel nodes are often clearly visualized with planar images and the levels of these nodes can be localized using external radioactive markers, such as a cobalt-57-source pen. Interpretation of planar images can be difficult because the anatomy information is limited to outlining

the body contour. Especially sentinel nodes in the head and neck region can be difficult to localize, as a result of complex anatomy, interlacing lymphatic vessels, unexpected drainage patterns and because the three-dimensional surface of the structures of the head is not visualized on planar images. Furthermore, sentinel nodes in proximity to the injection area, for example, in the preauricular or submandibular region, can easily be missed on planar images.

Intraoperative sentinel node detection with the gamma ray detection probe can be challenging as well. More than 95% of the administered radioactivity stays behind at the injection site and may cause nearby located sentinel nodes to be missed. Distinguishing sentinel nodes from second-echelon nodes with the probe is not possible and, as no overview can be provided, certainty about removal of all radioactive sentinel nodes is not provided by the gamma probe.

The complexity of the anatomy in the head and neck region requires optimization of sentinel node detection and localization in this area. First, we will report on the clinical indication for sentinel node mapping in head and neck malignancies. Then we will outline recent innovations to improve radioguided lymph node surgery in the head and neck area and we will report on our own preliminary results with those innovative imaging techniques.

2. Indication for Sentinel Node Mapping in the Head and Neck Region

As sentinel node mapping in the head and neck region is used for lymph node staging, only patients with clinically and radiologically negative lymph node assessment (stage N0) are considered for this procedure. If enlarged lymph nodes are found by physical examination or ultrasound aspiration cytology, the presence of nodal metastases can be confirmed and patients can proceed to selective or (modified) radical neck dissection.

Controversy exists regarding the appropriate indication for sentinel node biopsy in melanoma and squamous cell carcinoma. The indication for sentinel node mapping in melanoma generally depends on the Breslow thickness of the tumor, although ulceration and other prognostic factors might also be taken into account [7]. In thin lesions, the low risk of finding nodal metastases can be a reason to omit sentinel node staging and many authors do not use sentinel node biopsy in such lesions (e.g., less than 0.75 mm or less than 1.0 mm) [8]. In thick lesions, the high risk of synchronous distant metastases may outweigh the possible therapeutic and prognostic benefits of lymphadenectomy or sentinel node mapping [9, 10].

The exact role of lymph node staging with sentinel node biopsy in oral cancer has not been clearly defined yet, though several authors use the procedure to stage T1 or T1 and T2 lesions [4–6]. A diagnostic meta-analysis by Paleri et al. showed a good sensitivity (92.6%) of the sentinel node procedure in squamous cell cancer of the oral cavity an oral pharynx [5], while Civantos Jr. et al. found a negative predictive value of 96% for this procedure [4]. The lesser morbidity of sentinel node biopsy is often used as an argument against elective neck procedures; however some authors find selective neck dissection more appropriate in

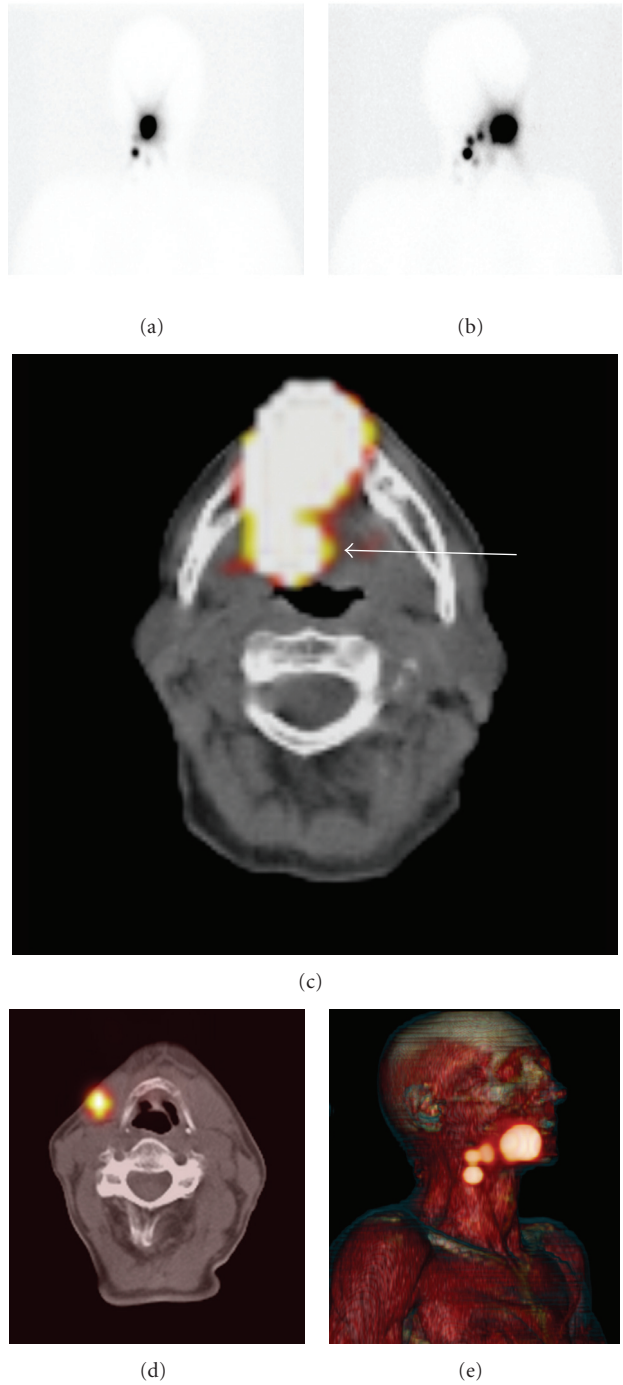
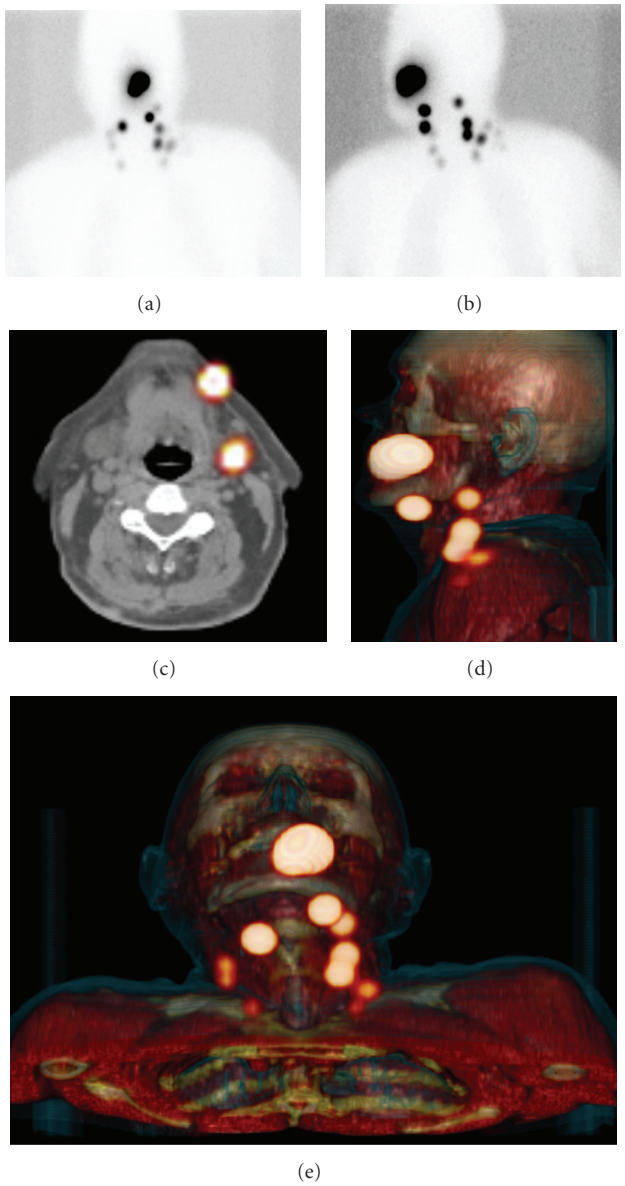
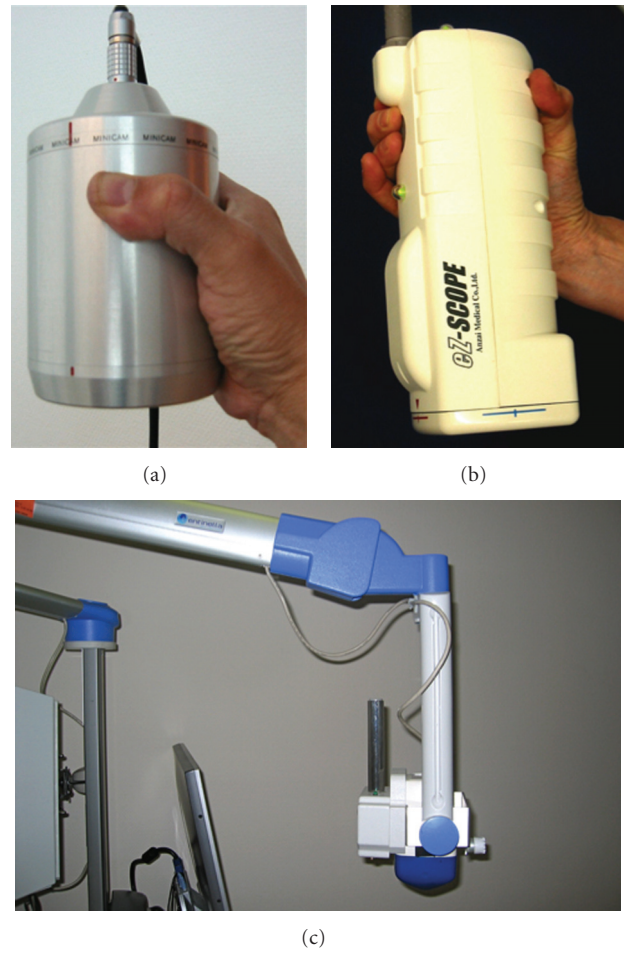


Figure 1: SPECT/CT to rule out a presumed sentinel node. Anterior (a) and oblique (b) planar static images after 2 hours show drainage to the right neck on the basis of which 3 sentinel nodes were marked. SPECT/CT (c) demonstrates the cranial hotspot located at the base of the tongue in the oropharynx (arrow), due to leakage of the tracer from the injection area. The sentinel nodes are clearly visualized with SPECT/CT (d), while three-dimensional reconstruction (e) shows an anatomic overview of all hotspots.



