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Review

Molecular Markers of Occult Lymph Node Metastasis in Head and Neck Squamous Cell Carcinoma (HNSCC) Patients

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Abstract

The accurate diagnosis of regional lymph node metastasis is critical for guiding treatment decisions in head and neck cancer patients. Despite advances in imaging techniques, detecting nodal metastasis using radiology remains challenging, leading to potential undertreatment or overtreatment. This review aims to identify molecular markers associated with occult metastasis in head and neck squamous cell carcinoma (HNSCC) patients. We divided the results by subsite for markers: lymph node analysis (microRNAs, myosin-5a (MYO5A), ring finger protein 145 (RNF145), F-box only protein 32 (FBXO32), CTONG2002744, cytokeratin 14 (CK14), eukaryotic initiation factor 4E (eIF4E), desmoglein-3 (DSG3), microsatellite D9S171, squamous cell carcinoma antigen, cytokeratin, tumor budding score, human papillomavirus-DNA (HPV-DNA), tumor infiltrating lymphocytes, sentinel lymph node analysis techniques, single fiber reflectance spectroscopy, radiological techniques), tumor tissue analysis (activin A, carcinoma-associated fibroblasts, cyclins, β-catenin, histopathology, genetic amplifications, DNA methylation, ecotropic viral integration site 1, CC-chemokine receptor 7, melanoma associated-A antigens, vascular endothelial growth factor-C (VEGF-C), panitumumab, epidermal growth factor receptor (EGFR), cornulin, total protein analysis, CD133, NANOG homeobox, neurogenic locus notch homolog protein 1 (NOTCH1), metastasis-associated protein 1, 14-3-3-zeta, E-cadherin, focal adhesion kinase, p-epithelial-mesenchymal transition (EMT), small proline rich protein 1B (SPRR1B), transcription factor NKX3-1, DNA copy number aberrations, microfibril-associated protein 5 (MFAP5), troponin C1, slow skeletal and cardiac type (TNNC1), matrix Gla protein (MGP), fibroblast growth factor binding protein 1 (FBFBP1), F-box protein 32 (FBXO32), fatty acid binding protein 5, B cell-specific Moloney murine leukemia virus integration site 1, podoplanin, p53, Bcl-2, epidermal growth factor receptor (EGFR), Ki67, cyclin D1, cox-2, semaphorin-3F, neuropilin-2, histologic features, cellular dissociation grade, prospero homeobox protein 1, radiologic features, Ki-67, poly (ADP-ribose) polymerase (PARP), Bcl-2 associated agonist of cell death (BAD), caspase-9, vascular endothelial growth factor A (VEGF-A), HPV, p16, methylation status of long interspersed element 1 (LINE-1) and Alu elements, mesenchymal-epithelial transition (MET), gene expression analyses, molecular subtypes) and blood markers (standard blood analysis indexes and ratios, circulating tumor cells, HPV-DNA, CD-31, bone marrow analysis). Several promising markers were identified, including miR-205, desmoglein 3 (DSG3), pan-cytokeratin (CK) AE1/AE3, HPV-16, activin-A, cyclin D1, E-cadherin, and neural progenitor lineage (NPL) that demonstrated effectiveness across multiple studies. Future research should focus on exploring combination scoring systems to improve diagnostic precision and optimize treatment selection in HNSCC patients.

Keywords: head and neck squamous cell carcinoma; meastasis; lymp node; regional metastasis; cancer marker; occult metastasis

1. Introduction

Head and Neck Squamous Cell Carcinoma (HNSCC) has an annual incidence of 600,000 new cases globally [1]. Lymph node metastasis profoundly impacts patient outcomes, drastically reducing 5-year overall survival (OS) rates from 63–86% to 20–36%, making it the paramount prognostic indicator [2–5]. Regrettably, existing imaging techniques often fail to detect occult nodal metastasis smaller than 2 mm, complicating accurate diagnosis and treatment planning [6,7].

Therapeutic approaches for HNSCC typically involve prophylactic neck dissection followed by radiotherapy with or without chemotherapy for clinically positive lymph nodes [8,9]. However, 15–20% of cases present with occult metastasis in clinically negative (cN0) lymph nodes, challenging treatment decisions [10]. Failure to detect such

metastases may lead to suboptimal therapeutic strategies, potentially resulting in cancer recurrence and compromised patient outcomes [11].

The National Comprehensive Cancer Network (NCCN) Guidelines underscore the importance of neck dissection for high-risk or clinically positive nodal metastasis. Moreover, histological confirmation of nodal metastasis warrants adjuvant therapy [12]. Detection of occult metastasis is pivotal, as it informs tailored treatment strategies, sparing patients unnecessary systemic chemotherapy while ensuring adequate management of distal metastases [12].

Identifying occult metastasis remains a daunting task, with significant implications for treatment decisions and patient prognosis. This review aims to comprehensively survey the literature to identify molecular markers associated with occult metastasis in HNSCC patients. By elucidating these markers, we aim to enhance diagnostic precision, optimize therapeutic selection, and ultimately improve patient outcomes in this challenging clinical scenario.

2. Results

To better categorize the results, we divided them into three main sections based on the site of marker research: lymph nodes, tumor tissue, and blood markers.

2.1 Lymph Nodes Markers

2.1.1 MicroRNAs in Lymph Nodes

MicroRNAs (miRNA, miR) are non-coding RNA molecules that regulate gene expression by interacting with messenger RNA (mRNA) [13,14]. Specific miRNAs play pivotal roles in orchestrating gene expression patterns in various tumors, including HNSCC [15–19]. For instance, Fletcher *et al*. [20] observed tumor-specific expression of miR-205 in metastatic HNSCC lymph nodes, demonstrating significant differential expression compared to benign mucosal samples ($p < 0.05$). While miR-205 levels may not serve as a marker for cancer transformation in epithelial tissues, they show promise in detecting lymph node metastasis. In their study, miR-205 expression was significantly different in histologically metastatic lymph nodes compared to non-metastatic ones ($p < 0.01$), indicating its potential as a marker for micro-metastatic disease. Additionally, quantitative real-time polymerase chain reaction (qRT-PCR) analysis demonstrated acceptable sensitivity in identifying metastatic HNSCC within lymph nodes, suggesting the utility of miR-205 as a diagnostic marker [20].

The pooled analysis of miRNA expression profiles in HNSCC lymph nodes revealed a spectrum of potential markers, encompassing miR-200a, miR-200c, miR-203, miR-205, miR-382, miR-628-5p, and miR-758. Notably, miR-628-5p, miR-758, and miR-382 exhibited limited sensitivity, detecting only 26.3%, 31.6%, and 52.6% of metastatic samples, respectively, underscoring their suboptimal diagnostic utility. Conversely, miR-200a, miR-200c, miR-203, and miR-205 demonstrated maximal specificity (100%) and high sensitivity levels (84.2%, 94.7%, 100%, and 100%, respectively), indicative of their potential as robust diagnostic markers. The expression profiles of these miRNAs were significantly associated with the presence of metastatic disease. However, miR-200a and miR-200c exhibited limited efficacy in detecting micrometastases, identifying only 40% and 80%, respectively, of such cases and failing to detect isolated tumor cells. In contrast, miR-203 and miR-205 displayed exceptional sensitivity, accurately classifying lymph nodes containing macrometastases, micrometastases, or isolated tumor cells with 100% sensitivity. The diagnostic accuracy of miR-200a was 84.2% (95% confidence interval (CI), 68.1–93.4%), with a positive predictive value of 100% and a negative predictive value of 68.4% (95% CI, 43.5–87.3%). Similarly, miR-200c exhibited an accuracy of 92.1%, with positive and negative predictive values of 100% and 81.2% (95% CI, 77.5–97.9%), respectively. For miR-203 and miR-205, both positive and negative predictive values, along with accuracy levels, were 100%, with an area under the curve (AUC) of 1.0. Given their high accuracy, miR-203 and miR-205 were further evaluated by comparing fine needle aspiration biopsies (FNAs) with cytological assessments, revealing complete concordance between molecular and cytological approaches. Notably, both markers exhibited 100% sensitivity, specificity, negative predictive value, and positive predictive value. Furthermore, the diagnostic accuracy of miR-203 and miR-205 in distinguishing positive and negative FNAs was remarkably high, with AUC values of 0.963 and 0.966, respectively, and accuracy levels of 97.3% (95% CI, 92.1–99.4%). Moreover, the negative predictive values were 95.9% (95% CI, 88.6–99.1%), and the positive predictive values were 100% (95% CI, 90.9–100%) for both miRNAs. These findings underscore the clinical potential of miR-203 and miR-205 as reliable diagnostic markers for HNSCC lymph node metastasis, offering enhanced accuracy and precision in patient management [21].

2.1.2 Genetic Analysis

Gene expression profiles of oral squamous cell carcinoma (OSCC) were scrutinized by comparing 70 metastatic and 40 non-metastatic lymph nodes. Utilizing multivariate linear regression analysis, several genes showed differential expression between these nodes: *REEP1*, *RNF145*, *CTONG2002744*, *MYO5A*, and *FBXO32*. Subsequently, employing stepwise logistic regression, a predictive model based on four of these genes-*MYO5A*, *RNF145*, *FBXO32*, and *CTONG2002744*-was identified. Comparison of this gene-based predictive model with tumor size-a conventional predictor of locoregional metastasis-revealed a significantly superior AUC at the Receiver Operating Characteristic (ROC) curve (AUC = 0.85 vs. 0.61, respectively; $p < 0.011$). Interestingly, the incorporation of tumor size into the gene-based model did not enhance its predictive

value. Notably, this investigation was conducted using the Affymetrix Platform, with quantitative PCR (qPCR) subsequently employed on a separate set of 31 metastatic vs. 13 non-metastatic lymph nodes. Observations from qPCR analysis indicated a correlation between *CTONG2002744* and *FBX032* only. However, it's noteworthy that statistical power analysis revealed sufficient power (at alpha = 0.05) for *CTONG2002744* exclusively. These findings underscore the potential significance of *CTONG2002744* as a key gene associated with metastatic processes in OSCC. As reported, further validation studies are warranted to consolidate and expand upon these preliminary findings, shedding more light on the role of *CTONG2002744* and other implicated genes in the metastatic cascade of OSCC [22].

qPCR was also used to assess the expression levels of CK14, eIF4E, and desmoglein 3 (DSG3) in 44 oral tongue squamous cell carcinoma (SCC) patients. These results were then compared with histological and immunohistochemical analyses. The sensitivity of each marker for detecting lymph node cancer involvement was: CK14: 0.6, eIF4E: 0.92, and DSG3: 0.88. Specificity values were: CK14: 0.9, eIF4E: 0.74, and DSG3: 0.8. Combining markers enhanced diagnostic accuracy, with CK14 and DSG3 together showing a sensitivity of 0.88 and a specificity of 0.85. A comprehensive evaluation of all markers achieved maximum sensitivity (sensitivity = 1). Immunohistochemical investigation of DSG3 alone had higher sensitivity than cytokeratin (0.9 vs. 0.7). The authors concluded that using multiple markers improves the diagnosis of occult metastasis in HNSCC patients, enhancing the sensitivity and specificity of intraoperative pN0 neck staging, potentially improving clinical outcomes and patient management [5].

