Gene Expression Comparison Between the Primary Tumor and its Lymph Node Metastasis in Head and Neck Squamous Cell Carcinoma: A Pilot Study

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Abstract. Background/Aim: In metastatic head and neck squamous cell carcinoma (HNSCC) the metastatic tumor does not always keep the same gene expression profile as the parental tumor, which may influence the course of the disease. The aim of this study was to compare the expression of genes implicated in HNSCC carcinogenesis between the primary tumor and the corresponding lymph node metastasis. Materials and Methods: Eighteen HNSCC, their corresponding node metastases and non-neoplastic tissues were studied by RT-qPCR for the expression of EGFR, VEGF, claudin7, maspin, survivin and SCCA. The levels of expression were correlated with histological characteristics and patients' prognosis. Results: All genes except for survivin displayed different expression in node metastasis compared to the primary tumor. The expression of EGFR, survivin, maspin, and claudin7 in node metastasis and SCCA in the primary tumor affected the prognosis. SCCA expression is associated with the expression of claudin7 and maspin. P16-positive tumors expressed low levels of VEGF and SCCA, while keratinizing tumors over-expressed VEGF. Conclusion: Differential gene expression levels in node metastases compared to the primary tumor is linked to the prognosis of HNSCC patients. The histological/immunohisto-chemical characteristics of the tumor are associated with these genes expression changes.

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Head and neck squamous cell carcinoma (HNSCC) harbors several genetic alterations, mostly affecting the p53 pathway, which is compromised in 80% of HNSCCs. Human Papilloma Virus (HPV)-related tumors do not show p53 abnormalities, but the viral proteins E6 and E7 functionally inactivate p53 and retinoblastoma protein (1). Beyond genetic alterations, HNSCCs have also been classified depending on their gene-expression profile into: i) SCCs characterized by hypoxia and over-expression of epithelial markers, ii) SCCs expressing immune response genes and showing epithelial-mesenchymal transition and iii) SCCs exhibiting a higher proliferation ratio and an activated detoxification pathway (2).

Furthermore, comparison of gene expression profiles between primary tumors with no metastatic potential and isolated lymph node metastases in vitro has shown important differences related to the tumor-stromal cell interactions rather than to the sole function of well-known oncogenes, tumor-suppressor genes, or genes encoding transcription factors/cell cycle regulators (3). This reflects the importance of differential gene expression profiling in the metastatic potential of tumors.

Interestingly, in carcinomas that have already metastasized, it is not known whether the metastatic tumor has kept the same pool of genes under active expression as the parental tumor or how an altered gene expression profile may influence the course of the disease. Thus, the aim of this study was to compare the expression of genes implicated in different aspects of HNSCC carcinogenesis between the primary tumor and the corresponding lymph node metastases. These include the epidermal growth factor receptor (EGFR), a well-known receptor tyrosine kinase (4), the vascular endothelial growth factor (VEGF) implicated in lymphangiogenesis and angiogenesis (5), claudin7 (6) and maspin, (7) two major tumor cell-environment interaction factors, survivin (8), an anti-apoptotic regulator, and squamous cell carcinoma antigen (SCCA) (9), a serological marker of squamous cell carcinomas.
Materials and Methods

Eligibility criteria. The eligibility criteria included: i) primary head and neck cancer, ii) pathological diagnosis of SCC before surgery, iii) presence of lymph node metastasis, iv) no distant metastasis, v) previously untreated tumors, vi) treatment with curative intent and complete remission following primary treatment, vii) a minimal follow-up of 24 months or until death.

Study design. Thirty patients were included in this prospective study; all samples were acquired with patients’ consent. The local ethics committee of the University Hospital of St-Etienne approved the study. Samples of the primary tumor (T), of the neighboring non-neoplastic tissue (NNT), and of the corresponding lymph node metastasis (N) were obtained during and at the site of operation, in order to optimize the RNA content. The samples were separated into two parts: i) one for RT-qPCR, which was snap-frozen in liquid nitrogen and was stored at −80°C until RNA extraction and ii) one submitted for frozen sectioning at the Department of Pathology, to assure the quality of the tissue. Cases were eliminated from further investigation when: i) the primary or metastatic tumor samples did not contain adequate cancerous tissue, ii) the tissue included large areas of necrosis or inflammatory cells compromising the tumor cell content, which should be >80%, and iii) the non-neoplastic tissue was inadequately sampled or showed areas of dysplasia. Based on these parameters, eighteen samples were eligible to proceed to RT-qPCR.