(a) (b) (c) (d) (e)

Figure 2: SPECT/CT localizing sentinel nodes and providing anatomic overview. Anterior (a) and oblique (b) planar static images after 2 hours show several hotspots. Two-dimensional SPECT/CT reconstruction exactly localizes each node, for example, localizing 2 sentinel nodes in the submandibular region (c). Three-dimensional SPECT/CT reconstruction shows an anatomic overview of all sentinel nodes (d) and (e).



(a) (b) (c)

Figure 3: Development of portable gamma cameras. (a) First generation portable gamma camera with a weight of approximately 2 kg. (b) Portable gamma camera with a weight < 1 kg but without support system. (c) Last generation portable gamma camera with improved ergometrical details and adequate support system for intraoperative use.

view of the high risk of nodal metastasis [11]. This especially is true in patients with more advanced lesions.

3. SPECT/CT for Preoperative Sentinel Node Detection

Hybrid single-photon emission computed tomography with integrated computed tomography (SPECT/CT) is a multimodal imaging device and can be used to visualize and localize sentinel nodes [12–20]. SPECT/CT can optimize sentinel node visualization which may lead to improved intraoperative detection [21]. A SPECT/CT system generally consists of a dual-head variable-angle gamma camera equipped with low-energy high-resolution collimators and a multislice spiral CT optimized for rapid rotation. A matrix 128×128 is used for SPECT acquisition and 25 seconds per view using 4–6-degree-angle steps enable adequate images. The CT settings are aimed at obtaining a low-dose CT (e.g., 130 KV, 40 mAs, B30s kernel), which is appropriate for attenuation correction and mapping. A major advantage of the hybrid SPECT/CT system is that the patient need not be moved between the SPECT and the CT data acquisition. After reconstruction, the SPECT images are corrected for attenuation and scatter. Axial 5 mm SPECT and CT slices are usually generated. Subsequently, the SPECT and CT images are fused and can be displayed using multiplanar reconstruction projection with two-dimensional orthogonal reslicing in axial, sagittal, and coronal orientation and maximum intensity projection. A three-dimensional image

Table 1: Technical details of SPECT/CT in head and neck malignancies in various studies.

Study	N^*	Malignancy	Dose	Injection	Planar Imaging	Timing SPECT/CT	SPECT	Surgery	Details
Even-Sapir et al. [12]	9	3 HNM 6 OCC	74 MBq	Intradermal or submucosal injection 4 peritumoral deposits of 0.4 mL each	Sequential images within minutes after injection until visualization (up to 24 hours)	Not specified	3° angle/20 s to 25 s steps Matrix: 128 × 128	Next day	Total number of patients: 34 (9 head and neck malignancy)
Wagner et al. [13]	30	OCC	20 MBq	Intra-mucodermal injection 2 peritumoral deposits of 0.05 mL each	Static image 60 minutes after injection	60 minutes after injection	6° angle/30 s steps Matrix: 128 × 128	Same day	Sentinel node biopsy performed in 13/30
Lopez et al. [14]	10	OCC	22.2 MBq	4 peritumoral deposits Total volume <0.5 mL	Sequential images 4 to 24 hours after injection	Not specified	6° angle/10 s steps Matrix: 128 × 128	Same day	Image registration performed manually by reprojection
Thomsen et al. [15]	37	OCC	20 MBq	4–6 peritumoral submucosal deposits Total volume 0.2 mL	Static images 30–60 minutes after injection	Not specified	6° angle/8 s steps Matrix: 128 × 128	Same day	SPECT/CT in 37 out of 40 patients
Terada et al. [16]	12	OCC	18.5 MBq	4 peritumoral submucosal deposits, volume unclear	A static lym-phoscintigram (anterior and bilateral oblique) was performed	After planar images	Not specified	Same day	Results of SPECT/CT are not compared to results of planar imaging
Bilde et al. [17]	34	OCC	120 MBq or 60 MBq[§]	4 peritumoral submucosal deposits Total volume 0.2 mL	Dynamic imaging (lateral and anterior) during 20 minutes Static images after 30 and 90 minutes	After planar images	3° angle/30 s steps Matrix: 128 × 128	Some the next day, some the same day	
Khafif et al. [18]	20	OCC	74 MBq	Injection at the border of the primary tumor 4 deposits of 0.4 mL each	Sequential images within minutes until visualization (up to 24 hours)	Not specified	3° angle / 20 s–25 s steps Matrix: 128 × 128	Next day	
Keski-Säntti et al. [19]	15	OCC	74 MBq	Peritumoral injection in 1 or 2 deposits Total volume 0.2 mL	Planar lym-phoscintigraphy with anterior and lateral projections	Not specified	Not specified	Next day	

Table 1: Continued.

Study	N^*	Malignancy	Dose	Injection	Planar Imaging	Timing SPECT/CT	SPECT	Surgery	Details
Covarelli et al. [20]	12 versus 11[±]	HNM	50 MBq or 10 MBq[§]	Peritumoral intradermal injection in 4 deposits In case of excision: 2 deposits around surgical scar Total volume 0.1 mL	Dynamic planar imaging for 20 minutes Sequential static images up to until 3 hours	45 minutes after injection	4° angle / 30 s steps Matrix: 256 × 256	Some the next day, some the same day	Patients received either planar imaging or SPECT/CT

*number of patients with a head and neck malignancy that received SPECT/CT.
HNM: head and neck melanoma.
OCC: oral cavity carcinoma.
§ the first dose was injected if patients were operated the next day; the last dose was injected if patients were operated the same day.
± 12 patients received SPECT/CT only; 11 patients received planar imaging only.

display can also be constructed, based on volume rendering of fused SPECT/CT images with a 16-bit color look-up table with defined opacity for soft tissue, bone, and skin. Based on this three-dimensional view, the opacity can be manually adjusted to visualize these structures with different colors: red for muscle, ochre for bone, and blue for skin. The sentinel node is displayed in yellow (Figures 1 and 2) in both two-dimensional and three-dimensional display. Sentinel lymph nodes can be identified and localized on the two-dimensional images, while three-dimensional reconstruction gives an anatomic overview of all lymph nodes in relation to the injection area.

Several authors have reported on the use of SPECT/CT in head and neck malignancies; previous study details are summarized in Table 1. The injected dose varies among the different studies as well as planar imaging protocols and timing of SPECT/CT and sentinel node excision. The injected dose depends on the time to operation. A larger dose of radioactivity is needed with a longer interval because of the physical half-life of the radionuclide. A dosage of 10 MBq–60 MBq radioactive colloid is used when patients are operated on the same day, while 50 MBq–120 MBq is used when operation takes place the next day. If low dosages are used, lymphatic vessels may not be visualized, which leads to difficulty in identifying sentinel nodes.

The results of additional imaging with SPECT/CT are summarized in Table 2. Numbers of patients studied are rather small in all studies and exact drainage visualization numbers are not mentioned in every study. Recorded visualization of lymphatic drainage on planar images ranges from 83% to 100%. SPECT/CT has proven to visualize additional sentinel nodes in more than half of the studies [12, 13, 15, 17–19], although the authors of one study conclude that SPECT/CT rarely reveals sentinel nodes that are not detected on planar images [19]. Especially nodes adjacent to the injection area appear to be detected by SPECT/CT, while these are easily missed on planar images [13, 15]. In 3 out of 9 studies, presumed sentinel nodes could be interpreted as nonnodal tracer uptake (tracer leakage or contamination) on the basis of SPECT/CT images [12, 18, 19]. All authors agree that SPECT/CT provides useful localization information [12–20]. Covarelli et al. have proven the clinical relevance of this localization information, since the operation time was significantly less when sentinel node surgery was performed on the basis of SPECT/CT images compared to planar images [20].