Further genetic analysis of lymph nodes for occult metastasis detection focused on the loss of heterozygosity (LOH) at the D9S171 microsatellite locus on chromosome 9 (9p21). 182 lymph nodes and 20 supraglottic cancer samples were examined for LOH at D9S171, alongside immunohistochemistry for CK19 and histological analysis. The outcomes of these techniques varied significantly, with detection rates of 34.4%, 23.6%, and 16.5%, respectively. Notably, none of the methods demonstrated high accuracy, with 45% of the population having micro-metastasis [23].

2.1.3 Squamos Cell Carcinoma Antigen and Cytokeratins

In 2004, Onishi *et al*. [24] conducted a study to identify occult metastasis in cervical lymph nodes of patients with oral cancer using PCR. Their investigation focused on assessing the expression of squamous cell carcinoma antigen (SCCA) and cytokeratin 13 (CK13) as potential markers. While CK13 was initially considered a candidate marker for occult metastasis detection, its expression was also found in control lymph nodes, rendering it unsuitable for this purpose. Conversely, SCCA demonstrated promising results, with expression observed in 4 out of 30 control lymph nodes and significantly elevated levels detected in metastatic lymph nodes. Based on their findings, the authors concluded that SCCA mRNA expression, detected through real-time qPCR, holds clinical utility for the detection of occult tumor cells in cervical lymph nodes. This underscores the potential of molecular techniques, such as PCR, in improving the accuracy and sensitivity of lymph node metastasis detection in oral cancer patients, thereby informing treatment decisions and enhancing patient outcomes [24].

Cytokeratin 14 (CK14) expression was investigated using real-time (RT) PCR in 153 cervical lymph nodes from 13 patients with HNSCC. These lymph nodes were also subjected to semi-step sectioning and immunohistochemistry for CK14 analysis. A cutoff value of 50 molecules of CK14-RNA per nanogram was utilized for the RT-PCR analysis. CK14-RNA was detected in 33 nodes, 14 of which had nodal metastasis upon pathological examination. Notably, 2 metastatic nodes with occult metastasis tested positive for CK14-RNA, and 2 additional nodes without micrometastasis exhibited CK14 levels above the cutoff value. The study concluded that RT-PCR for CK14-RNA in lymph nodes is sensitive in detecting micro-metastasis but has a relatively high false-positive rate. These findings highlight the need for cautious interpretation of RT-PCR results for CK14-RNA in lymph node evaluation, emphasizing the importance of corroborating findings with other diagnostic methods to minimize misdiagnosis and ensure accurate clinical management of HNSCC patients [25].

Cytokeratin 19 (CK19) has been found to be an inadequate marker for occult lymph node metastasis in oral squamous cell carcinoma (OSCC), as well as lacking specificity for SCC. This conclusion was drawn based on observations that glandular tissue adjacent to the analyzed lymph nodes exhibited positive expression of CK19. In a study involving tissue microarrays from 212 patients, the authors investigated the correlation between CK19 expression in tumors and lymph nodes. They found that in 65 cases, there was a correlation between tumor and lymph node CK19 expression, albeit this correlation was deemed only "fair" (kappa 0.391; $p = 0.001$). Notably, for early-stage OSCC, this correlation was not statistically significant (kappa 0.422; $p = 0.064$). Furthermore, CK19 was also evaluated through CK19 mRNA expression in cervical lymph nodes. Amplification of CK19 mRNA, as demonstrated by RT-PCR, was associated with the presence of carcinoma cells in lymph nodes, with significantly higher values observed in metastatic nodes ($p < 0.0001$). This approach exhibited higher sensitivity for nodal involvement compared to histology alone (16.3% vs. 36%; $p < 0.0001$). In summary, CK19 has limitations as a marker for occult lymph node metastasis in OSCC, with its expression not being specific to SCC. However, CK19 mRNA expression analysis via RT-PCR shows promise as a more sensitive method for detecting nodal involvement, highlighting the importance of employing advanced molecular techniques in clinical prac-

tice for improved diagnostic accuracy and patient management [26]. CK19 mRNA was further investigated using a one-step nucleic acid amplification method to identify occult metastasis from OSCC, achieving an accuracy of 95%. Oka *et al*. [27] analysed gene expression profiles from metastatic lymph nodes, identifying 36 genes, including annexin A8-like 2 (ANXA8L2) and desmoglein 3 (DSG3), which were consistently detected at significantly higher levels in metastatic lymph nodes compared to benign ones. A retrospective analysis of 330 lymph nodes was performed, with 62 testing positive for metastatic involvement. The individual accuracy of each marker—CK19, ANXA8L2, and DSG3—was approximately 90%. Notably, combining these markers improved sensitivity to 96–100%. Additionally, ANXA8L2 and DSG3 expression was detected in about 3% of histopathological metastasis-negative lymph nodes. These findings suggest that ANXA8L2 and DSG3 hold promise as molecular markers for enhancing the detection rate of occult metastasis in OSCC. Utilizing a combination of these markers can help clinicians achieve higher sensitivity in identifying metastatic involvement, leading to more accurate staging and treatment planning for OSCC patients [27].

Broader analysis of cytokeratin expression was conducted using pan-cytokeratin (pan-CK) (AE1/AE3). 133 lymph nodes obtained from 10 patients diagnosed with OSCC were studied. In addition to pan-CK analysis, these lymph nodes underwent serial sectioning at 100 µm intervals to detect micro-metastases or single cancer cells. The analysis resulted in the upstaging of 3 out of 10 patients (3.33%), with cancer positivity detected in 2.25% of the examined lymph nodes [28]. Similarly, Barrera *et al*. [29] utilized pan-CK AE1/AE3 in a study involving 1012 lymph nodes from 50 patients with HNSCC. They compared the efficacy of metastasis detection using serial sectioning at 5- to 6-µm intervals. Notably, unexpected micrometastases were identified through pan-CK AE1/AE3 immunohistochemistry, leading to an upstaging in 29% of N0 patients and 45% of N1 patients. The authors observed that in 8 cases where serial sectioning analysis yielded negative results, immunohistochemical analysis with pan-CK AE1/AE3 revealed positive findings, while the opposite occurred in 3 cases. Consequently, they concluded that the combined utilization of serial sectioning and immunohistochemistry with pan-CK AE1/AE3 could enhance the detection of micro-metastases. This approach holds promise for improving the accuracy of nodal staging in patients with HNSCC, potentially leading to more tailored and effective treatment strategies [29]. Rhee *et al*. [30] utilized the monoclonal antibody cocktail AE1/AE3 for cytokeratin in their investigation of occult nodal metastasis. Their study uncovered 5 micro-metastases among 10 patients that were not detected by standard analysis methods. This underscores the potential of utilizing monoclonal antibody cocktails for enhancing the sensitivity of nodal metastasis detection, providing valuable insights into the presence of micrometastases that may otherwise go undetected using conventional approaches [30]. Immuno-histochemistry assays utilizing antibodies were also used for molecules against CK5/14, a broad spectrum of cytokeratin (CK 1–8, 10, 14–16, and 19), and CD44v6 for investigating regional lymph node metastasis. Among 50 cN0 subjects with HNSCC, the authors detected seven micro-metastases in 5 patients and 31 disseminated tumor cells in 12 patients [31].

Building on the observation that Sentinel lymph node biopsy is recommended by NCCN guidelines for HNSCC and breast cancer, researchers noted the enhanced reliability of the one-step nucleic acid amplification (OSNA) method in breast cancer and explored its application for HNSCC. In a study involving 26 cN0 HNSCC patients, 157 lymph nodes were analyzed using immunohistochemistry for CK19, RT-qPCR for CK19 (the target of the OSNA assay), and two additional markers, EPCAM and PVA. OSNA provided intraoperative results for all patients, detecting 21 metastases. Of the 157 lymph nodes, 139 were concordant (88.5%). There were 18 initially discordant lymph nodes (11.5%), with 13 (8.3%) being OSNA positive but pathologically negative, and 5 (3.2%) being OSNA negative but pathologically positive. After the elimination of allocation bias, the false-negative rate was reduced to 1.3%, with a sensitivity and specificity of 90% and 95.6%, respectively. The positive predictive value and negative predictive value were calculated at 75% and 98.5%, respectively. These findings underscore the potential utility of OSNA as an effective intraoperative diagnostic tool for assessing lymph node metastasis in HNSCC patients, providing valuable insights for treatment decision-making and patient management [32].

2.1.4 Desmoglein 3

Desmoglein-3 (DSG3) was studied as a potential maker of occult metastasis. Antibodies against DSG3 were used within lymph nodes with the Lateral Flow Test assay system. This technique demonstrated a sensitivity of 72.5% and a specificity of 55.6% in detecting nodal metastasis. These findings indicate that while the assay system has moderate sensitivity, its specificity is relatively low, highlighting the need for further refinement or complementary approaches to improve accuracy [7]. DSG3 immunohistochemical analysis was also conducted by Nagvekar *et al*. [33] in a study involving 47 lymph nodes from 10 patients with OSCC. The researchers identified DSG3 positivity in 6 nodes using 3 µm histological sections. However, the identification of additional micro-metastatic deposits was complicated by the presence of numerous activated macrophages exhibiting DSG3 immunoreactivity. These DSG3-positive macrophages were distributed throughout various regions, including the subcapsular sinuses, interfollicular areas, medullary sinuses, and lymphoid follicles. Further characterization revealed that these DSG3-positive

cells expressed CD68, confirming their macrophage phenotype. Based on these observations, the authors concluded that while DSG3 is overexpressed, its utility as a marker for detecting micro-metastasis is limited due to the confounding presence of DSG3-positive macrophages. This finding underscores the complexity of interpreting DSG3 immunoreactivity in lymph nodes and emphasizes the importance of considering potential cellular heterogeneity and non-neoplastic expression patterns when assessing its diagnostic significance in OSCC metastasis detection [33]. Patel *et al*. [34] introduced a nanostructured immunoarray system tailored for the ultrasensitive detection of DSG3 in lymph node tissue lysates. Through their research, they observed that DSG3 exhibits high expression levels in all head and HNSCC lesions and their corresponding metastatic cervical lymph nodes, while being conspicuously absent in non-invaded lymph nodes. This finding underscores the potential of DSG3 as a discriminatory biomarker for metastatic disease in HNSCC [34].

