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 contrast, suppressed tumour transcription of VEGF-D was associated with poorer postrelapse 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.

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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°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 assessed 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: CACCAGCATGATTATGCGGAATCCAACCT
- Forward Primer: GCCCAGTGAAGGTCCTCAAA
- Reverse Primer: CTGGCTCGCTAGAGATTT

- VEGF-B Probe: GACATCTTACATGAAACCTACTCCCT
- Forward Primer: TGGCAGGAAGGTCCTCTG
- Reverse Primer: TCTCTAGGGCTGCACTGAGTTCT

- VEGF-C Probe: TTGATGTCATTTCAAGCAGGAAA
- Forward Primer: CACAGAATGTCTGATCC
- Reverse Primer: TCTCTAGGGCTGCACTGAGTTCT

- VEGF-D Probe: TGACATTGAAACACTAAAAGTTATAGA-
- Forward Primer: CACCAGCATGATTATGCGGAATCCAACCT
- Reverse Primer: CGCTGTCGGCCCTGGTTGATCTCAAA

- FLT1 Probe: TGGCAGGAAGGTCCTCTG
- Forward Primer: TCTCTAGGGCTGCACTGAGTTCT
- Reverse Primer: TCTCTAGGGCTGCACTGAGTTCT

- FLT4 Probe: TGCCCTGCTTCCTGCTGGTTAGTCC
- Forward Primer: GCACCCCTTATACCCCGC
- Reverse Primer: GAGTTTACACTGAGGTCACCTTTGA

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 means 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
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 biopsy 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 summarises 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 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, \( P = .41 \)). 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**                                             |
| **Age**                                                      |
| Median (range)                                               |
| 65 (45–77)                                                   |
| **Relapse-free interval (months)**                           |
| Median (range)                                               |
| 15 (5–221)                                                   |
| **Size (cm)**                                                |
| Median (range)                                               |
| 2 (0.3–6)                                                   |
| **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                                                            |
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
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 post-surgery 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 of the HER family genes with clinicopathological parameters.

|                | EGFR | HER2 | HER3 | HER4 |
|----------------|------|------|------|------|
|                | Low  | High | Low  | High | Low  | High | Low  | High | Low  | High | Low  | High | P     | P     | P     | P     |
| Alcohol        |      |      |      |      |      |      |      |      |      |      |      |      |       |       |       |       |
| Consumption    | .577 | .404 | .259 | .239 | .999 | .501 | .812 | .545 | .425 | .256 | .145 | .716 | .999  | .999  | .999  | .999  |
| 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|      |      |      |      |      |      |      |      |      |      |      |      |       |       |       |       |
| 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|      |      |      |      |      |      |      |      |      |      |      |      |       |       |       |       |
| <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 | .999 | .999 | .999 | .999 | .999 | .999 | .999 | .999 | .240  | .417  | .999  | .448  |
| 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 (0) | 1 (5) | 0 (0) | 1 (5) | 0 (0) |        |      |      |      |       |       |       |       |
| Differentiation grade at relapse | .240 | .417 | .999 | .448 | .999 | .999 | .999 | .205 | .217 | .094 | .538 | .752 | .530  | .305  | .341  | .748  |
| 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 | .999 | .999 | .999 | .205 | .217 | .094 | .538 | .752 | .530  | .305  | .341  | .748  |
| <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 | .530 | .305 | .341 | .748 | .999 | .999 | .999 | .999 | .999  | .999  | .999  | .999  |
| <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 | .530 | .305 | .341 | .748 | .999 | .999 | .999 | .999 | .999  | .999  | .999  | .999  |
| <12 months     | 7 (33) | 9 (45) | 9 (50) | 13 (72) | 11 (52) | 14 (70) | 11 (55) | 12 (63) |        |      |      |      |       |       |       |       |
| >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
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 tumoral VEGF-C/VEGFR3 signalling in nodal/soft tissue relapse and poor
VEGF-A ligand, was shown to exert antiangiogenic e
important for the fine-tuning of angiogenesis. Recently,
VEGF-A, B, and C and modulating the activity of the
neoplastic neovascularisation, forming heterodimers with
VEGF-C/VEGFR3 signalling may include dissemination of
tumour cells in the systemic circulation and arrest in
node/skin tumour 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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