Our own preliminary results with SPECT/CT in head and neck malignancies are in line with the literature findings. The visualization rate was 100% for planar imaging as well as SPECT/CT in the first 33 patients, but SPECT/CT visualized additional sentinel nodes in 18% of the patients and excluded hotspots as being sentinel nodes in 9% of all patients. Figure 1 shows an example of a hotspot that was assumed to be a sentinel node but which SPECT/CT showed to be caused by tracer leakage in the oral cavity on SPECT/CT. The exact anatomic localization could be realized with SPECT/CT in all patients. An example of anatomic localization with SPECT/CT is given in Figure 2. In our hospital, surgical incisions are based on SPECT/CT images, because of the anatomic localization and overview SPECT/CT images provide.

Combining our results with literature findings, SPECT/CT appears to be very useful for exact anatomic localization of the sentinel nodes. In the head and neck area it is of considerable importance to identify the relation of sentinel nodes to several vital vascular and neural structures in order to be able to safely remove these nodes. SPECT/CT also detects sentinel nodes that are missed on planar lymphoscintigraphy in a substantial number of patients. In head and neck cancer, many sentinel nodes are located in close proximity to the injection area and are easily overlooked on planar images. Cases of nonnodal tracer uptake (e.g., tracer leakage in the oral cavity after injection or contamination on the skin) can be identified with SPECT/CT, while distinguishing between leakage and a sentinel node on planar images is often impossible.

After tracer administration (intradermally in melanoma, submucosally in oral cavity carcinoma), sequential planar

Table 2: SPECT/CT results in various studies.

Study	Visualization with planar imaging	Visualization with SPECT/CT	Additional sentinel nodes visualized with SPECT/CT	Main conclusions with regards to imaging
Even-Sapir et al. [12]	Multiple drainage basins: 11%	Multiple drainage basins: 33% Additional clinical relevant information with SPECT/CT: 44%	In 3 out of 9 patients 1 false positive node excluded	SPECT/CT provides additional data of clinical relevance in patients with trunk or head and neck melanoma and patients with mucosal head and neck tumor.
Wagner et al. [13]	38 sentinel nodes	Sentinel node visualization with planar imaging and SPECT/CT: 90% 49 sentinel nodes	11 sentinel nodes	SPECT/CT is feasible for sentinel node detection. SPECT/CT enhances topographic orientation and diagnostic sensitivity. SPECT/CT is necessary to identify nodes adjacent to the primary lesion.
Lopez et al. [14]	Sentinel node visualization: 100%	Localization of the sentinel nodes in 9/10 patients		Multimodal registration is an effective method for anatomic localization of the sentinel nodes in N0 oral squamous cell carcinoma.
Thomsen et al. [15]	99 sentinel nodes	SPECT/CT and added oblique planar images: 123 sentinel nodes	24 extra sentinel nodes found with SPECT/CT in combination with added oblique planar images	Added oblique planar images and/or SPECT/CT detect extra clinical relevant hotspots in 38% of the patients. Sentinel lymph nodes close to injection area are difficult to find.
Terada et al. [16]		Sentinel node visualization with planar imaging and SPECT/CT: 100%		Intraoperative sentinel node biopsy based on SPECT/CT images is an easy, accurate, and reliable method. Analysing the three hottest sentinel nodes reliably predicts a patients neck status.
Bilde et al. [17]	88 sentinel nodes	Sentinel node visualization: 94% 107 sentinel nodes	19 sentinel nodes In 15 out of 32 patients (47%)	Correction of anatomic level with SPECT/CT in 22%. Reclassification of anatomic level during surgery in 22%. SPECT/CT detects more sentinel nodes and provides additional anatomical and spatial information.
Khafif et al. [18]		Sentinel node visualization with planar imaging and SPECT/CT: 95% SPECT/CT added significant anatomical preoperative information in 6 out of 20 patients (30%)	Additional sentinel nodes seen in 2 patients (metastatic sentinel node in 1) Exclusion of sentinel nodes in 4 patients (all activity at injection site)	SPECT/CT sentinel node mapping provides additional preoperative data of clinical relevance.

Table 2: Continued.

Study	Visualization with planar imaging	Visualization with SPECT/CT	Additional sentinel nodes visualized with SPECT/CT	Main conclusions with regards to imaging
Keski-Säntti et al. [19]	Sentinel node visualization: 100%	Sentinel node visualization: 100% Additional data provided by SPECT/CT was considered clinical relevant in 6 out of 15 patients (40%)	1 additional sentinel node visualized 2 false positive nodes excluded	SPECT/CT enables more accurate localization of sentinel nodes. SPECT/CT rarely reveals sentinel nodes that are not detected on planar images.
Covarelli et al. [20]	Sentinel node visualization: 83% 12 sentinel nodes in 12 patients	Sentinel node visualization: 100% 13 sentinel nodes in 12 patients		SPECT/CT is more effective and reliable than planar lymphoscintigraphy. Sentinel node biopsy takes significantly less time in the SPECT/CT group.

Table 3: Requirements for intra-operative imaging.

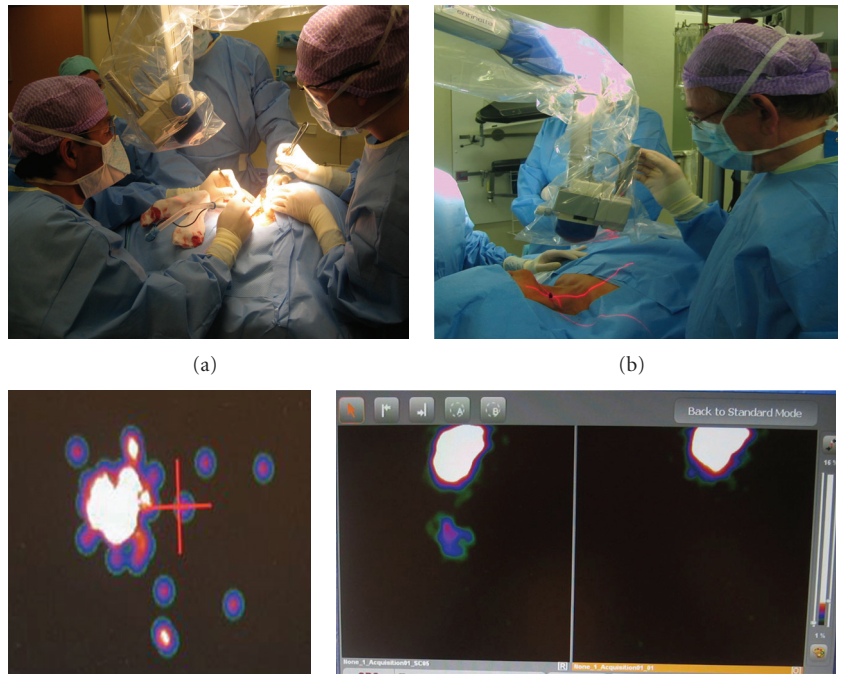
Portable gamma camera	(1) Manageable design (portable and stable)
	(2) Sufficient resolution and detection sensitivity
	(3) No delay between image acquisition and display (real-time imaging)
	(4) Adequate field of view
Intra-operative situation	(5) No interference with field of operation
	(6) Possibility for continuous monitoring
	(7) Spatial orientation on screen
	(8) Possibility to use pointers for position and localization
	(9) Real-time quantification of the number of counts per second
Sentinel node	(10) Sufficient uptake of the radiotracer by the sentinel node

images can identify nodes that are on a direct drainage pathway from the primary tumor (sentinel nodes). Subsequently, SPECT/CT can localize these sentinel nodes, giving anatomical reference points for the planning of the surgical approach.

4. Portable Gamma Cameras for Intraoperative Imaging

Sentinel node surgery is based on the combination of gamma probe detection and blue dye mapping. Surgeons localize sentinel nodes combining the auditive signal (probe) to the visual one (blue dye). In head and neck patients, the use of blue dye can be problematic. The injection of blue dye in the mouth can lead to obscured tumor edges and thus interfere with resection [18]. Furthermore, blue dye shifts very fast. For these reasons the application of blue dye in head and neck patients, with a high density of lymph nodes and short distances between injection site and sentinel node, is limited. The incorporation of another visual element in sentinel node surgery of the head and neck can facilitate the procedure. Against this background, portable gamma cameras have been designed to facilitate radioguided surgery. The development of such cameras is illustrated in Figure 3. While the first prototypes were heavy hand-held devices, the new generation of portable gamma cameras is equipped with a stable support system.

The use of portable gamma cameras has been described in parathyroidectomy [22, 23]. The parathyroid adenomas can be located intraoperatively by means of sestamibi imaging using a portable gamma camera [22, 23]. Another possible application of the portable gamma camera can be localizing lymph nodes [24–26]. Exact localization of sentinel nodes by the portable gamma camera was shown in an animal model [27], and recently the synchronous use of a portable gamma camera and gamma probe showed exact localization of sentinel nodes in 11 breast cancer patients [28]. Furthermore, the depth of the sentinel nodes was successfully estimated preoperatively using the portable gamma camera [28]. Several conditions are required to optimize intraoperative imaging with the portable gamma camera. A summary of these requirements is given in Table 3.



(a) (b) (c) (d)

Figure 4: Localization and postexcision monitoring. Continuous monitoring (a) provides the possibility to record the whole procedure. With stepwise monitoring (b), the sentinel nodes are localized first, then excision takes place, and afterwards the portable gamma camera is used to screen for remaining activity. The laser pointer is positioned above the previous marked sentinel node level and the camera displays the technetium-signal (c), indicating that the node is located just right from the laser pointer. The portable gamma camera can also give an overview of the surgical field (d). It shows the injection area with a sentinel node located more caudally. After excision, the camera clearly shows no remaining radioactivity (d).