2.1.5 HPV-DNA in Lymph Nodes

In a study investigating HPV-16 positivity in cervical lymph node metastases of HPV16+ oropharyngeal SCC, researchers conducted RT-PCR analysis on cervical lymph nodes from 11 patients with oropharyngeal SCC and 3 controls with HPV-negative oropharyngeal SCC. The results revealed a significantly higher viral load in metastatic lymph nodes compared to tumor-free nodes in the experimental group ($p < 0.01$). Among the tumor-free lymph node samples, 16 had undetectable viral load values, 8 showed low or medium levels ($<10^5$ copies per million cells), and 3 exhibited high levels ($>10^5$ copies per million cells). This finding led the researchers to conclude that the detection of HPV-16 DNA in lymph nodes of patients with HPV-16(+) oropharyngeal cancer is indicative of metastatic involvement. Moreover, they suggested that tumor-free lymph nodes with a high viral load value may signify the presence of occult lymph node metastasis, thereby proposing HPV-16 DNA as a potential marker for metastasis in these cases [35]. HPV-DNA was also searched using RT-PCT by Mirghani *et al*. [35] for HPV-16 as a potential marker of occult metastasis. Their study included 11 patients with HPV16+ oropharyngeal SCC and 3 patients with HPV16-OSCC. Notably, HPV16 was not identified in the HPV16-patients, while metastatic lymph nodes from HPV16 oropharyngeal SCC exhibited a high viral load. Among 27 pathologically tumor-free lymph node (PTFLN) samples, 16 had no detectable viral load, while the viral load was low or medium ($<10^5$ copies/million cells) in 8 samples and high ($>10^5$ copies/million cells) in 3 samples. Interestingly, in the latter group where high viral load was detected in PTFLN, no metastatic cells were identified, and the viral DNA was found to be located in immune cells. Based on these findings, the authors concluded that HPV16 detection in lymph nodes can be attributed to its presence within either metastatic cells or immune cells. Furthermore, they suggested that HPV16 detection in PTFLN may not necessarily correlate with occult lymph node metastases [36].

2.1.6 Use of Multiple Markers

Matsuzuka *et al*. [37] utilized a microfluidics-based molecular assay system for the intraoperative detection of nodal metastasis, selecting markers based on meta-analysis. They developed both polyclonal and monoclonal antibodies, establishing a lateral flow assay system to screen lymph nodes from patient samples—both positive and negative for metastasis—to determine sensitivity and specificity. In their preliminary study focusing on DSG3, five monoclonal and one polyclonal antibodies were developed and validated in positive (17) and negative (7) lymph nodes. The marker demonstrated a sensitivity above 80% and specificity above 71%. Subsequently, a sandwich ELISA indicated the optimal combination of antibodies, and the Lateral Flow test (LFT) assays developed with this combination for DSG-3 exhibited a sensitivity of 72.5% and specificity of 55.6% in detecting positive lymph node samples (11 positive & 9 negative lymph nodes). Moving forward, DSG3, along with the newly identified markers, will undergo validation in larger patient cohorts, with the aim of selecting the best combination of markers for developing the diagnostic assay. The authors concluded that incorporating multiple markers on a proficient platform like microfluidics holds the potential to enhance the clinical utility of the assay system, offering improved accuracy and reliability in the intraoperative detection of nodal metastasis [37].

2.1.7 Histology

Several histological techniques were compared for lymph node analysis. In stage I (T1-2cN0) tongue cancer patients, sentinel lymph nodes were pathologically evaluated using frozen section, imprint cytology, hematoxylin-eosin staining, serial step sectioning (SSS) with hematoxylin-eosin, and immunohistochemistry (IHC). Metastases were classified based on size: macro-metastasis ($>$2.0 mm), micro-metastasis (0.2 mm–2.0 mm), and isolated tumor cells ($<$0.2 mm). Out of 80 patients, occult metastasis was detected in 20. Frozen section and imprint cytology identified metastasis in 10 patients, while hematoxylin-eosin stain detected it in 13 patients. SSS further upstaged the disease in 7 additional patients (9%). While frozen section successfully detected macro-metastasis in 7 out of 8 cases, it missed micro-metastasis in 4 out of 7 cases and isolated tumor cells in all 5 cases. SSS was particularly effective, upstaging the disease by 10%, with a sensitivity and negative predictive value of 90% and 97%, respectively, when combined with hematoxylin-eosin stain. The authors concluded that frozen section and imprint cytology are inadequate for identifying occult metastasis, while IHC and SSS are necessary to detect micro-metastasis and isolated tumor cells [38].

Tumour budding score was also studied and compared with the AE1/AE3 cocktail. In a study involving 97 patients with cT2N0 tongue SCC, researchers investigated the potential of tumour budding score and the AE1/AE3 cocktail as predictors of occult nodal metastasis. Their findings confirmed the significance of both markers in predicting the occurrence of occult neck metastasis. Specifically, a tumor budding score of $\geq$4 emerged as a significant independent predictive factor for occult neck metastasis [39].

Tumor infiltrating lymphocytes (TILs) were investigated in a study involving 14 patients treated with transoral robotic surgery and neck dissection for HPV+ oropharyngeal SCC. Immunohistochemistry targeting CD3, CD8, forkhead box P3 (FOXP3), programmed death-ligand 1 (PD-L1), and cytotoxic T-lymphocyte antigen 4 (CTLA-4) was conducted. The analysis revealed that occult primary tumors exhibited a higher percentage of CD3+ and CD8+ TILs compared to tumors in lymph nodes. Specifically, the percentage of CD3+ T cells was 61% in occult primary tumors versus 42% in nodal tumors (p = 0.006), and the percentage of CD8+ T cells was 36% versus 23%, respectively (p = 0.01). Additionally, there was a higher concentration of FOXP3+ TILs in primary tumors compared to nodal tumors, with percentages of 8% and 5%, respectively (p = 0.01). Although not statistically significant, there was a trend towards a higher percentage of CTLA-4+ cells in primary tumors compared to nodal tumors (52% versus 34%, p = 0.09). Based on these findings, the authors concluded that occult primary HPV+ oropharyngeal SCC tumors tend to have a higher concentration of CD3+ and CD8+ TILs compared to their associated regional lymph node metastases. This observation may potentially contribute to the clinical presentation observed in these patients [40].

2.1.8 Single Fiber Reflectance Spectroscopy

In a cohort of nine patients, intraoperative single-fiber reflectance (SFR) spectroscopy was utilized to evaluate its predictive capability for nodal metastasis. Analysis of the data identified three key parameters—blood volume fraction (BVF), microvascular saturation (StO2_22), and Rayleigh amplitude—that were significantly reduced in lymph nodes with metastases. These parameters were integrated into a composite score, termed the "delta" score, using discriminant analysis. The "delta" score demonstrated a marked decrease in positive lymph nodes, showing substantial performance metrics: sensitivity of 90.0%, specificity of 88.9%, positive predictive value (PPV) of 90.0%, and negative predictive value (NPV) of 88.9%, with a p-value of 0.0006. Furthermore, the area under the ROC curve was notably high at 96.7% (95% confidence interval: 89.7%–100.0%) [41].

2.1.9 Radiology

cN0 HNSCC cases are typically identified following clinical and radiological assessments and positron emission tomography (PET) and magnetic resonance imaging are often conducted for patient staging. The combination of PET and MRI has shown superior performance compared to PET or MRI alone. Specifically, PET/MRI demonstrated higher sensitivity, specificity, negative predictive value, positive predictive value, and accuracy when compared to PET or MRI alone. These values were as follows: 83.3%, 92.1%, 97.2%, 62.5%, and 90.9% for PET/MRI, 50%, 89.5%, 91.8%, 42.8%, and 84% for PET alone, and 83.3%, 68.4%, 96.2%, 29.4%, and 70.4% for MRI alone, respectively [42]. The combination of PET with CT scan was also studied, the sensitivity of PET-CT in detecting nodal disease in recurrent laryngeal cancer was found to be 16.7% (95% CI, 3.5–46.0%), with a specificity of 97.1% (95% CI, 83.8–99.9%). The PPV was calculated at 66.7% (95% CI, 20.2–94.4%), while the NPV stood at 76.7% (95% CI, 62.1–87.0%). These findings suggest that PET-CT exhibits poor sensitivity and NPV, indicating its limitations as a predictor of nodal disease in recurrent laryngeal cancer [43].

2.2 Tumour Tissue Marker Analysis

2.2.1 Activin A and Carcinoma-Associated Fibroblasts

In oral tongue squamous cell carcinoma (SCC), research has explored the role of Activin A and its relationship with carcinoma-associated fibroblasts (CAFs). Activin A, a member of the transforming growth factor beta family, is secreted by CAFs and is known to play a critical role in cancer progression. It was demonstrated that Activin A promotes cell proliferation and increases tumor volume in xenograft models of oral tongue SCC. Additionally, Activin A has been shown to enhance cancer cell migration, invasion, and epithelial-mesenchymal transition, potentially contributing to the aggressiveness of the disease. Notably, high levels of Activin A expression were significantly correlated with regional recurrence (p = 0.01), regional metastasis (p = 0.034), and occult lymph node metastasis (p = 0.006) [44]. Additionally, multivariate analysis has revealed that activin A overexpression serves as an independent marker for overall survival in early-stage oral tongue SCC. When comparing populations with Activin A overexpression to those with low levels, the 5-year overall survival rates were 76.5% versus 89.7%, respectively (HR: 2.44, 95% CI, 1.55–3.85, p = 0.012) [45].

2.2.2 Cyclins

Several studies have investigated Cyclin D1 as a potential marker for occult metastasis in HNSCC. In a cohort of 158 patients with early-stage tongue or floor of mouth cancers, all clinically negative for neck lymph node metastasis, Cyclin D1 expression was found to be significantly associated with occult nodal metastasis (e.g. [6]). Specifically, Cyclin D1 amplification and immunohistochemical positivity were strongly correlated with occult nodal metastasis in early-stage floor of mouth SCC (p = 0.020) [6]. The

predictive value of Cyclin D1 as a marker for occult lymph node metastasis has been validated in various studies, including the trial conducted by Capaccio *et al*. [46]. In this trial involving 96 cN0 HNSCC patients, 32 were found to have pN+ status, while 64 were pN0. Cyclin D1 expression was detected in 42 patients (43.7%) using immunofluorescence. Univariate regression analysis showed a significant correlation between Cyclin D1 expression and occult lymph node metastasis ($p = 0.007$). This correlation remained significant in the multivariate regression analysis, where Cyclin D1 was identified as an independent predictor of occult metastasis ($p = 0.0059$) [46]. Numerical aberrations of the Cyclin D1 gene were found to be associated with occult lymph node metastasis in a study involving 45 patients with OSCC [47]. These patients underwent primary tumor surgical excision without neck dissection. Fluorescence *in situ* hybridization (FISH) was used to detect numerical aberrations in the Cyclin D1 gene from fine-needle aspiration biopsies of cN0 patients. Among the cohort, 15 patients (33.3%) exhibited CCND1 aberrations. Notably, 12 out of these 15 patients (80%) developed cervical lymph node metastases within 2 years. Multivariate analysis highlighted that numerical aberration of the CCND1 gene independently predicted late cervical lymph node metastasis (RR = 8.685, 95% CI, 2.232–33.802, $p = 0.002$) [47]. Further evidence supporting Cyclin D1's predictive role comes from a study involving 75 cases of laryngeal SCC. In this study, Cyclin D1 and E-Cadherin were assessed via immunohistochemistry to predict various outcomes, including lymph node metastasis [48]. Cyclin D1 emerged as a significant independent prognostic factor for lymph node metastasis ($p = 0.000$). Consequently, the researchers concluded that Cyclin D1 could serve as an independent prognostic marker for lymph node metastasis in patients with laryngeal SCC. Moreover, they suggested that Cyclin D1 expression could aid in identifying patients with clinically negative lymph nodes who may still harbor a considerable risk for occult metastasis [48].