Histopathological evaluation. Tumor characteristics were examined in all available histological sections of the formalin-fixed paraffin-embedded tissue. Squamous cell carcinomas were categorized into keratinizing (K) and non-keratinizing (NK) (10-12). This categorization is of clinical importance as the second type is the one usually associated with HPV infection (13). The Brandwein-Gensler histological risk assessment model was used to semi-quantify the aggressiveness of the tumor, and particularly, the lymphocytic host response (LHR) and the pattern of invasion (POI) (10-12). Last, an abundant tumoral stroma reaction, defined as tumors having equal or more than 50% of fibrous stroma (14) and the presence of large areas of necrosis were also recorded.

Quantitative analysis (qPCR). Total RNA was extracted using RNeasy mini kit (Qiagen, Courtaboeuf, France) according to the manufacturer’s instructions. The quality and quantity of the extracted RNA were evaluated using spectrophotometric analysis with Nanodrop (ThermoFisher Scientific, Waltham MA). Then, Agarose gel electrophoresis (2%) was performed in order to identify ribosomal RNA that indicates a successful preparation.

Complementary DNA (cDNA) was synthesized from total RNA. The RNA was further treated with DNASE I (Invitrogen, ThermoFisher Scientific, Carlsbad, CA) to ensure that all genomic DNA was removed. First-strand cDNA synthesis was carried out with 1 μg of RNA, 1 μL of oligo(dT) primers (0.5 μg/μL), 1 μL of a solution with all four deoxyribonucleoside triphosphates (each at 10 mM), and 10X Superscript III reverse transcriptase (Invitrogen).

Quantitative real-time PCR was performed using the SYBR Green chemistry in a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with 100 ng of cDNA. The primers used to perform the real time PCR are shown in Table 1. Calculations were made with the use of the comparative Ct (2−ΔΔCT) method. RPLPO was used as an internal control gene to normalize the PCRs for the amount of RNA added to the reverse transcription reactions. Reactions were performed in duplicates for each sample and primer set. Two studies were done. The comparison was first performed between non-neoplastic tissue and primary tumor (T/NNT) or node metastasis (N/NNT) and second between lymph node metastasis and primary tumor (N/T).

For the first study, non-neoplastic tissue was used as a calibrator for making PCRs from distinct comparable runs. ΔΔCT represents the difference between the mean ΔCT value of a primary tumor or the node metastasis and the mean ΔCT of the calibrator, both calculated after the same PCR run. ΔCT is the difference between the threshold cycle (Ct) of the target gene (EGFR, VEGF, Claudin7, Maspin, Survivin or SSX) and the Ct of the endogenous reference gene RPLPO of the same sample. For the second study primary tumor (T) became the calibrator and was compared to lymph node metastasis (N).

For the analyses we considered Ct values<35 as acceptable for further interpretation. Then, values of 2−ΔΔCT between 0.5 and 2 were considered as of no alteration, 2−ΔΔCT<0.5 as under-expressed and 2−ΔΔCT>2 as over-expressed.

Immunohistochemical study. Four-μm thick full sections were used for immunohistochemistry performed using an automated staining system (Leica Biosystems, Newcastle Upon Tyne, UK). Positive immunoreactions were visualized using 3,3'-diaminobenzidine (Dako REAL™ DAB+ Chromogen, Glostrup, Denmark) as the chromogenic substrate. Mouse monoclonal antibody against p53 (1:50, Dako, Clone DO-7, Glostrup, Denmark) and mouse monoclonal antibody against p16INK4a (1:50, LabVision, Clone 16P07, Fremd CA, USA) were used as primary antibodies.

Regarding p16, a surrogate marker for HPV infection (10-12, 15) cases were classified in a binary manner as either positive (nuclear/cytoplasmic diffuse staining of >75% of cells, regarding intensity) or negative (absence of expression or partial staining <75% of cells) (11, 12).

P53 staining was nuclear. The percentage of positive cells was recorded. Three patterns of p53 expression were recognized: i) over-expression (strong nuclear staining by at least 50% of the cells), ii) negative when there was a complete absence of staining in the tumor, with normal expression in neighboring normal tissue serving as internal positive control, and iii) normal p53 expression when a weak expression of few tumor cells (weak expression by no more than 49%) was found (11, 12).

Values comparison. Each gene’s status of expression (no change, under-expression or over-expression) of the primary tumor (T/NNT) and that of metastasis (N/NNT and N/T) was compared to all histopathological and immunohistochemical values, the overall survival (OS) and the progression-free survival (PFS), as well as the status of the rest of the genes.