In our centre, the use of a portable gamma camera was introduced for laparoscopic lymph node localization in urological malignancies [29] and has recently been commenced to aid lymph node localization in oral cavity carcinoma as well. The portable gamma camera (Sentinella, S102, GEM imaging, Valencia, Spain) is equipped with a 4 mm pinhole collimator and uses a CsI(Na) continuous scintillating crystal. The pinhole collimator enables visualization of the whole surgical field and the field of view depends on the distance between the camera and the imaging plane. The field of view is 4×4 cm when placed at 3 cm from the imaging plane and increases to 20×20 cm when placed at a distance of 15 cm. The intrinsic spatial resolution is 1.8 mm. The extrinsic spatial resolution values are 7 mm and 21 mm for a distance of 3 cm and 15 cm, respectively. The detection sensitivity for this collimator depends on the distance to the imaging plane, being 708 and 41 cpm/uCi for distances of 3 cm and 15 cm respectively. These and other technical details of this portable gamma camera are described by Sánchez et al. [24].

Intraoperative real-time imaging with the portable gamma camera provides an overview of all radioactive hotspots in the whole surgical field. Its position can be adjusted to also show sentinel nodes near to the injection area, which are easily overlooked using the probe. The differentiation between sentinel nodes and secondary tier is facilitated, because the amount of radioactivity within each node can be quantified with the portable gamma camera and the intraoperative images can be related to the preoperative scintigraphic images. Furtheremore, sentinel nodes can be exactly localized by on screen visualization, besides the audiological localization by the laparoscopic gamma probe. Figure 4 shows 2 possibilities for intra-operative use of the portable gamma camera. Continuous monitoring can be used to record the whole procedure and stepwise monitoring enables localization of sentinel nodes and detects remaining activity afterwards. If the laser pointer signal matches the technetium signal on screen, this indicates that the sentinel node has been exactly localized. Another clear advantage of the portable gamma camera is the certainty it can provide about the completeness and accuracy of the sentinel node excision, since it shows remaining radioactivity. Figure 4 also shows the comparison of the situation before and after excision.

As experience with radioguided surgery is gained, the need for advanced imaging modalities will increase. The portable gamma camera is an innovative tool that can improve nodal excision in areas with complex anatomy. Further research in our institute is underway to define the exact value and indication of the intraoperative use of a portable gamma camera.

5. Future Directions

Over the next years, the use of SPECT/CT might become routine procedure for patients with difficult to interpret planar images. The major challenge remains to optimize intraoperative visualization of sentinel nodes. The development of a gamma camera with a multiplanar detection system (e.g., two heads) might enable real-time three-dimensional visualization. Furthermore, the development of new tracers may improve intraoperative visualization as well. A slower migrating alternative to patent blue might improve the direct intraoperative localization of sentinel nodes in the neck. Another option may be the development of a dual-tracer, which contains radioactivity as well as color and can be used for lymphoscintigraphy and intraoperative visual and auditive (gamma probe) at the same time.

6. Conclusion

Sentinel node biopsy is increasingly being used to provide accurate staging in early stage head and neck malignancies, such as melanoma and oral squamous cell carcinoma. Lymphatic mapping in the head and neck area can be complicated because of the complex anatomy and variable drainage patterns in this area and easy obscuration of sentinel node by the primary injection site. The need for accurate imaging extends beyond planar lymphoscintigraphy.

SPECT/CT is a new imaging modality that improves preoperative lymphatic mapping by exactly localizing sentinel node. It depicts sentinel nodes that are missed on planar images, especially nodes in close proximity to the injection area, and can exclude presumed sentinel nodes that are based on tracer leakage or contamination. Sequential planar images remain essential to distinguish sentinel nodes from second-echelon nodes.

A portable gamma camera can be used to improve intraoperative search for sentinel nodes. Such a camera provides real-time imaging of the sentinel node and can detect and localize those nodes, even if located near the injection area. A gamma camera increases certainty about the accuracy and completeness of the excision of the radioactive nodes, since it facilitates postexcision monitoring.

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Review Article

Medullary Thyroid Carcinoma: Targeted Therapies and Future Directions

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Medullary thyroid cancer (MTC) is a rare neuroendocrine neoplasm that accounts for approximately 5% of all thyroid malignancies. The natural history of MTC is characterized by early lymph node and distant metastases, making complete surgical cure often impossible. Conventional chemotherapy and external beam radiation have been largely ineffective in altering the natural history of MTC. Therefore, there is a great need to develop novel therapeutic strategies to affect symptom control and reduce tumor burden in patients with widely disseminated disease. Here, we review several pathways which have been shown to be vital in MTC tumorigenesis and focus on the pathways of interest for which targeted drug therapies are currently being developed.

1. Introduction

Medullary carcinomas of the thyroid are neuroendocrine neoplasms derived from the parafollicular cells, or C cells, of the thyroid and account for nearly 5–10% of thyroid malignancies [1]. In nearly all cases of medullary thyroid cancer (MTC), the C cells secrete calcitonin, a specific and highly sensitive biomarker whose measurement plays an important role in the diagnosis and postoperative followup of patients [2, 3]. Less common, MTC cells elaborate other polypeptide hormones, including vasoactive intestinal peptide (VIP), serotonin, somatostatin, and carcinoembryonic antigen (CEA), the latter of which has been shown to herald contralateral lymph node and distant metastases [4].

The majority of MTCs are sporadic, but up to 25% of MTC cases result from a germ-line activating mutation in the rearranged during transfection (*RET*) proto-oncogene [5, 6]. Hereditary MTCs occur in the setting of the multiple endocrine neoplasia (MEN) syndrome type 2 (2A or 2B) or as familial MTC (FMTC) without an associated MEN syndrome. In sporadic MTC, patients most commonly present in the fifth or sixth decade with a palpable cervical lymph node or a solitary thyroid nodule. Fine needle aspiration (FNA) biopsy of the mass and the presence of an elevated serum calcitonin are diagnostic of MTC. Unlike sporadic MTC, most patients with hereditary disease are identified by genetic testing of at-risk family members for the germline mutation of the *RET* gene. As such, hereditary MTC tends to present at an earlier age than sporadic disease and is typically multifocal and bilateral [1].

The management of MTC relies heavily on surgical resection, consisting of total thyroidectomy and lymph node dissection; however, recurrent disease develops in approximately 50% of patients with MTC [7]. Similarly, biochemical documentation of persistent or recurrent MTC by serum calcitonin levels is often associated with unresectable recurrence in distant locations, including lung and liver [8]. Therefore, although MTC tends to be a slow-growing tumor primarily treated with surgical resection, it frequently metastasizes early in the disease course to the liver and regional lymph nodes, precluding patients from a curative resection. It is thus necessary to develop alternative therapeutic strategies to control tumor growth, possibly through manipulation of various cellular signaling pathways [9].

Table 1: Targeted therapies currently in clinical trial development for MTC.

Drug	Target	Mechanism of action
Zactima (ZD6474)	VEGFR, RET, EGFR (HER1)	Tyrosine kinase inhibitor
XL184	VEGFR2, MET, RET	Tyrosine kinase inhibitor
Imatinib (STI571)	*bcr-abl*, PDGFR, C-KIT	Tyrosine kinase inhibitor
Sorafinib (BAY-43-9006)	BRAF, CRAF, VEGFR, RET, PDGFR	Tyrosine kinase inhibitor
Sunitinib (SU11248)	VEGFR, RET, PDGFR, C-KIT, CSF-1R, flt3	Tyrosine kinase inhibitor
AMG-706	VEGFR1-3, PDGFR, C-KIT	Tyrosine kinase inhibitor
Gefitinib	EGFR (HER1)	Tyrosine kinase inhibitor
Axitinib (AG-013736)	VEGFR, PDGFR, C-KIT	Tyrosine kinase inhibitor
Pazopanib (GW786034)	VEGFR1-3, PDGFR, C-KIT	Tyrosine kinase inhibitor
SAHA	Notch1, TNF-α, IL-1-β, IL-6, IFN-γ	HDAC inhibitor
Lithium	GSK-3ß	Unknown, suspected GSK3 inhibition, inositol depletion

2. Molecular Pathogenesis and Cytogenetics

Although MTC is rare, there has been considerable interest in the molecular pathways that regulate MTC cellular growth, differentiation, survival, and hormone expression. We and others have previously shown that manipulation of these pathways may be a potential therapeutic strategy to control the growth and hormone production of NE tumors like MTC [9–12]. With the application of current molecular techniques, decades of research have begun to elucidate a genetic model that contributes to MTC tumorigenesis that includes three important processes: mutated proto-oncogenes which result in altered receptor protein production and concomitant accelerated tumor growth, alterations in signal transduction pathways which regulate the NE phenotype, and variations in tumor suppressor genes that facilitate unregulated cell growth [13]. Although a comprehensive review of all pathways which have been studied is beyond the scope of this paper, we aim to focus on new pathways of interest in MTC for which targeted drug therapies are currently in development (Table 1).