Other cyclins, such as L1 and B1, have also been investigated in individual studies (e.g. [49]). Cyclin L1 amplification has been associated with higher stages in HNSCC. A logistic regression analysis based on tissue microarray data using fluorescence *in situ* hybridization in 280 cases of HNSCC revealed a correlation between CCNL1 gain and lymph node metastasis, independent of stage or subsite ($p = 0.049$). Additionally, this amplification was linked to shorter overall survival, as indicated by the log-rank test ($p = 0.006$). These findings highlight the potential prognostic significance of Cyclin L1 amplification in HNSCC, particularly regarding lymph node involvement and overall survival [49]. Cyclin B1 expression in the cytoplasm of tumor cells has been associated with occult cervical lymph node metastasis in a case series involving 40 patients with oral tongue SCC. Additionally, Cyclin B1 levels were found to be positively correlated with Ki67 levels in cancer cells. This suggests that Cyclin B1 expression may serve as a potential marker for predicting occult lymph node metastasis in oral tongue SCC patients, highlighting its potential role in assessing tumor aggressiveness and metastatic potential [50].

2.2.3 E-cadherin

E-cadherin has been identified as a promising marker for occult nodal metastasis. In a 2002 study, Rodrigo *et al*. [51] quantified E-cadherin levels in supraglottic laryngeal SCC and examined its association with various outcomes, including nodal metastasis. They discovered that low levels of E-cadherin in SCC of the supraglottic larynx were significantly correlated with nodal metastases ($p = 0.007$). Based on these findings, the authors concluded that E-cadherin is an independent predictor of nodal metastases in supraglottic SCC [51]. The predictive value of E-cadherin was further confirmed in a study involving 95 patients with supraglottic laryngeal cancer. In this study, E-cadherin and Focal Adhesion Kinase (FAK) were investigated as markers for nodal metastasis [52]. Reduced E-cadherin expression was significantly associated with the presence of nodal metastases ($p = 0.006$). Additionally, combining the assessment of E-cadherin and FAK expression resulted in improved accuracy in detecting nodal metastasis ($p = 0.001$). Histological grade also showed a significant association with nodal metastases ($p = 0.005$). Multivariate analysis confirmed that these parameters were independent predictors of nodal metastases [52]. Furthermore, in an analysis of 120 patients with HNSCC affecting the oral cavity or oropharynx, the Intensity Reactivity Score for E-cadherin expression was quantified and compared to lymph node status obtained via sentinel lymph node biopsy. The study revealed a significant correlation between differentiation grade and E-cadherin expression with positive lymph node status ($p = 0.018$ and $p = 0.005$, respectively) [53].

2.2.4 Other Molecular Markers

The expression of β-catenin in OSCC has been found to be significantly associated with nodal stage ($p = 0.02$), suggesting its potential role as a marker for identifying occult metastases in OSCC patients. This finding highlights the importance of β-catenin as a potential biomarker for assessing metastatic potential and disease progression in OSCC. Further research is needed to validate its clinical utility for identifying occult lymph node metastases in OSCC patients [54].

The expression of CC-chemokine receptor 7 (CCR7) and its ligand, CCL21, plays a crucial role in tumor cell chemotaxis, particularly in the context of lymph node metastasis. In OSCC, the expression of CCR7 has been investigated in relation to cervical lymph node metastasis. Analyzing paraffin-embedded samples from previous patients using hematoxylin and eosin staining and anti-cytokeratin AE1/AE3 antibodies, researchers found that

CCR7 expression in tumors was not significantly associated with cervical metastasis (p = 0.058). However, they concluded that lymph node sectioning combined with pan-CK AE1/AE3 staining remains an important complementary tool in detecting lymph node metastasis. Despite the lack of a significant correlation, the authors stated that the higher immunoexpression of the chemokine CCR7 in tumors of patients with cervical metastasis suggests its potential role as a prognostic biomarker, which warrants further investigation [55].

Some authors also examined the expression of connexins (Cx) 37, Cx40, Cx45, Pannexin 1 (Panx1), and Vimentin in the cancer tissue of 32 patients with SCC using immunofluorescence. The results revealed significant associations between these markers and neck metastatic status. Notably, the median Immunoreactive Score (IRS) for Panx1 was significantly higher in patients with negative neck status (IRS = 4.5) compared to those with metastatic neck disease (IRS = 2), with a p-value of 0.045. Conversely, Vimentin expression was higher in patients with positive neck status, showing a median score of 7.65 compared to 3.83 in those with negative neck status (p = 0.048). Logistic regression analysis identified Panx1 as an independent prognostic factor for regional metastatic disease in laryngeal SCC (p-value = 0.049, 95% CI, 0.563–0.980, odds ratio = 0.76, regression coefficient = –0.271). Additionally, other significant risk factors for positive neck disease included higher histological grade, advanced T stage, and positive lymphovascular invasion, all with p-values < 0.05 [56].

Melanoma-associated antigens (MAGE-A) are generally inactive in normal tissues, except for the testis, but their expression in other tissues is often indicative of tumor presence. In a study of OSCC, RT-PCR analysis for MAGE-A12 demonstrated gene expression in 49.1% of the 57 cancer tissue samples, while it was undetectable in normal tissues. These results led the authors to propose that MAGE-A12 may serve as a useful diagnostic marker for occult metastasis [57].

Vascular endothelial growth factor-C (VEGF-C) is a key regulator of lymphangiogenesis and has been implicated in the progression of various cancers, including OSCC. Its role in lymphatic metastasis makes it a potential biomarker for predicting occult metastasis. In a study involving 87 patients with OSCC and T1-2cN0M0 tumors, researchers analyzed VEGF-C expression to assess its association with occult metastasis [58]. After elective neck dissection, which revealed an incidence of 22% for occult metastasis, the study aimed to determine whether VEGF-C expression correlated with lymph node metastasis. Surprisingly, the findings indicated that VEGF-C expression did not show a significant relationship with lymph node metastasis in this patient cohort [58].

Panitumumab is a monoclonal antibody targeting the epidermal growth factor receptor (EGFR), which is expressed in up to 90% of HNSCC cases. In a study involving six patients, intravenous administration of panitumumab-IRDye800 was followed by evaluation using a high-sensitivity fluorescence imaging system [59]. The researchers assessed the correlation between fluorescence intensity and tumor localization as determined by pathologists. During neck dissections, a total of 172 lymph nodes were excised, eight of which were confirmed to be cancer-positive. Fluorescence imaging with panitumumab-IRDye800 accurately reflected the lymph node status in all instances. Specifically, the imaging identified 164 nodes as true negatives (non-fluorescent and tumor-negative), eight nodes as true positives (fluorescent and tumor-positive), and did not yield any false positives or false negatives. This resulted in sensitivity, specificity, positive predictive value, and negative predictive value of 100%. The authors concluded that panitumumab-IRDye800 is highly effective in identifying lymph node metastasis in HNSCC with exceptional specificity and negative predictive value [59]. In 2021, a study explored the use of Panitumumab-IRDye800CW for sentinel lymph node biopsy in 27 patients with OSCC, including 18 with clinical node-negative disease [60]. The technique involved intravenous administration of Panitumumab-IRDye800CW prior to surgical resection of the primary tumor, which included neck dissection and/or SLNB. A total of 960 lymph nodes were examined, with 34 (3.5%) found to contain metastatic disease. Panitumumab-IRDye800CW preferentially localized to metastatic and sentinel lymph nodes, showing a higher fluorescent signal compared to non-metastatic nodes. The median fluorescent intensity (MFI) was significantly greater in metastatic lymph nodes than in benign ones (0.06 versus 0.02, $p < 0.05$). Selecting the five lymph nodes with the highest fluorescence intensity from each specimen achieved 100% sensitivity, 85.8% specificity, and 100% negative predictive value for detecting occult metastases, as well as 100% accuracy in clinically staging the neck. In the cN+ cohort, evaluating the top five fluorescent lymph nodes per patient resulted in 87.5% sensitivity, 93.2% specificity, and 99.1% NPV for identifying metastatic nodes. These results suggest that Panitumumab-IRDye800CW-enhanced SLNB provides high sensitivity and accuracy for detecting occult metastases and staging the neck in OSCC patients [60].

Instead of targeting a specific marker, some researchers have focused on identifying potential biomarkers by analyzing protein expression profiles. In a study comparing protein expression between snap-frozen tumor tissues and adjacent normal tissues from patients with HNSCC, the goal was to uncover biomarkers linked to occult nodal metastases [61]. The researchers employed laser microdissection and saturation-labeling 2D difference in-gel electrophoresis (2D-DIGE) for total protein analysis,

and used the Significance Analysis of Microarray (SAM) method to evaluate differential protein expression. Proteins of interest were further analyzed using liquid chromatography and tandem mass spectrometry. The study found no significant differences in protein expression between tumor tissues from patients with and without occult nodal metastases [61]. However, notable differences were observed in the adjacent normal tissues: 60 protein spots showed significant variations between the two patient groups. Among these, 31 proteins were underexpressed and 29 were overexpressed in patients with occult metastases. The most notable finding was that the top underexpressed protein in the occult metastasis group was 11.9-fold lower, while the top overexpressed protein was 6.6-fold higher. Cornulin, a 53 kDa calcium-binding protein from the S100 family, emerged as a significant overexpressed protein in the occult metastasis group. Previously recognized as a novel biomarker for HNSCC, cornulin was found to be overexpressed in the adjacent normal tissue of patients with occult nodal metastases. Elevated cornulin levels in normal adjacent tissue may signal ongoing epithelial injury, potentially increasing the likelihood of aggressive tumor development. These findings suggest that cornulin overexpression in adjacent normal tissue could be a novel biomarker for identifying tumors with occult metastases in clinically node-negative HNSCC patients [61]. Additionally, the study highlights the importance of tumor-stroma interactions in the early development of nodal metastases. Overall, this research offers valuable insights into the molecular mechanisms of occult nodal metastases in HNSCC and points to the potential utility of cornulin as a biomarker for high-risk patients. Further research is needed to validate these findings and explore the clinical implications of cornulin expression in HNSCC [61]. A proteomic approach was also employed to analyze lymph nodes and identify predictors of occult metastasis in early-stage buccal mucosa SCC involving 90 patients. Among the molecules considered, higher expression of SFN was associated with a lower risk of nodal metastasis ($p = 0.03$), while higher expression of TCTP was also linked to a lower risk of nodal metastasis ($p = 0.003$). Additionally, these markers, along with 14-3-3-zeta, exhibited significant differences in expression between well-differentiated tumors and others. These findings highlight the potential utility of SFN and TCTP as markers for identifying occult nodal metastasis in early-stage buccal mucosa SCC [62].