Statistical analysis. Data were analyzed using the StatView software (Abacus Concepts, Berkley Ca, USA). A relationship between two groups was investigated using Fisher’s exact test for categorical data. Survival probability was estimated using Kaplan–Meier analysis. For all analyses, statistical significance was indicated at a p-value of ≤0.05.
Results

Characteristics of the study group. Patients’ characteristics are shown in Table II. Most patients were male with a median age of 53 years (range 40-74). Median follow up was 31 months. Twelve SCCs were keratinizing, 11 were negative for p16 (all of them were keratinizing) and 13 (of which the 12 were keratinizing) showed abnormal (either over-expression or completely negative) p53 expression. Ten SCCs had abundant stroma reaction and 11 showed an aggressive pattern of invasion.

Gene expression profiles. Results regarding gene expression levels are shown in Table III. Except for Survivin, all other genes showed differential expression at the site of metastasis when compared to the parental tumor.

Correlation of gene expression with patient prognosis. Median overall survival (OS) and progression-free survival (PFS) were 31 and 27 months, respectively (Figure 1A and B). The association of gene expression profiles with overall and progression-free survival is presented in Tables IV and V, respectively.

Correlation of gene expression profiles with histopathological/immunohistochemical features. All three types of evaluation (T/NNT, N/NNT, and N/T) were analyzed in comparison with the available histological and immunohistochemical characteristics. P16 positivity was inversely correlated with VEGF expression in the primary tumor (p=0.02), while at the same time, p16-positive tumors showed under-expression of SCCA (p=0.05). Most keratinizing tumors over-expressed VEGF (p=0.06) and EGFR (p=0.02). Primary tumors with a more aggressive pattern of invasion showed a trend in over-expressing Maspin in the primary tumor (p=0.09), but also in metastasis (p=0.05). Tumors with an abundant stroma reaction tended to under-express claudin7 (p=0.06). Laryngeal tumors over-expressed EGFR (p=0.006) and tended to over-express Survivin (p=0.06) in comparison to hypopharyngeal and oropharyngeal tumors. All other parameters studied did not reveal any significant correlations.

Comparison between various genes’ expression. In all three types of evaluation (T/NNT, N/NNT, and N/T), all gene expression levels were correlated with the expression of the rest of the genes. Primary tumors under-expressing SCCA (tumor versus normal tissue) had an unchanged claudin7 (p=0.03). Furthermore, in primary tumors, maspin and EGFR both tended to be overexpressed (p=0.08), while
when Maspin was over-expressed, claudin7 tended to be unchanged ($p=0.08$).

In lymph node metastasis claudin7 and SCCA both appeared under-expressed ($p=0.03$). Maspin is positively correlated with SCCA ($p=0.005$) and while Survivin was unchanged, claudin7 was under-expressed ($p=0.01$). Furthermore, claudin7 tended to be inversely correlated with VEGF ($p=0.06$), while Survivin tended ($p=0.06$) to show a positive correlation with EGFR.

**Discussion**

Claudins are a family of proteins that regulate tight junctions in epithelial cells (6). They are differentially expressed in different tissues and are down-regulated in several cancers, leading to loss of tight junction function (6). Claudin-7 expression is lost in esophageal SCC leading to reduced E-cadherin expression and increased invasiveness (6). In the present study, claudin7 is under-expressed in 29.4% of the primary tumors, mostly in those with an abundant stroma reaction; however, it is under-expressed in most of the corresponding node metastases. These data show that the primary tumor is heterogeneous in its content, at least in cells with a metastatic potential. It may be that it is the clone under-expressing claudin7 that finally reaches the distant focus. This is based on the fact that despite the lack of correlations with the level of claudin7 expression in the primary tumor, metastases under-expressing Claudin7 showed a significantly worse survival compared to the parental tumor. This suggests that the loss of Claudin7 continues to offer an advantage to the tumor even when metastatic. This is further supported by the fact that metastases with high levels of claudin7 had lower levels of VEGF and survivin, implying that these tumors may need fewer pro-tumoral factors.

Similar are the findings for maspin, as almost half of the primary tumors over-expressed it, while it was under-expressed in most of the node metastases, suggesting that its suppression is important for metastasis. Maspin is a protein implicated in the regulation of actin cytoskeleton leading to a less invasive phenotype and a lower metastatic spread. In esophageal SCC, lower immunoreactivity for maspin has been associated with worse prognosis (7). However, the effect on prognosis differs depending on its localization, with the nuclear Maspin acting as a suppressor of metastasis (16),