3. Receptor Proteins

3.1. The RET Receptor Tyrosine Kinase (RTK). Activating germline mutations in familial MTC involve the *RET* proto-oncogene, which is mapped to chromosome 10q11.2 [1]. The *RET* gene encodes a 120-kDa transmembrane receptor tyrosine kinase (RTK) that functions as a target for the glial-derived neurotrophic factor (GDNF) family of growth factors [14]. Mutations in the *RET* proto-oncogenes have been implicated in nearly 95% of cases of hereditary MTC associated with MEN types 2A and 2B and FMTC. Interestingly, in hereditary MTC, recent data suggest that specific germline *RET* mutations are associated with age-specific penetrance of cancer development and lymph node metastases [2, 15]. The most common mutation in MEN 2A (codon 634) occurs in up to 80% of MEN 2 families and nearly half of affected children develop MTC by ages 5–10. The codon most frequently associated with MEN 2B (codon 918) confers a significantly higher risk of MTC, often beginning in the first 6 months of life. Patients assigned to this highest risk category clearly benefit from prophylactic thyroidectomy in the first year of life, if possible.

While MTC displays a slow-growth pattern and indolent disease course, frequent metastases to the liver and regional nodal basins plague patients with hereditary disease, precluding these patients from a curative resection. Clearly, there is a great need for novel therapeutic and palliative strategies to treat these patients with metastatic MTC.

Several small molecule RTK inhibitors have been developed against RET and show promising results both in vitro and in vivo as emerging therapies for the treatment of MTC. These molecules include ZD6474 (Vendetanib), SU11248 (sunitinib), BAY 43-9006(sorafenib), CEP-751 and CEP-701, XL-880, XL-184, and RPI-1 [2, 16]. Much is known about vandetanib, a low molecular weight tyrosine kinase inhibitor that has demonstrated effective inhibition of RTK in vitro [17, 18]. Likewise, Carlomagno et al. [17] have shown that vandetanib blocks in vivo phosphorylation and signaling of the *RET*/MEN2B oncoprotein and prevents the growth of two human cancer cell lines that carry spontaneous *RET* rearrangements. Currently, promising data in patients with MTC have led to vandetanib being assigned orphan drug designation by the US Food and Drug Administration. These data are based on studies in which vandetanib has demonstrated clinical activity in a single-arm Phase II study in patients with metastatic hereditary MTC [19]. In this study, thirty patients (21 female; median age 50 years) received initial treatment with vandetanib 300 mg with a median duration of treatment of 172 days. Based on site investigator assessments, 20% (6/30) of patients experienced a partial response (duration of response 59–260 days) and another 30% (9/30) of patients experienced stable disease at 24 weeks, yielding a disease control rate of 50% (15/30). Similarly, in 19 patients, plasma calcitonin levels showed a >50% decrease from baseline that was maintained for at least 6 weeks. Based on these and other data, an international, randomized, placebo-controlled Phase III study of vandetanib monotherapy in metastatic MTC has been initiated and is currently ongoing.

In addition to vandetanib, several other novel RTK inhibitors are under investigation for treatment of RET-dependent thyroid carcinomas. Recently, Kim et al. have

shown that sorafenib, a multikinase inhibitor of RTK, VEGFR, and BRAF kinase, inhibits proliferation of ATC cell lines and inhibits tumor angiogenesis via induction of endothelial apoptosis in an orthotopic anaplastic thyroid carcinoma xenograft model in nude mice [20]. Similarly, the orally administered multitarget tyrosine kinase inhibitor, sunitinib, has been shown to be a novel potent inhibitor of thyroid oncogenic RET/papillary thyroid cancer kinases [21]. Many of these agents which are earlier in the development pipeline are capable of inhibiting RET at subnanomolar concentrations and hold significant promise for the treatment and palliation of hereditary MTC [2].

3.2. EGFR as a Therapeutic Target. Epidermal growth factor (EGF) is a 6-kDa polypeptide which has been demonstrated to stimulate the proliferation of normal and malignant thyroid cells and inhibit cellular differentiation [22]. Overexpression of the EGF receptor (EGFR), a 170-kDa transmembrane glycoprotein tyrosine kinase, has been documented in various differentiated thyroid carcinomas [23, 24] and is thought to be essential in thyroid carcinoma proliferation and metastasis. Recently in a proteomics study of MTCs expressing *RET* germ-line mutations, Gorla and colleagues [25] demonstrated high-level expression of minimally phosphorylated EGFR in two separate MTC cell lines. These data, taken together with studies demonstrating that gefitinib and erlotinib, well known EGFR inhibitors, have resulted in objective tumor responses in patients with EGFR-overexpressing tumors, suggest that EGFR inhibitors might be beneficial for therapy of refractory or metastatic MTC. Interestingly, several of the novel RTK inhibitors—vandetanib, sorafenib, and sunitinib, for example—appear to be multitarget kinase inhibitors capable of nonspecific inhibition of multiple critical signaling pathways critical in MTC tumorigenesis. While vandetanib is currently in phase II clinical trials to measure efficacy and clinical response in patients with hereditary MTC, the question of whether the multitarget kinase inhibitors' lack of receptor specificity will be advantageous or disadvantageous remains unanswered.

3.3. Angiogenesis Inhibitors. Many proteins appear to be involved in the formation and maintenance of new blood vessels which support primary tumor growth and metastatic tumor deposits. One of these proteins, vascular endothelial growth factor (VEGF), stimulates angiogenesis by attaching to VEGF receptors on the endothelial cells, supporting new vessel stability [26]. Interestingly, several recent studies suggest that thyroid cancer cells demonstrate elevated levels of VEGF compared to normal controls.

To date, several phase II clinical trials have been completed in MTC for single agent small molecules which target the VEGF receptor. AG-013736 (Axitinib) is a potent small molecule inhibitor of VEGF receptors 1 through 3 which has been shown in a single arm multicenter trial to be associated with an overall partial response rate of 20% in patients with thyroid cancer of any histology that was resistant or not appropriate for ^{131}I. Among patients with MTC, nearly 20% (2/11 patients) demonstrated radiographic partial responses while almost half (5/11 patients) of all MTC patients exhibited stabilization of disease. Therapy was well tolerated, with reported side-effects of fatigue, hypertension, and proteinuria [27]. A similar phase II trial of the multikinase inhibitor AMG-706 in MTC has been recently completed. In this study, patients with differentiated thyroid cancer or MTC received AMG-707 until disease progression or drug toxicity occurred. Although the results of the MTC cohort have not yet been reported, the results for the differentiated group have been presented and demonstrate partial tumor response by Recist criteria in 12% of patients while another 70% of patients demonstrated stable disease [28]. Clearly, these studies offer significant insight into the idea that targeting the soluble VEGF receptor may be a potential target for effective therapy in MTC.

4. Signal Transduction Proteins

4.1. Glycogen Synthase Kinase-3ß (GSK-3ß) in MTC. GSK-3ß is a multifunctional serine/threonine protein kinase that was first described as playing a role in the regulation of glycogen synthesis [29] and has since been shown to be an important regulator of cell proliferation and survival. In contrast to other kinases, GSK-3ß is highly active and nonphosphorylated in unstimulated cells; however, activity of the kinase is inhibited by phosphorylation of a single serine residue (Ser^9) in response to signaling cascades, including the Raf-1/mitogen-regulated extracellular kinase (MEK)/extracellular regulated kinase (ERK) signaling pathway [29]. Recently, we have shown that Raf-1 activation in human MTC cells results in phosphorylation and subsequent inactivation of GSK-3ß [30]; likewise, inactivation of GSK-3ß by phosphorylation results in MTC growth inhibition both in vitro and in vivo.

Several small molecule inhibitors of GSK-3ß are available and demonstrate promising results both in vitro and in vivo as potential targeted therapies for the treatment of MTC. We have recently shown that inactivation of GSK-3ß with lithium chloride (LiCl) and SB216763 results in MTC differentiation and cell growth inhibition; likewise, these small molecules have been shown to be associated with a significant decrease in NE markers such as human achaete-scute complex-like l (ASCL1) and chromogranin A (CgA) in cultured MTC cells [30]. In vivo studies in LiCl-treated MTC xenograft mice demonstrated a significant reduction in tumor volume compared with those treated with control [30]. LiCl has been utilized clinically for more than fifty years as an adjunctive psychiatric medication for the treatment of bipolar disorder and has shown only minimal adverse side effects. As such, the efficacy of LiCl therapy in patients with MTC is currently being investigated at our institution in phase II clinical trials.

4.2. The Notch1/Achaete-Scute Complex-Like 1 (ASCL1) Pathway. Notch1 is a multifunctional transmembrane receptor that regulates cellular differentiation, proliferation, and survival [31–33]. Binding of any one of the Notch ligands

(e.g., DLL-1 or JAG-1) promotes a sequence of proteolytic cleavages resulting in the activated Notch intracellular domain (NICD). This activated form of Notch1 then translocates to the nucleus and binds with the DNA-binding protein complex CSL (C promoter-binding factor 1, suppressor of hairless and Lag-1), resulting in transactivation of various target genes like hairy enhancer of split-1 (HES-1).

In human cancer cells, Notch1 acts as either a tumor suppressor or an oncogene. In many types of cancer, including pancreatic, colon, nonsmall cell lung cancer (NSCLC), cervical and renal cell cancer, Notch1 is upregulated; furthermore, it has been suggested that expression of Notch1 signaling prevents cellular differentiation and inhibits apoptosis in these cancer types. Conversely, Notch1 signaling is very minimal or absent in NE tumors such as small-cell lung cancer (SCLC), carcinoid cancer, and MTC [34]. In recent studies, we have shown that activation of doxycycline-inducible Notch1 in MTC cells significantly reduced MTC cellular growth and regulated calcitonin level in a dose-dependent fashion; furthermore, these changes were dependent on the amount of Notch1 protein [35]. These observations clearly suggest that Notch1 signaling proteins are conserved in MTC cells and support the idea that Notch1 activation may be a potential target to treat patients with MTC tumors.