Further research explored the relationship between cancer stem cell markers—CD133, NANOG, and NOTCH1—and lymph node metastasis in 144 patients with T1-2cN0 OSCC. High expression levels of CD133, NANOG, and NOTCH1 were detected in 72.91%, 59.02%, and 56.94% of tumor samples, respectively. Significant associations were observed between the expression of these markers and lymph node metastasis in early-stage OSCC, with p-values of 0.035 for CD133, 0.024 for NANOG, and 0.043 for NOTCH1. These results indicate that CD133, NANOG, and NOTCH1 may serve as potential biomarkers for predicting lymph node metastasis in early-stage OSCC [63].

The role of Metastasis-associated protein (MTA) 1 has been investigated as a potential marker for predicting occult nodal metastasis [64]. In a study involving 43 patients with tonsillar SCC, the overexpression of MTA1 was found to be a significant predictor of occult nodal metastasis. MTA1 was expressed in 41.9% of the patients, and its presence was significantly associated with lymph node metastasis ($p = 0.034$). The diagnostic performance of MTA1 for occult metastasis showed a sensitivity of 53.3% and a specificity of 84.6%. These findings suggest that MTA1 expression could be a valuable marker for identifying occult nodal metastasis in patients with tonsillar SCC. Further research is needed to confirm these results and explore the clinical utility of MTA1 in managing tonsillar SCC [64].

Understanding the molecular mechanisms underlying lymph node metastasis in HNSCC is crucial for improving diagnostic and therapeutic strategies. One area of interest is the epithelial-mesenchymal transition (EMT) process, which is implicated in tumor progression and metastasis. A study by Parikh *et al.* [65] aimed to explore the role of partial epithelial-mesenchymal transition (p-EMT) markers in predicting nodal involvement in OSCC. In their study involving 99 OSCC patients, Parikh *et al.* [65] examined tumor tissues for three validated p-EMT markers—PDPN, LAMB3, and LAMC2—and one marker associated with well-differentiated epithelial cells, SPRR1B. The results revealed that the p-EMT score correlated with node positivity, showing a significant difference (2.09 vs. 1.87, $p = 0.02$), including a higher incidence of occult node positivity (56% vs. 19%, $p = 0.005$). Multivariate analysis further demonstrated that p-EMT was independently associated with nodal metastasis (OR = 3.12, $p = 0.039$). These findings suggest that p-EMT markers could be valuable in predicting lymph node metastasis and occult nodal involvement in OSCC [65].

In a study involving 64 patients with laryngeal squamous cell carcinoma (SCC), researchers examined the expression of B cell-specific Moloney murine leukemia virus integration site 1 (BMI-1) using immunohistochemical methods on formalin-fixed, paraffin-embedded primary tissue specimens. The findings revealed that high nuclear BMI-1 expression was an independent prognostic factor for lymph node metastasis ($p = 0.0002$). Furthermore, elevated BMI-1 expression showed a significant correlation with distant metastasis ($p < 0.05$), whereas negative or low BMI-1 expression was associated with the absence of lymph node involvement ($p < 0.05$) [66].

In a prospective clinical trial involving 120 patients with early-stage HNSCC of the oral cavity and oropharynx undergoing sentinel lymph node biopsy, the predictive value of cancer cell-expressed podoplanin for sentinel

lymph node metastasis was evaluated. Podoplanin expression in cancer cells was assessed using immunohistochemistry on tissue microarrays, with expression levels quantified by intensity reactivity scores and categorized as either expressed or not expressed. Occult metastasis was detected in 45 patients (37.5%) upon sentinel lymph node examination, while 29 out of 120 (24.2%) primary HNSCC tumors showed podoplanin expression. There was a significant correlation between podoplanin expression and sentinel lymph node metastasis ($p = 0.029$), and podoplanin remained a significant predictor of lymph node status even after adjusting for tumor stage ($p = 0.028$). However, as a predictive marker for sentinel lymph node metastasis, podoplanin expression demonstrated low sensitivity (36%) and moderate specificity (83%) [67].

Apoptotic markers such as p53 and Bcl-2, proliferation markers including EGFR, Ki67, and Cyclin D1, as well as the inflammatory marker Cox-2, were evaluated. Cox-2 was found to be significantly associated with nodal positivity, suggesting its potential utility as a marker for occult nodal metastasis. Additionally, the expression of Cyclin D1 or Ki67 in node-negative patients may indicate the need for neck dissection or irradiation to manage the risk of occult nodal metastasis [68]. p53 was investigated aso in association with PCNA as a predictive marker of occult nodal metastasis with poor results [69]. A targeted spatial proteomic approach was used to analyze lymph node metastasis. Their observations revealed higher expression levels of Ki-67, PARP, BAD, and cleaved Caspase 9 within metastatic cells compared to primary cancer cells [70]. Ki-67 and vascular endothelial growth factor A (VEGF-A) expression were also investigated in pharyngeal and laryngeal SCC. Ki-67 expression was a significant risk factor for nodal involvement (N+) across all tumors ($p \leq 0.009$). Conversely, VEGF-A expression was associated with nodal involvement in oral and pharyngeal SCC exclusively ($p < 0.03$). Specifically, Ki-67 expression alone in oral and pharyngeal SCC was linked to a relative risk of nodal involvement of 3.83 (95% confidence interval, 1.22–11.99; $p = 0.009$), and the additional expression of VEGF-A increased this value to 6.12 (2.09–17.93; $p < 0.001$). Furthermore, the combined expression of both markers was 3.25 times more effective in predicting nodal involvement for T1,2 tumors compared to T3,4 tumors [71]. Despite previous research on Ki-67 status, some authors have contested its association with occult metastasis [68,70,71]. Researchers investigated the immunohistochemical assessment of PCNA and Ki-67, as well as mitotic counting in laryngeal SCC, and they found that proliferative markers were not reliable indicators for diagnosing occult neck metastasis [72].

In 53 patients with clinically node-negative HNSCC, the expression levels of semaphorin-3F (SEMA3F) and neuropilin-2 (NRP2) were investigated. It was found that SEMA3F expression was significantly lower in patients with lymph node involvement compared to those without (cN0/pN0). Based on these findings, patients were categorized into two groups according to their risk of occult nodal metastasis: Group 1 (n = 34): This group exhibited high SEMA3F expression and low NRP2 expression, demonstrating a low risk of occult nodal involvement. Only 14.7% of patients in this group progressed from cN0 to pathologically node-positive (pN+). Group 2 (n = 19): Patients in this group had either low SEMA3F expression or high SEMA3F expression along with high NRP2 expression. They showed a significantly higher risk of occult nodal involvement, with 78.9% of patients progressing from cN0 to pN+. Multivariate analysis further confirmed that patients in Group 2 had a substantially higher risk (26.2 times higher) of lymph node involvement compared to those in Group 1. These findings suggest that SEMA3F-NRP2 expression levels may serve as potential predictive markers for occult nodal metastasis in HNSCC [73].

Human papillomavirus (HPV) is a well-established risk factor for HNSCC. Its role in cancer development and nodal metastasis has been studied as an indicator of occult nodal metastasis. In a trial involving 93 cases of nodal metastatic (N+) SCC, researchers used *in situ* hybridization for high-risk HPV and immunostaining for p16 in both nodal tissues and primary tumors. The cohort included 32 cases of oropharyngeal cancer, 35 cases of oral cancer, and 26 cases of laryngeal or hypopharyngeal cancer. Of the total cases, 23 were found to be HPV-positive, with 22 of these originating from the oropharynx. The findings suggested that lymph node metastasis can be effectively assessed using a combination of *in situ* hybridization for HPV and p16 immunoreactivity, along with morphological evaluation [74].

In a study involving 151 lymph nodes from 20 cases of SCC, both in-depth histology and endpoint and real-time quantitative RT-PCR techniques were utilized. MET-encoding sequences were detected in 61 out of 151 nodes (40%), with 24 nodes (16%) identified as metastatic by in-depth histopathology. In comparison, routine histopathologic analysis of 654 lymph nodes from the same cases identified only 36 metastases (5%). RT-PCR was employed to measure MET gene-specific mRNA levels in normal tissues, primary tumors, and lymphatic metastases. The study concluded that the MET gene product serves as a valuable marker for detecting occult tumor cells in lymph nodes due to its high expression in metastatic cells [75].

Mermod *et al*. [11] investigated the potential of lymphatic vessel density as a predictor of occult lymph node metastasis, using the transcription factor Prospero homeobox protein 1 (PROX1) as a marker. They retrospectively analyzed 42 patients with clinically node-negative HNSCC and 10 patients with clinically node-positive HNSCC. A PROX1 nuclear cutoff value greater than 31.33 demonstrated a sensitivity of 0.6 (95% CI, 0.26–0.88), specificity of 0.98 (95% CI, 0.87–0.99), positive predictive value of 0.86 (95% CI, 0.42–0.99), negative predictive value of 0.91

(95% CI, 0.79–0.98), and overall accuracy of 0.88 (95% CI, 0.76–0.96). Reliability analysis, assessing inter-rater agreement, yielded an intraclass correlation coefficient of 0.83 ($p = 0.005$). Based on these findings, the authors concluded that PROX1 could serve as an independent predictor of occult metastasis [11].

2.2.5 DNA Modifications

DNA modifications are the base of tumorigenesis. Study of cancer DNA has been performed in many trials in order to detect genomic alteration predicitive for occult nodal metastasis. Amplification of the 11q13 region, which includes genes such as *CCND1*, *FGF4*, *FADD*, and *CTTN*, along with the loss of CSMD1, has shown a significant correlation with lymph node metastasis in a cohort of 355 patients with oropharyngeal SCC and OSCC. In a clinically relevant subgroup analysis, 11q13 amplification was the only factor that consistently detected occult metastasis ($p = 0.002$), with a negative predictive value of 81%. This finding suggests that 11q13 amplification may serve as a valuable marker for identifying occult lymph node metastasis in patients with oropharyngeal SCC and OSCC [76]. Thangaraj *et al*. [77] investigated a cohort of 100 patients with oral tongue SCC using RT-PCR and found that the upregulation of Tanancin C and Podoplanin genes was associated with occult lymph nodal metastasis ($p = 0.049$, $F = 6.76$; $p = 0.049$, $F = 0.5$).

Epigenomic modification of DNA can significantly influence genic expression. Those mechanisms are ofter used in physiologic and pathologic precesses. Cancer uses those mechanisms too in order to increase its proliferation and survival. Several authors studied those modifications in order to predict occult nodal metastasis. Clausen *et al*. [78] investigated the potential of DNA methylation analysis in cancer cells as a predictor of nodal metastasis. They compared methylation levels between six cases of OSCC with nodal metastasis (N+) and six cases without nodal metastasis (N0) using MethylCap-Seq. The isolated methylated DNA fragments were sequenced with Illumina GA II and computationally mapped back to the genome. The next step involves validating the most promising methylation markers identified in this study in a larger cohort of 463 cases, pending the completion of follow-up data collection [78]. Epigenomic analysis was conducted on lymph node tissues to explore their potential as markers for cancer metastasis. Hypomethylation of Long INterspersed Element 1 (LINE-1) and Alu elements (Alu) was investigated using the Combine Bisulfite Restriction Analysis (COBRA) technique. A total of 61 nodes were analyzed. LINE-1 and Alu loci were classified based on the methylation statuses of two cytosine-phosphate-guanine (CpG) dinucleotides in each allele, including hypermethylation (mCmC), hypomethylation (uCuC), and two forms of partial methylation (mCuC and uCmC). The results showed altered LINE-1 methylation, with lower LINE-1 methylation levels observed ($p < 0.001$). Additionally, there was a higher percentage of mCuC ($p < 0.01$), a lower percentage of uCmC ($p < 0.001$), and a higher percentage of uCuC ($p < 0.001$) in the analyzed samples. Receiver operating characteristic (ROC) curve analysis revealed that %uCmC and %mCuC values had high areas under the curve (AUC) of 0.806 and 0.716, respectively, in distinguishing lymph node (LN) from non-metastatic (NM) cases. Based on these findings, the authors concluded that the LINE-1 methylation changes in LN exhibited a similar pattern to that in primary tumors. This epigenomic alteration may be indicative of the presence of occult metastatic tumor cells in the lymph nodes analyzed [79]. Clausen *et al*. [78] explored the use of DNA methylation analysis in cancer cells as a predictor for nodal metastasis. They analyzed methylation levels in six cases of OSCC with nodal metastasis and six cases without nodal metastasis using MethylCap-Seq. The isolated methylated DNA fragments were sequenced with the Illumina GA II and mapped to the genome using computational tools. The researchers plan to validate the most promising methylation markers identified in this study with a larger cohort of 463 cases, pending the completion of follow-up data collection [80].

In a study involving 60 patients with OSCC, Affymetrix mapping arrays were utilized to analyze DNA copy number aberrations (CNAs) as a potential marker for occult nodal metastasis. Through correlation analysis between CNA data for genes and the presence of occult metastasis using Fisher's exact test, several gene clusters with loss/deletion or gain/amplification of genes were found to be significantly associated with occult metastasis ($p < 0.05$). Among these clusters, the authors focused on the loss of NKX3-1 (8p21.2), a homeodomain-containing transcription factor, based on findings from a literature review. Further analysis through quantitative RT-PCR and immunohistochemistry (IHC) confirmed significantly lower expression of NKX3-1 in cases with occult nodal metastasis. This observation was validated by IHC analysis in independent cases, where the Wilcoxon rank sum test revealed a significant difference in average positive rates between OSCC cases with and without occult lymph node metastasis (LNM) ($p < 0.001$). Additionally, the Wilcoxon rank sum test applied to the IHC results in the independent OSCC cases further confirmed the significance ($p = 0.004$). Thus, the authors concluded that loss of NKX3-1 may serve as a potential biomarker for occult LNM in OSCC [81].

93 cases of nodal metastatic SCC were studied using *in situ* hybridization for high-risk HPV and immunostaining for p16 in both nodal tissues and primary tumors. The cohort comprised 32 cases of oropharyngeal cancer, 35 cases of oral cancer, and 26 cases from the larynx or hypopharynx. Of the total cases, 23 were found to be HPV-positive, with 22 of them originating from the oropharynx. The findings suggested that lymph node metastasis could

be assessed using *in situ* hybridization and p16 immunoreactivity in conjunction with histomorphological evaluation [74].

Tissue samples from patients with tongue squamous cell carcinoma (TSCC) were analyzed using the Affymetrix HTA2.0 high-density oligonucleotide array to identify genes differentially expressed in relation to cervical lymph node metastasis. The study found 107 genes with differential expression ($p < 0.05$) in TSCC samples with cervical lymph node metastasis (n = 6) compared to those without (n = 6). Gene Ontology and Kyoto Encyclopedia of Genes and Genomes analyses indicated that these genes were associated with cell-matrix adherens junctions and migration functions. Five genes—*MFAP5*, *TNNC1*, *MGP*, *FBFBP1*, and *FBXO32*—implicated in these pathways were selected for further validation. RT-PCR was conducted on a larger cohort of TSCC samples (n = 32), confirming the relevance of MFAP5 and TNNC1. Subsequent immunohistochemical analysis in an additional cohort (n = 61) further validated the expression of MFAP5 and TNNC1. A significant positive correlation between MFAP5 and TNNC1 expression was observed ($p < 0.001$). Notably, overexpression of MFAP5 and TNNC1 was linked to cervical lymph node metastasis, metastasis relapse-free survival, and overall survival. These findings suggest that MFAP5 and TNNC1 could serve as potential biomarkers for predicting occult cervical lymphatic metastasis and prognosis in patients with oral tongue carcinoma [82].

Identifying specific gene expressions that differentiate primary tumors from metastatic lesions is crucial for understanding cancer progression and developing targeted therapies. Uma *et al*. [83] conducted a study to investigate gene expression differences in tongue cancers using the mRNA Differential Display system (DD-PCR), aiming to uncover potential biomarkers for distinguishing between primary tumors and metastases. In their study, 30 tongue cancer samples were analyzed using DD-PCR. From these, 15 signals displaying differential expression between primary tumors and metastatic samples were selected. Among these, only two signals were successfully reamplified, producing a single band of 180 bp using primer pair AP18 and T12MC. These signals exhibited higher expression levels in the primary tumors compared to the metastases. Subsequent sequence analysis revealed that these signals had 100% homology with the gene encoding Homo sapiens fatty acid binding protein 5 (FABP5), which is associated with psoriasis. Northern blot analysis confirmed that FABP5 expression was up to four times higher in primary tumors than in metastatic lesions. Specifically, three metastatic samples showed no FABP5 expression, while one case exhibited similar levels of FABP5 in both primary and metastatic tissues. A paired *t*-test comparing the expression levels between primary tumors and metastases indicated a significant difference ($p = 0.011$), with mean expression values of 0.8741 for primary tumors and 0.5309 for metastatic samples. These findings suggest that FABP5 expression is significantly higher in primary tongue tumors compared to metastases, highlighting its potential as a biomarker for distinguishing between these stages of cancer [83].

2.2.6 Histologic Features

Tumours are evolving diseases, their structure varies during the pathology history. Several authors analysed the histologic features of HNSCCs as predictors of nodal metastasis. Sparano *et al*. [84] examined histologic and staging characteristics in early-stage (T1-2cN0) oral tongue cancers and explored their association with occult metastasis. They found that several factors were significantly linked to occult metastasis, including greater tumor thickness, deeper muscle invasion, T2 stage, poorly differentiated tumors, an infiltrating-type invasion front, presence of perineural invasion, and presence of angiolymphatic invasion. Using a multivariate analysis, they constructed a model to predict the likelihood of occult neck disease, which incorporated greater tumor thickness, presence of perineural invasion, infiltrating-type invasion front, poorly differentiated tumors, and T2 stage [84]. Oral tongue SCC were also studied for muscle invasion and depth of invasion (DOI) in oral tongue squamous cell carcinoma (SCC). 61 T1N0 cases served as a reference group for assessing their predictive value for occult metastasis over a 2-year follow-up period. Among cases with muscle invasion, there was a 23.3% positive predictive value for occult lymph node metastasis. Similarly, cases with a DOI greater than 3 mm exhibited a 29.7% PPV for occult lymph node metastasis [85]. Several further researchers focused on histological features of oral tongue SCC. A retrospective review of 48 patients with early oral tongue SCC studied histopathological factors such as depth of tumor, differentiation, blood vessel invasion, lymphatic invasion, and tumor budding for their association with late lymph node metastasis. Univariate analysis revealed that blood vessel invasion, lymphatic invasion, and high-grade tumor budding were predictive factors for neck recurrence ($p < 0.001$). However, the Cox proportional hazards model identified high-grade tumor budding as an independent predictive factor ($p < 0.01$). Notably, the combination of a tumor depth $\geq$3 mm and high-grade tumor budding showed high diagnostic accuracy [86]. Tumor budding grade for oral tongue SCC was also analysed in patients with cT1/2N0 along with tumor–stroma ratio (TSR) to predict lymph-node metastases. Among 70 patients, 35 had positive neck lymph node metastasis. Univariate analysis revealed correlations between lymph node metastasis and pathological depth of invasion ($p < 0.001$), TBG ($p = 0.008$), and TSR ($p < 0.001$). In multivariate analysis, pDOI ($p = 0.01$) and TSR ($p = 0.02$) remained significant predictors of lymph node metastasis [87]. Digital analysis of tumor budding and minimal cell nest size was conducted in 331 cases of HNSCC, both HPV-positive and HPV-negative. The analysis encompassed 1 and 10 high-

power fields. High cellular dissociation grading was found to be linked with clinically occult lymph-node metastases [88]. In an article published in the British Journal of Cancer, a novel grading system termed cellular dissociation grade (CDG), based on Tumour Budding and Cell Nest Size, was proposed as a predictor of occult metastasis. In a subgroup of HNSCC patients with clinically negative cervical lymph nodes (cN0 necks; n = 40), occult metastases were detected by pathological evaluation of neck dissection specimens in 8 out of 40 cases (20.0%). All cases with occult metastases had a histopathological grading of nG2/3, while none of the nG1 cases showed presence of lymph node metastases. This finding suggested a positive predictive value of 100% for nG1 grading in predicting nodal negativity upon pathological examination in cN0 necks [89].