Until recently, methods for the delivery of activated Notch1 to tumor cells, aside from gene therapy, had been nonexistent. Recently, we have demonstrated that the histone deacetylase inhibitors valproic acid (VPA) and suberoyl *bis*-hydroxamic acid (SBHA) act as strong Notch1 activators in MTC cells [36, 37]. HDAC inhibitors represent a class of diverse molecules that modulate gene transcription by increasing histone acetylation; the resulting alteration in chromatin structure is believed to possess antineoplastic effects in preclinical and clinical studies in neuroblastoma cells and a variety of other cancers [38]. In our studies, SBHA and VPA treatment of MTC tumor cells resulted in dose-dependent induction of the Notch1-intracellular domain, the active form of the protein [36, 37]. Furthermore, with Notch1 activation there was a concomitant decrease in ASCL1 in vitro, a downstream target of Notch1 signaling. Currently, we are seeking to develop phase II clinical trials at our institution to determine the efficacy of these HDAC inhibitors as part of a comprehensive therapy in patients with MTC.

4.3. The Raf-1/Mitogen-Regulated Extracellular Kinase (MEK)/Extracellular-Regulated Kinase (ERK) Signaling Pathway. The Raf-1/MEK/ERK pathway has long been recognized for its role in tumor biology and specifically for its role in MTC tumor development [11]. Utilizing an estradiol-inducible estrogen receptor fused with the catalytic domain of the Raf-1 fusion protein, we have shown that activation of the Raf-1 pathway is associated with a reduction in NE hormones such as calcitonin and CgA [39]. More importantly, ectopic raf-1 expression led to significant MTC growth suppression both in vitro and in a mouse xenograft model of MTC [40–43]. More recently, Ning et al. [44] showed that the Raf-1 pathway regulates essential cell-cell contact molecules and the metastatic phenotype of MTC cells. These data clearly demonstrate that although activation of the Raf-1 signaling pathway is growth promoting in several cancers, in certain cell-specific subtypes, Raf-1 activation inhibits tumorigenesis.

Despite the fact that methods to deliver activated Raf-1 to MTC cells are limited, pharmacologic activators of the Raf-1 pathway are currently under investigation. ZM336372, originally identified as a potent and specific inhibitor of Raf isoforms [45], has been shown to paradoxically demonstrate a >100-fold induction of Raf-1 activity in NE cell culture systems [45, 46]. In MTC cells in vitro, Kunnimalaiyaan et al. [47] have demonstrated that ZM336372 treatment resulted in increasing Raf-1 activation as measured by phosphorylation of ERK1/2. Importantly, treatment with ZM336372 in the presence of small interfering RNA against Raf-1 resulted in an increase in Raf-1 production, suggesting that ZM336372 upregulates Raf-1 at the transcriptional level. While this is the first description of a novel compound capable of regulation the Raf-1 pathway in vitro, further studies into the in vivo effects of ZM336372 are ongoing.

5. Tumor Suppressor Genes and Nuclear Oncogenes

5.1. The p53 Tumor Suppressor Genes. It is believed that nearly 50% of all human malignancies are due to inactivating mutations of the p53 tumor suppressor gene; however, the significance of the p53 gene in MTC carcinogenesis is less clear [48]. In a cohort of nearly 100 patients, Saltman et al. clearly demonstrated the presence of aberrant p53 expression in more aggressive phenotypes of thyroid carcinoma; the study demonstrated a gradual increase in p53 immunopositivity rate along the spectrum of thyroid carcinoma progression with a statistically significant difference between well-differentiated and anaplastic phenotypes (0% versus 31.8%, resp.; $P < .001$) [49]. Similarly, using microdissection and genotyping, Sheikh et al. studied 11 cases of MTC for allelic losses in a panel of known tumor suppressor genes in an attempt to elucidate the molecular basis for clinical outcome. Among the tumor suppressor genes with the most frequent allelic losses, p53 demonstrated a 44% frequency of allelic loss. When combined with high-risk clinical variables, including advanced patient age and cervical lymph node status, this genotype accurately predicted nearly all patients in whom recurrence was likely [50]. Although frequency of allelic loss in tumor suppressor genes like p53 may provide a useful adjunctive prognostic test in MTC, there are currently no pharmacologic methods of selectively targeting this genotype.

5.2. The c-myc, c-jun, and c-fos Nuclear Proto-Oncogenes. The proto-oncogenes *c-myc*, *c-jun*, and *c-fos* regulate the production of nuclear transcription factors which activate the expression of target genes involved in the control of thyroid cell growth and differentiation [51]. Although no evidence for gene rearrangements or amplification of the nuclear proto-oncogenes has been noted in thyroid carcinomas, in

human MTC samples, both a high frequency of *c-myc* allelic losses [50] and elevated levels of *c-myc* and *c-jun* mRNA have been demonstrated [52]. Correlation between the c-myc transcript levels and the thyroid carcinoma differentiation was reported, demonstrating that the less differentiated tumors are associated with a greater abundance of c-myc mRNA [53].

6. Conclusions

Although current treatment options for patients with metastatic and refractory MTC are limited, recent advances in molecular oncology have fostered the development of novel small molecules which target specific pathways that are thought to be essential in MTC tumorigenesis. Inhibitors of the activated *RET* proto-oncogene and other RTK inhibitors appear particularly promising, based on the high prevalence of mutated oncogenes and specific expression patterns in MTC [2]. Likewise, targeting angiogenesis in MTC with small-molecule VEGF inhibitors and multitarget kinase inhibitors is currently under investigation and has demonstrated promising results. Many of these newer RTK inhibitors are currently under investigation in a series of randomized trials. Furthermore, alterations in various cellular signaling pathways like the Notch1 and Raf-1/MEK/ERK pathways offer potential targets for novel small molecule regulators. As molecular techniques continue to be developed and the human genome sequenced, other target therapies will undoubtedly be developed and will enter the clinical setting, prompting patients with metastatic and refractory MTC to participate in clinical trials evaluating these novel therapies.

Abbreviations

VEGFR: Vascular endothelial growth factor receptor
RET: Rearranged during transfection
EGFR: Epidermal growth factor receptor
HER: Human epidermal growth factor receptor
MET: Hepatocyte growth factor receptor tyrosine kinase
C-KIT: Cluster of differentiation (CD) 117
BRAF: B-raf
CRAF: C-raf
PDGFR: Platelet-derived growth factor receptor
CSF-1R: Colony-stimulating receptor-1R
flt3: fms-like tyrosine kinase receptor-3
TNF: Tumor necrosis factor
IL: Interleukin
IFN: Interferon
GSK-3ß: Glycogen synthase kinase-3ß
HDAC: Histone deacetylase
SAHA: Suberoylanilide hydroxamic acid.

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Research Article

Prognostic Factors in Patients with Multiple Recurrences of Well-Differentiated Thyroid Carcinoma

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Introduction. Patients with multiple recurrences of well-differentiated thyroid carcinoma (WDTC) have markedly reduced overall survival when compared with those who have ≤1 recurrence of their disease. The purpose of this investigation is to identify prognostic factors for mortality in this subgroup. *Methods.* Patients with multiple recurrences of WDTC were retrospectively identified from the thyroid cancer database at Mount Sinai Hospital, Toronto (1963–2000). Data on patient, tumor, and recurrence characteristics were collected, and each patient was given aMACIS score. *Results.* A total of 31 patients were identified (11 male, 20 female; 16–83 years). Using univariate analysis, age >45, stage III/IV disease, distant metastasis, vascular invasion, MACIS score >6, and time to recurrence of <12 months were found to be significant predictors for mortality in this subgroup. *Conclusions.* Patients with multiple recurrences of WDTC follow a distinct clinical course, marked with multiple treatment failures and a substantial risk of mortality.

1. Introduction

Although well-differentiated thyroid carcinoma (WDTC) is the most commonly diagnosed thyroid malignancy, it accounts for only 2% of all cancers in the body and is responsible for less than 0.5% of cancer-related deaths [1]. Combination therapy with thyroidectomy and adjuvant I^{131} is the treatment of choice at most institutions. The majority of patients have an excellent prognosis, with disease-specific survival rates at 10 years greater than 90% [1]. However, 8%–23% of patients will fail initial therapy and go on to develop a recurrence of their disease [1–4]. Mortality rates among patients with disease recurrence have been reported to be as high as 38%–69% [4–6]. In a previous study, Palme et al. [7] showed that WDTC patients who had either no recurrence of their disease or only one recurrence after initial therapy had no difference in disease-specific or overall survival (100% versus 94%, 89% versus 83%, resp.). In addition, patients with multiple treatment failures had significantly reduced survivals (60% and 58%, resp., $P < .001$).