Cellular populations in tumor mass are also a potential marker of cancer behaviour. In a study involving 152 patients with cT1-T3N0 oral squamous cell carcinoma (OSCC), the presence of stromal myofibroblasts was examined as a potential marker for occult nodal metastasis. Immunohistochemical analysis of surgical resection specimens revealed that 84.2% of OSCC cases (n = 128) were positive for myofibroblasts in the tumor stroma. Importantly, an increased presence of myofibroblasts in the tumor stroma was significantly correlated with the presence of occult neck metastasis ($p < 0.001$) [90].

Worst pattern of invasion is associated with worst prognosis in OSCC [91]. In a study involving 323 patients with stage I OSCC (cT1-2N0), the Worst Pattern of Invasion-type 5 (WPOI-5) was evaluated as a risk model outcome. High-risk classification according to WPOI-5 was associated with regional metastasis ($p = 0.052$; HR, 3.27; 95% CI, 1.42–7.5). Moreover, WPOI-5 was found to be significantly predictive of occult cervical metastases ($p < 0.0001$) [92].

Machine learning is a new frontier of medical research that is progressively growing. Those algorithms have been studied in several fields. The Modified Polsby-Popper (MPP) score, implemented as a semi-automated image analysis workflow, was explored as a potential predictor of cervical lymph node metastases in tongue cancer. Machine learning models were constructed to forecast both survival outcomes and the likelihood of occult cervical metastases. The findings indicated that higher MPP scores correlated with an elevated incidence of distant metastasis, particularly in early-stage tongue cancer [93].

2.2.7 Radiologic Features

Despite radiology has a too low resolution for occult nodal metastasis [6,7]. But macroscopic radiological features of cancer may be related to locoregional metastatization of disease. MRI is a widespread technique for HNSCC staging. Kwon *et al*. [94] investigated tumor contrast MRI thickness measurements in the axial (mediolateral), coronal (superoinferior), and sagittal (anteroposterior) views of oral tongue SCC as predictors of occult lymph node metastasis in 53 patients. Among the 39 patients classified as cN0, 15 (38.5%) were found to have occult metastasis. Using ROC curves, the authors developed a predictive model for occult lymph node metastasis, achieving an area under the curve (AUC) of 0.750 in the mediolateral direction on the axial view, 0.753 for tumor thickness (TT) in the superoinferior direction on the coronal view, and 0.750 for TT in the anteroposterior direction on the sagittal view. They identified cutoff values of 6.7 mm, 7.2 mm, and 12.3 mm in the axial, coronal, and sagittal planes, respectively, as significant predictors of occult lymph node metastasis ($p < 0.05$) [94].

Nuclear medicine impact was combined with standard radiology for increase diagnositic capacity.

SPECT/CT, an imaging technique for lymphatic metastasis detection, was evaluated in 44 cases of OSCC, including 13 with occult nodal disease confirmed by histopathology and elective neck dissection (END). Occult nodal disease was present in 29.5% (n = 13) of patients based on END histopathology. Sentinel node biopsy (SNB) demonstrated a sensitivity of 76%, specificity of 100%, negative predictive value (NPV) of 91%, and positive predictive value (PPV) of 100%. A total of 183 sentinel nodes were identified, with a mean of 8.13 per patient. Planar lymphoscintigraphy (PL) and SPECT revealed ipsilateral neck hotspots in 95% (n = 42) of patients and contralateral neck hotspots in 9% (n = 4). PL identified 77 hotspots (mean 1.75 per patient) and SPECT identified 92 hotspots (mean 2.5 per patient). SPECT/CT detected additional hotspots in 8 patients, including 3 where PL failed to detect any nodes. In 2 patients, both PL and SPECT were negative. Detection rates by PL, SPECT, and gamma probe were 93%, 95%, and 97%, respectively. Good concordance was observed between anatomical localization on SPECT/CT and gamma probe findings. Although SPECT/CT allowed better anatomical characterization, the authors concluded that given the excellent accuracy of combined planar imaging and intra-operative gamma probe, SPECT/CT did not offer clear advantages [95]. Xu *et al*. [96] investigated whether the maximum standardized uptake value (SUVmax) measured on PET/CT could serve as a predictor of occult nodal metastasis in stage I (cT1-2N0) tongue SCC. The study included 120 patients for analysis. Among 60 patients with an SUVmax of $\leq$9.7, 5 patients had occult metastasis. In contrast, among 60 patients with an SUVmax of $>$9.7, 13 patients had occult metastasis. This difference was found to be statistically significant ($p = 0.041$) [96]. Kuźmińska *et al*. [97] also considered PET/CT alone as a potential tool for detecting occult metastasis.

Norling *et al*. [98] investigated the benefits of incorporating ultrasonography into the standard imaging protocol for OSCC. They found that the short axial diameter was the most effective size criterion for metastasis detection. However, they observed that sonographic characteristics were better predictors than size alone. Specifically,

the presence of at least four sonographic characteristics—hypoechoic or heterogeneous appearance, irregular border, spherical shape, absence of nodal hilum, and peripheral nodal blood flow—yielded a sensitivity of 43.8%, specificity of 91.4%, positive predictive value (PPV) of 70.0%, and negative predictive value (NPV) of 78.0%. With this approach, the number of patients with occult metastases decreased from 16 out of 51 (31%) to nine out of 51 (18%) [98]. Another study focused on ultrasonography was conducted on 60 patients with laryngeal SCC who had negative neck nodes on CT scan. The respective values for ultrasound-guided fine needle aspiration cytology (USg FNAC) demonstrated high sensitivity, specificity, positive predictive value, negative predictive value, and accuracy (92%, 100%, 100%, 96%, and 97%, respectively). However, the size, shape, and vascularity showed significantly lower values for these statistical parameters [99].

2.2.8 Gene Expression Analyses and Molecular Subtypes

In a retrospective cohort study, gene expression subtypes in OSCC and laryngeal SCC were examined to determine their predictive value for nodal metastasis. The study identified four molecular subtypes: basal (BA), mesenchymal (MS), atypical (AT), and classical (CL). In OSCC, the mesenchymal (MS) subtype was significantly associated with a higher risk of nodal metastasis. Furthermore, it was predictive of occult nodal metastasis in a subset of T1-2cN0M0 patients, with a relative risk of 3.38 (95% CI, 1.08–10.69) [100].

2.3 Blood Markers

2.3.1 Indexes and Rations from Standard Blood Analysis

Standard blood tests are usually performed preoperatively to HNSCC patients. Some authors hypothesized that variations or over/under expression of circulating molecules could be indicators of locoregional metastasis. Some researchers also studied the variations of circulating cellular populations proportions as predictor of occult lymphatic metastasis. In a multicentric retrospective analysis by Gaudioso *et al.* [10] involving 472 patients with cN0 neck, various baseline blood parameters including neutrophil-to-lymphocyte ratio (NLR), platelet-to-lymphocyte ratio (PLR), lymphocyte-to-monocyte ratio (LMR), systemic inflammatory marker (SIM), and systemic immune-inflammation index (SII) were evaluated, along with stage and differentiation grade. The study focused on oral, oropharyngeal, and hypopharyngeal cancers, which were found to have a greater risk of occult metastasis compared to other head and neck subsites. Additionally, locally advanced stages and moderate to poor differentiation grades (G2 and 3) were associated with a higher risk of lymph node involvement. Multivariate analysis revealed a significant association between NLR, LMR, PLR, SIM, and SII, with NLR greater than 2.12 emerging as the most reliable parameter (OR = 5.22; 95% CI, 2.14–12.75) for predicting lymph node metastasis. Based on these findings, the authors developed a predictive score for lymph node metastasis incorporating cancer subsite, local stage, and NLR value [10]. NLR and PLR were widely studied in several more trials for their predictive value. In a retrospective analysis of 108 patients with laryngeal SCC, the NLR and PLR were assessed as predictors of occult metastasis. High values of NLR, but not PLR, were found to significantly correlate with the probability of occult metastasis. Through an iterative algorithm, an NLR value of 2.26 was determined to correspond to a probability of occult metastasis of 20%. As a result, the authors concluded that there is a statistical correlation between high pre-treatment NLR values and occult metastasis in patients with laryngeal SCC [101]. Furthermore, a retrospective study involving 110 patients with early-stage oral tongue SCC (T1-2cN0), the NLR analysis revealed a statistically significant relationship between high levels of pre-treatment NLR and the probability rate for neck occult metastases ($p = 0.000496$). A cutoff value of NLR >2.93 was determined, above which the probability of finding metastasis in a clinically negative neck increased exponentially according to their model [102]. In a recent study by Yamagata *et al.* [103], the NLR was confirmed as a predictor of occult metastasis. Similarly, Ventura *et al.* [104] found a significant association between NLR ($p = 0.001$) and monocyte-lymphocyte ratio ($p = 0.011$) with neck status on univariate analysis. However, multivariate analysis revealed that only NLR ($p = 0.02$) was an independent risk factor for occult metastasis among inflammatory blood markers [104]. In a cohort of 110 patients with oral tongue SCC, the NLR and DOI were analyzed as predictors of occult nodal metastasis using a logistic regression model. The study found that a DOI greater than 5.4 mm and an NLR greater than 2.93 are associated with an increased risk of presenting occult cervical metastases. Additionally, a positive correlation was observed between the variables NLR and DOI, as indicated by Spearman's rank correlation coefficient rho of 0.64. Specifically, a unitary increase in the DOI of 1 mm was directly associated with an increase of 0.47 in the NLR [105].

2.3.2 Circulating Tumour Cells and Circulatinh Hybrid Cells

Lymphatic vessels eventually drain into the bloodstream, allowing metastatic cells to spread through the blood. In a study involving 152 patients using the OncoDiscover technique for detecting circulating tumor cells (CTCs), comparisons were made with 40 non-HNSCC controls. The study measured several outcomes, including the presence of nodal metastasis. The results indicated that CTC counts above 20.5 were strongly associated with nodal metastasis ($p < 0.0001$). Additionally, a linear trend was observed for the detection of occult metastasis ($p = 0.061$) [106]. Circulating hybrid cells (CHCs) are characterized by their fusion of genetic material from cancer cells and

host leukocytes. These hybrid cells exhibit increased tumorigenic potential compared to standard circulating tumor cells and have been found to correlate with disease stage and progression in cancer [107,108]. In a study involving 20 patients with clinically node-negative OSCC, researchers investigated CHC levels and compared them with the pathological nodal status. The protocol also included positive controls (patients with clinically positive nodal metastasis, cN+) and negative controls (volunteers without cancer, T0). The findings revealed a significant difference in CHC levels between patients with cN0 OSCC who later developed positive nodes and those who remained negative ($p = 0.005$) [107]. Henn *et al.* [108] also found a significant relationship between CHC and occult nodal metastasis ($p = 0.006$) in patients with clinically node-negative OSCC.