There has been a large body of research compiled over the past few decades examining various prognostic factors for both recurrence and mortality in patients with WDTC. Factors such as age >45, male sex, large tumor size, histological type, advanced stage of disease, extrathyroidal extension, lymphatic invasion, and presence of distant metastasis have all been cited as indicators of poor outcome [1–4, 6, 8–20]. Several groups have attempted to classify patients into low-, intermediate-, and high- risk groups based on the presence of these factors [10, 17]. Prognostication is therefore used to identify patients at high risk who require close follow-up and prompt therapy for any evidence of disease recurrence. To our knowledge, there are no reports in literature delineating prognostic factors to predict disease outcome in patients who have suffered with multiple treatment failures of WDTC. Thus, the purpose of this investigation is to examine patient, tumor, treatment, and recurrence factors that may predict for mortality among patients with multiple recurrences of WDTC.

2. Materials and Methods

Thirty-one patients with multiple recurrences of WDTC were retrospectively identified from the thyroid cancer

database at Mount Sinai Hospital, Toronto (1963–2000). Recurrence was defined as any evidence of disease requiring further treatment after initial curative therapy. Patient (age, sex), tumor (histology, size, stage, solitary/multifocal, extrathyroidal spread, vascular invasion, lymphatic invasion), and treatment (extent of initial surgery, adjuvant I^{131}, and external beam radiation) characteristics were collected. Information about the site of each recurrence (local, regional, distant, unspecified), mode of detection (clinical, imaging, thryoglobulin estimation), and treatment (surgery, I^{131} therapy) were also recorded. Extent of disease at presentation was staged according to the American Joint Committee on Cancer (AJCC) staging system for WDTC [21]. In addition, each patient was scored according to the Metastasis, Age, Completeness of Resection, Invasion, and Size (MACIS) prognostic index [22]. Extent of initial surgery was recorded as either a subtotal or total thyroidectomy with or without an accompanying neck dissection. A recurrence was classified as unspecified if thyroglobulin levels were elevated in the presence of a negative clinical exam and failure of localization with available imaging modalities (i.e., ultrasonography, I^{131} scanning, CT, and MRI). Final outcome was recorded as alive, no evidence of disease (ANED), alive with disease (AWD), dead, no disease (DND), and dead of disease (DOD). Follow-up was counted from completion of initial therapy to the last known clinical encounter or date of death.

Statistical analysis of survival data was performed using the Kaplan-Meier method, and curves were compared using the log-rank test. $P < .05$ was considered statistically significant. Univariate analysis was performed in order to identify prognostic factors significant for the development of a poor outcome (i.e., death) in patients with multiple recurrences of WDTC. Multivariate analysis using the Cox proportional hazards model was not possible due to the limited number of events in this study. All statistics were carried out using SPSS software (SPSS Inc, Chicago, Ill).

3. Results

Thirty-one patients with multiple recurrences of WDTC were identified from treatment records at the Department of Otolaryngology — Head & Neck Surgery, Mount Sinai Hospital (Toronto), with a median follow-up of 12.6 years (range 9 months–35.5 years). There were 20 (64.5%) female patients and 11 (35.5%) male patients (median age 43, range 16–83 years; Table 1). The final histopathologic diagnosis was papillary carcinoma in 19 (61.3%), tall cell variant in 5 (16.1%), follicular carcinoma in 4 (12.9%), and mixed in 3 (9.7%) cases. The median size of the dominant nodule was 3.3 cm (range 0.5–5.5 cm). Seven of the charts did not contain a report of tumor size and thus could not be scored with the MACIS prognostic index. These patients were excluded from further analysis. Of the 31 patients identified, 6 (19.4%) had evidence of distant metastasis at diagnosis. Other tumor characteristics present at first surgery included multifocal disease in 21 (67.7%) patients, extrathyroidal spread in 18 (58.1%) patients, lymphatic invasion in 18 (58.1%) patients, and vascular invasion in 3 (9.7%) patients. According to the AJCC staging system, Stage I disease was present in 4 patients (12.9%), Stage II in 8 patients (25.8%), Stage III in 11 patients (35.5%), and Stage IV in 8 patients (25.8%). The MACIS prognostic index was applied to 24 cases in the series with complete pathology records (median score 6.03, range 3.25–11.82).

Table 1: Demographic data for patient with multiple recurrences of well-differentiated thyroid carcinoma.

N	31
Age, median (range), years	43 (16–83)
Sex, M:F	11:20
Histologic type	
Papillary	19
Follicular	4
Mixed	3
Tall cell	5
Tumour size, median (range), cm	3.3 (0.5–5.5)
Multifocal disease	21
Extrathyroidal extension	18
Lymphatic invasion	18
Vascular invasion	3
Distant metastasis	6
AJCC stage	
I	4
II	8
III	11
IV	8
MACIS, median (range)	6.03 (3.25–11.02)
Extent of initial surgery	
Subtotal thyroidectomy	12
Total thyroidectomy	19
Neck dissection†	15
Lodine 131 therapy	27
External beam radiation therapy	10
Site of recurrence	
Local	6
Regional	15
Distant	6
Unspecified	4
Outcome	
Alive, no evidence of disease	9
Alive with disease	12
Dead of disease	10
Dead, no evidence of disease	0
Follow–up, median (range), years	12.6 (9months35.5years)

AJCCAmerican JOINT Committee on Cancer.
Data is for the number of patients in each category.
† central +/- lateral neck dissection.
Unspecified recurrence-elevated thyroglobulin levels in the presence of a negative clinical exam and failure of localization with imaging modalities (i.e., ultrasonography, I131 scanning, CT and MRI).

Extent of initial surgery was dependent on both disease severity and the prevailing treatment philosophy at the time of diagnosis. A total thyroidectomy was performed in 19 (61.3%) patients, whereas a subtotal thyroidectomy was performed in 12 (38.7%) cases. A neck dissection accompanied thyroidectomy in 15 patients with evidence of nodal metastasis at initial surgery. Almost all patients in this series were treated with adjuvant I^{131} (87.1%). Ten patients had residual disease severe enough after initial operation to warrant external beam radiation therapy (ERT).

All patients in this series experienced multiple treatment failures. The average time to first recurrence was 25.4 months (range 0.2–185.4 months). Recurrences were classified as local (19.4%), regional (48.4%), distant (19.4%), and unspecified (12.9%). Neither the mode of detection (i.e., clinical, imaging, or elevated thyroglobulin) nor the method of treatment (surgery, I^{131}, or ERT) for recurrent disease was found to be a significant predictor of survival in this study population.

Thirty-two percent of patients with multiple recurrences of WDTC died of their disease (DOD). Other outcomes included alive, no evidence of disease (ANED) in 29% and alive with disease (AWD) in 38.7%. No patients in this series died of causes unrelated to their thyroid carcinoma. Actuarially predicted disease-specific survival among patients with multiple treatment failures at 20 years was 60%, a significant reduction from that of patients with either no recurrences or only one recurrence of their disease [7]. Univariate analysis revealed that age >45, stage III/IV disease, distant metastasis, vascular invasion, MACIS score >6, and a time to recurrence of <12 months are all predictive factors for mortality in this group ($P < .01, < .01, < .001, < .001, < .01, < .03$, resp.; Figure 1). In addition, gender, histological type, initial surgery (total thyroidectomy vs. subtotal thyroidectomy), initial I^{131}therapy, multifocal disease, tumor size, lymphatic invasion, and neck dissection were shown to have no predictive utility in this subgroup of patients with WDTC (Table 2) .

Table 2: Patient, tumour, treatment, and recurrence data for patients with multiple recurrences of well-differentiated thyroid carcinoma.

Age > 45	$^{**}P = .0093$
Gender (M Versus F)	$P = .7876$
Extent of initial surgery (ST versus TT)	$P = .0964$
Neck dissection	$P = .1978$
Iodine 131 therapy	$P = .1749$
External beam radiation therapy	$^{*}P = .0167$
Histologic type—overall	$P = .1362$
Tall cell versus others	$^{\dagger}P = .0748$
Stage I/II versus IV	$^{**}P = .0052$
Size > 4 cm	$^{\dagger}P = .0621$
Multifocal disease	$^{\dagger}P = .0795$
Lymphatic invasion	$P = .9361$
Vascular invasion	$^{***}P = .0002$
Extrathyriodal extention	$^{\dagger}P = .0772$
Distant metastasis	$^{***}P = .0002$
MACIS > 6	$^{**}P = .0061$
Time to recurrence < 12 months	$^{*}P = .0270$
Site of recurrence	$^{\dagger}P = .0560$
Mode of detection	
Clinical	$P = .6328$
Imaging	$P = .5752$
Thyroglobulin	$P = .7497$
Treatment of recurrence	
Surgery	$P = .4128$
Iodine 131 therapy	$P = .6281$
External beam radiation therapy	$P = .8889$

ST: subtotal thyroidectomy; TT: total thyroidectomy
$^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$
† trend toward significance.

4. Discussion

Despite optimal treatment, patients with WDTC often experience disease recurrence, with rates reported in literature ranging from 8% to 23% [1–4]. Palme and associates [7], reported no significant difference in disease specific and overall survival among WDTC patients cured after initial therapy, and those with a single recurrence. However, patients with multiple recurrences are at a significantly increased risk of death, with mortality rates ranging between 12% and 69% [2, 4–6]. It appears that patients who develop multiple recurrences of WDTC follow a distinct course, marked by multiple treatment failures and a significant risk of mortality. It was the intention of the present study to delineate patient, tumor, treatment, and recurrence factors that may be used by physicians to predict for mortality in these patients.

A large body of research exists exploring various prognostic factors for recurrence and mortality among patients with WDTC. Characteristics such as age, sex, tumor size, stage, extrathyroidal spread (ETS), nodal metastases, distant metastases, and extent of initial surgery have all been cited as indicators of poor outcome [1–4, 6, 8–20]. In this investigation, we found that among patients with multiple treatment failures, age >45, stage III/IV disease, distant metastasis, vascular invasion, MACIS score >6, and a time to recurrence of <12 months predicted mortality in this group.

Several studies have cited advanced age as being one of the most significant predictors for recurrence and mortality among patients with WDTC. Shah et al. [8] reported that patients above the age of 50 have a 270% greater risk of death from differentiated thyroid cancer than their younger cohorts. In addition, several authors have cited that the greatest change in prognosis occurs at the age of 45, with older patients having significantly reduced total survival [6, 8–13]. This is in agreement with the results of the current study; patients with multiple recurrences of WDTC who are older than 45 years of age are at an increased risk of death from their disease ($P < .01$).

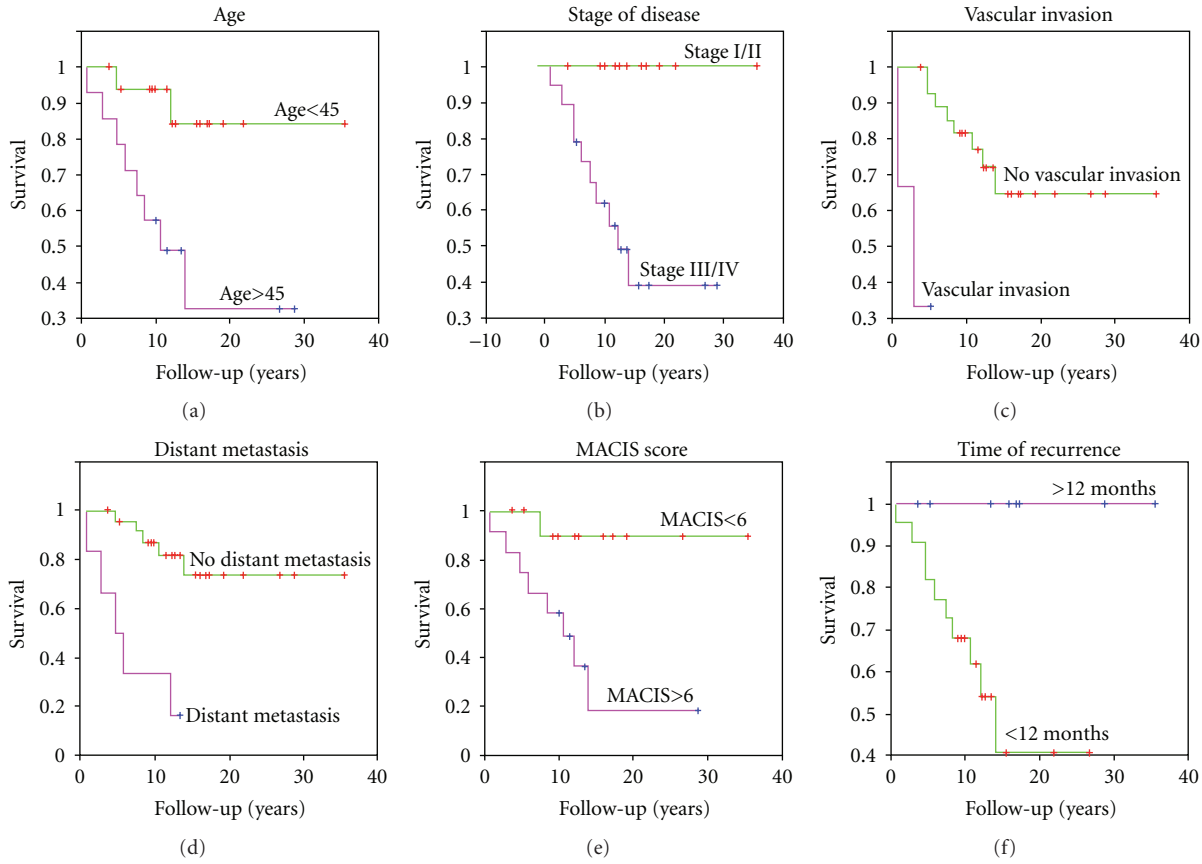

Figure 1: Prognostic factors significantly associated with mortality in patients with multiple recurrences of well-differentiated thyroid carcinoma. (a). age >45, (b). stage III/IV disease, (c). angioinvasion, (d). distant metastasis, (e). MACIS score >6, (F). time to recurrence <12 months.

Extent of disease at initial diagnosis strongly influences prognosis in patients with WDTC. Large tumor size, especially >4 cm, has been shown to adversely affect mortality in multiple trials [9–11, 14]. Although greater tumor size (i.e., >4 cm) was found to be a predictor of multiple recurrences in patients with WDTC [7], it did not appear to predict for mortality in this population.

The AJCC staging system, which incorporates the TNM (tumor, lymph nodes, metastases) classification, is the current standard in staging thyroid malignancies. Stage III/IV disease (III = ETS or nodal metastases, IV = distant metastases) appears to portend an increased risk of mortality among patients with multiple recurrences of WDTC. This is in agreement with other authors, who have found that advanced stage disease not only increases the risk of recurrence but aslo significantly reduces disease-specific survival [7–9, 12, 13, 15]. In the present study, there appears to be a trend toward significance for the adverse effect of extrathyroidal extension (T3) on mortality ($P = .07$). In addition, we found that neither lymphatic invasion nor initial neck dissection showed a statistical significance for mortality among patients with WDTC. However, the presence of vascular invasion did appear to portend a poor prognosis on survival in this cohort ($P = .002$). Lastly, distant metastases were found to be a highly significant predictor of mortality among patients with multiple recurrences of WDTC ($P = .0002$).

Patients who have their first treatment failure <12 months after initial therapy appear to have significantly shorter survival than those who recur after one year. The median time to first recurrence in the present study was 7.3 months, with one patient not showing evidence of any treatment failure until more than 15 years after initial surgery. Given the extensive length of time which may pass between initial treatment and recurrence, life-long follow up is necessary.

Several authors have shown that both the method of detection and the treatment modality used for a first recurrence can predict future treatment failures [7, 20]. That is, patients who have clinically detectable disease recur at a greater rate than those whose first recurrence is detected by thyroglobulin measurement or imaging modalities, stressing the need for early detection before tumor burden becomes significant. In the present investigation, neither the method of detection (i.e., clinical, imaging, I^{131}, thyroglobulin) nor the treatment modality (surgery, I^{131}, external beam radiotherapy) influenced the mortality rates among patients with multiple treatment failures of WDTC.

Because both multiple recurrences and mortality from WDTC are a rare event, it was necessary in the present study to collect data over several decades. In 37 years of clinical experience treating WDTC at our institution, only 31 patients were identified as having multiple recurrences of their disease. Of these, only 10 patients died from their disease. In addition, it is well known that patients with WDTC can be free from disease for many years before developing a first recurrence, a phenomenon that necessitates life-long follow-up in these patients. The long duration of follow-up in the present study allows for the identification of disease recurrences as well as an assessment of long-term clinical outcomes.

Despite changing treatment paradigms over the 4 decades analyzed in this study, all 10 patients who died of their disease had both surgical and adjunctive management of their initial disease that is comparable to current practices at our institution. All patients with advanced stage WDTC underwent either a total thyroidectomy ($n = 8$) or a completion thyroidectomy at the time of initial diagnosis ($n = 2$). Those with unresectable gross residual disease were treated with external beam radiation therapy. Patients who were considered to have microscopic residual disease or who had advanced stage disease were given postoperative I^{131} ablation. In 2002, Hay et al. reported 6 decades of experience treating papillary thyroid carcinoma. They found that despite evolving treatment paradigms, there was no difference in overall survival among patients treated with subtotal versus total thyroidectomy and no survival benefit to postoperative I^{131} ablation in low-risk patients (MACIS score <6) [23]. Given this, it is unlikely that changing treatment paradigms has significantly influenced the validity of the current results.

5. Conclusions

To our knowledge, this is the first paper to identify prognostic factors among patients with well-differentiated thyroid carcinoma who suffer multiple recurrences of their disease; patients with multiple recurrences of WDTC follow a poor clinical course, with multiple treatment failures and decreased survival. Among this subgroup, those aged 45 years or more with aggressive primary tumors (ETS and vascular invasion) and advanced stage disease (Stage III/IV, MACIS>6) have a significantly higher risk of mortality. In addition, time to first recurrence within 12 months of initial therapy conveys a worse prognosis. Interestingly, mortality rates in this study were not influenced by the method of detection nor the type of therapy chosen for first recurrences. One potential explanation is that these patients have a more biologically aggressive variant of WDTC which does not readily respond to treatment of the primary tumor or the initial recurrence. Although the failure to cure these cases after several attempts may cause frustration in both the treating physician and the patient, close follow-up and aggressive treatment of further recurrences is still warranted, as approximately 30% of these will go on to be free of disease after subsequent therapies. Further research is needed into the biological and molecular markers of tumor severity in order to provide an understanding of why some patients with WDTC have an excellent prognosis with complete cure, while others are plagued by multiple treatment failures and eventual death.

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