2.3.3 Circulating HPV DNA

Circulating HPV DNA in the serum of patients with HPV-positive HNSCC was investigated using conventional PCR, real-time quantitative assay, and Southern blotting for confirmation in case of positivity. Among the patients tested, conventional PCR using E7 primers and Southern blot hybridization detected circulating HPV DNA in 6 patients. Strikingly, 4 of these patients subsequently developed distant metastasis. This finding led the authors to hypothesize a potential relationship between circulating HPV DNA and tumor cells, suggesting a possible role in metastatic dissemination [109].

2.3.4 CD31

The density of the panvascular endothelial antibody CD31 was evaluated as a potential predictor of occult metastasis in patients with clinically node-negative (cN0) oral cavity and oropharyngeal SCC. A total of 56 cases of oral cavity SCC and 6 cases of oropharyngeal SCC were included in the analysis. The results revealed a significant correlation between CD31 density and occult lymph node metastasis ($p < 0.01$). Using recursive partitioning analysis, a cutoff value of 19.33 for CD31 density was determined, which demonstrated a sensitivity of 91%, a specificity of 65%, a positive predictive value of 40%, a negative predictive value of 97%, and an overall diagnostic accuracy of 71% for identifying occult lymph node metastasis [110].

2.3.5 Bone Marrow

In 2004, Wollenberg *et al.* [111] conducted a study investigating the predictive value of detecting free tumor cells in bone marrow aspirates of patients with HNSCC. They utilized monoclonal KS 19.1 antibodies to detect CK19 expression in bone marrow aspirates obtained from the iliac crest of 176 HNSCC patients. Among them, 54 patients tested positive for CK19 expression in bone marrow cells. Over a 60-month follow-up period, 60 patients (34.09%) experienced disease recurrence, with 34 cases (56.67%) involving locoregional recurrences and 26 cases (43.33%) involving distant metastases. Among the 54 patients with CK19-positive cells in the bone marrow, 27 (50%) had tumor recurrence, compared to 33 out of 122 patients (27.05%) in the CK19-negative group. This difference demonstrated a significant correlation between CK19 positivity in bone marrow aspirates and tumor recurrence ($p < 0.05$) [111].

3. Discussion

Accurate diagnosis of regional lymph node metastasis is critical for determining the appropriate treatment strategy in patients with head and neck cancer. Clinical staging, based on physical examination and imaging modalities, may not always detect occult metastasis accurately. Therefore, elective neck dissection is often recommended for N0 patients with a calculated risk of locoregional metastasis exceeding 20%, particularly in high-risk cases or when imaging results are inconclusive. This approach helps ensure that occult metastases are detected and managed appropriately, ultimately improving patient outcomes [12,112]. Postoperative histological analysis often reveals that a significant proportion of patients who undergo elective neck dissection do not have metastatic disease in their lymph nodes. A study has reported that 50% to 80% of patients with clinically node-negative necks ultimately show no evidence of metastasis in the dissected lymph nodes [107]. This underscores the potential for over-treatment and the associated morbidity of unnecessary surgical intervention. Consequently, there is a growing interest in refining the selection criteria for elective neck dissection to minimize unnecessary procedures and reduce associated morbidity while still ensuring appropriate management for those at risk of occult metastasis [107,113–116].

The sensitivity of conventional pathologic evaluation, which typically involves examining hematoxylin and eosin (H&E)-stained tissue sections under a microscope, is limited in detecting small metastatic deposits within lymph nodes. This limitation can lead to false-negative results, where small metastases are missed during routine histopathological examination. Consequently, patients with occult metastasis may be incorrectly classified as node-negative, resulting in potential undertreatment. This highlights the need for more sensitive diagnostic approaches to accurately identify occult metastases in lymph nodes, particularly in patients with clinically node-negative necks [117]. Absolutely, identifying reliable markers of locoregional or distal metastasis is crucial for several reasons. Firstly, it helps avoid subjecting patients to unnecessary and potentially harmful treatments, such as elective neck dissection or adjuvant therapy, if they do not have metastatic disease. This reduces the risk of treatment-related morbidity and improves patients' quality of life. Secondly, accurate identification of metastasis allows for more tailored and precise treatment strategies, ensuring that patients receive appropriate therapy based on their disease stage and prog-

nosis. Lastly, early detection of metastasis enables timely intervention and monitoring, which can improve outcomes and overall survival rates for patients with head and neck cancer.

Therefore, finding reliable markers of metastasis is essential for optimizing patient care and treatment outcomes in this population. Furthermore, clinical staging of lymph nodes is far less accurate than pathological staging. Even pathological staging has limitations, as it may fail to detect micrometastases in a subset of nodal specimens [20].

Markers of occult metastasis are not necessarily binary variables. In many cases, it is essential to define a threshold value. Fletcher *et al*. [20] emphasize the importance of establishing this threshold for metastasis markers. The threshold value differentiates patients into positive and negative groups, guiding treatment decisions. Determining the optimal threshold involves balancing sensitivity and specificity, this is not an easy procedure but it is essential especially for molecules or cells with quantitative alterations due to locoregional diffusion [20]. The investigation into metastasis markers presents several considerations. Blood markers offer a less invasive approach, allowing surgeons to plan operations and conduct concurrent procedures like neck dissection and tumor excision. Markers derived from cancer mass should be analysed from the initial biopsy of the cancer, rather than post-surgery specimens. This is because suspected nodal metastasis may necessitate a second surgery, leading to treatment delays and increased risks due to repeated anesthesia exposure. Moreover, it's important to note that many studies included in this systematic review focused solely on neck nodal metastasis that need to be excised to be studied. Paratracheal lymph nodes are not usually removed during prophylactic neck dissection. However, paratracheal lymph nodes should also be considered as a potential site of occult metastasis. In a cohort of laryngeal cancer patients undergoing salvage laryngectomy, 14% had paratracheal involvement, with 55% showing no lateral neck disease. Neglecting to consider lateral neck disease may result in incomplete evaluation and mismatches in identifying molecular markers [118]. Identifying micrometastases through molecular techniques in lymph node specimens may seem inconsequential unless we consider its impact on post-surgical therapy. A positive lymph node status (pN+) typically prompts healthcare providers to recommend adjuvant chemotherapy or radiotherapy to the patient [12]. Hence, investigating micro-metastases preoperatively through blood markers aids in surgery planning, while examining nodal specimens post-neck dissection is pivotal for adjuvant therapy planning. In our review, we noted various potential markers of occult metastasis. Although the concept of identifying a single powerful marker to predict occult nodal involvement is appealing, it may overlook the influence of other factors. Combining multiple markers could enhance predictive accuracy [5,7,37]. Indeed, the concept of devising a scoring system that incorporates multiple factors, similar to the approaches taken by Gaudioso *et al*. [10] and Matsuzuka *et al*. [37], enhances predictive capability. Integrating the various molecules and markers discussed could pave the way for the development of a novel scoring system with enhanced predictive value for occult lymph node metastasis [5,37].

Advancements in imaging technology are inherently tied to technological progress. As new imaging techniques enhance their diagnostic capabilities, it becomes essential to correlate molecular findings with the latest radiological insights. However, a significant challenge lies in the slow dissemination of costly equipment. Despite the promising outcomes of new imaging modalities, their widespread adoption, like PET/magnetic resonance imaging (MRI), remains limited due to cost and accessibility issues [42]. While numerous researchers have explored promising markers, the limited sample sizes often hinder conclusive findings. For instance, in the case of HPV DNA research as a marker for circulating tumor cells, only six patients tested positive, with four of them later developing distant metastasis. As reported, such small sample sizes underscore the need for larger, more comprehensive research to draw meaningful conclusions about these markers [41]. With such a small number of patients studied, generalizing the findings becomes challenging. However, these preliminary results can serve as a valuable starting point for larger-scale clinical trials, providing a foundation for further investigation and potentially uncovering more robust associations between the marker and metastasis. Some of the techniques mentioned could prove useful intraoperatively, aiding surgeons in making clinical decisions regarding neck dissection during surgery. For instance, light reflectance spectroscopy has shown promise as a predictor of nodal metastasis, offering real-time information that can guide surgical interventions [41]. It's crucial to critically analyze the practical impact of such promising results on clinical practice. While techniques like light reflectance spectroscopy offer real-time guidance during surgery, it's important to consider their limitations. Since neck dissection already exposes patients to significant morbidity, the decision to perform it should be carefully weighed. While predictive models may not achieve 100% accuracy in detecting metastasis, factors like DOI remain important predictors for occult lymph node metastasis in OSCC [119–121]. Indeed, indicators like a DOI exceeding 2.5 mm or the presence of poorly differentiated OSCC are strong signals for considering elective neck dissection. These factors provide valuable guidance in determining the appropriate course of action to manage potential occult lymph node metastasis [122]. The adoption of this marker, as endorsed by NCCN guidelines, establishes it as the current gold standard in clinical practice [12]. Hence, we contend that any novel marker should undergo comparison with DOI or be integrated with it before being incorporated into any clinical protocol. For example, Méndez *et al*. [22] compared their model with tumor

size, which is a distinctly different parameter compared to DOI. Indeed, the relationship between tumor size and DOI can vary significantly. Hence, it's crucial to compare these parameters alongside DOI for a more comprehensive understanding of their predictive value. Several of the aforementioned markers have been tested in multiple studies, consistently showing efficacy as predictors of occult cervical lymph node metastasis. However, the studies reviewed here often involve small populations, limiting their statistical power. We advocate for large multicenter trials that focus on markers demonstrating effectiveness across multiple studies. Promising candidates for such validation trials include microRNAs, notably miR-205, as well as DSG3, pan-CK AE1/AE3, HPV-16, Activin-A, Cyclin D1, E-Cadherin and neural progenitor lineage (NPL).

4. Conclusions

The accurate diagnosis of regional lymph node metastasis is pivotal for guiding treatment decisions in head and neck cancer patients. Markers serving as diagnostic tools hold promise in averting overtreatment of negative necks and ensuring appropriate treatment for metastatic patients. To solidify their efficacy, future research efforts should involve larger populations. This validation process is particularly crucial for markers like miR-205, DSG3, pan-CK AE1/AE3, cytokeratins, HPV-16, Activin-A, Cyclin D1, E-Cadherin, and NPL, which have demonstrated effectiveness across multiple studies. Combining multiple markers into a scoring system could enhance their predictive accuracy.

Author Contributions

PGM: designed the research study, performed the research and wrote the manuscript. SP: performed the research and wrote the manuscript. LP: literature research. CF, GB and EC: manuscript revision, project supervision and literature research. CB: provided help and advice on molecular aspects, manuscript revision and supervision. AM: provided help and advice on head and neck cancer management; supervised manuscript. AM and MdV: provided help and advice on head and neck cancer management; supervised manuscript. PGM, EC: contributed to original draft. All authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity. All authors read and approved the final manuscript. All authors contributed to editorial changes in the manuscript.

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