**Table III. Comparison of gene expression between metastasis (N) and the corresponding primary tumor (T).**

| Gene       | Primary tumor vs. normal | Node metastasis vs. normal | Node metastasis vs. primary tissue |
|------------|--------------------------|----------------------------|-----------------------------------|
| EGFR       | No change: 6, 5, 6       | Under-expression: 3, 3, 5  | Over-expression: 9, 10, 7         |
| VEGF       | No change: 4, 0, 6       | Under-expression: 4, 6, 4  | Over-expression: 10, 12, 8        |
| Survivin   | No change: 6, 6, 12      | Under-expression: 1, 1, 3  | Over-expression: 11, 11, 3        |
| Maspin     | No change: 6, 0, 5       | Under-expression: 5, 11, 9 | Over-expression: 7, 7, 4          |
| SCCA       | No change: 4, 2, 3       | Under-expression: 10, 13, 12 | Over-expression: 4, 3, 3          |
| Claudin7   | No change: 10, 2, 4      | Under-expression: 5, 11, 14 | Over-expression: 3, 5, 2          |

EGFR: Epidermal growth factor receptor; N: lymph node metastasis; NNT: non neoplastic tissue; SCCA: squamous cell carcinoma antigen; T: primary tumor; VEGF: vascular endothelial growth factor.

![Figure 1. Overall survival (A) and progression-free survival (PFS) (B) curves.](image-url)
while cytoplasmic expression is associated with more aggressive tumors (17). This is in accordance with our results, as we found that metastases with under-expressing *maspin* had better overall survival. This is also compatible with the more aggressive pattern of invasion observed in tumors over-expressing *Maspin* (17).

Survivin is an inhibitor of apoptosis, leads to DNA repair through PARP (Poly-ADP-ribose polymerase) through binding and inhibition of caspase 9 (8). Normally, it is expressed only during fetal life and it is no longer present in adult mature tissues, with few exceptions, such as melanocytes or colonic mucosa (8). Survivin is over-expressed in various types of cancer and is associated with worse prognosis (8). Here we found that survivin is over-expressed in the majority of primary HNSCC and their corresponding metastases, suggesting that it continues to offer a survival potential even in advanced tumors.

Squamous cell carcinoma antigen (SCCA) is a serine protease inhibitor, a serpin, found to be over-expressed in SCCHN as well as in cervical cancer, where it was initially described (9). SCCA levels are increased in the blood of these patients and decrease after therapy, however, the exact role of this molecule in SCC is unclear. It has been mostly investigated in cell lines; in tissue samples from oral SCCs, where it has been shown to be over-expressed, without having an impact on prognosis as a unit, but a high SCCA2/SCCA1 ratio (the two isoforms of SCCA) have been associated with disease recurrence (9). In the current study, SCCA is under-expressed in most of the metastases, suggesting that it is probably no longer used by the tumor for metastasis. This is further supported by the fact that its levels in the metastatic tumor do not affect prognosis. However, its over-expression in the primary tumor is associated with a better overall survival. This implies an important role of SCCA in the primary tumor that should be further investigated. We also show here, for the first time, a strong association between SCCA with *claudin-7* and *Maspin*, implying a role of cell adhesiveness on SCCA expression. Furthermore, we find that it is under-expressed in p16-positive tumors, thus, its function should be investigated mostly in non HPV-related tumors.

EGFR, a member of the well-known human epidermal growth factor receptor (HER) family of receptor tyrosine kinases, has an essential role in proliferation, differentiation, anti-apoptosis, angiogenesis and metastasis of various malignancies (4). EGFR over-expression in HNSCC is associated with worse prognosis, but at the same time its overexpression is associated with a better response to irradiation.

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**Table IV. Association of gene expression with survival in months (log-rank test).**

| Gene   | Primary tumor (T/NNT) | p-Value | Node Metastasis N/NNT | p-Value | Node Metastasis N/T | p-Value |
|--------|-----------------------|---------|-----------------------|---------|---------------------|---------|
| EGFR   | No change             | 11      | Not reached           | 11      | Not reached         | 11      |
|        | Under-expression      | 11      | 0.4                   | 4       | 0.008               | 5       | 0.06 |
|        | Over-expression       | 48      | Not reached           | 48      | 0.3                 | 9       | 0.8  |
| VEGF   | No change             | 23      | 0.09                  | 4       | 0.09                | 3       | 0.9  |
|        | Under-expression      | 4       | 0.09                  | 4       | 0.1                 | 4       | 0.009 |
| Survivin | No change           | 11      | Not reached           | 11      | Not reached         | 11      |
|        | Under-expression      | 4       | 0.4                   | 4       | 0.4                 | 4       | 0.004 |
|        | Over-expression       | 11      | Not reached           | 11      | 0.03                | 35      | 0.03 |
|        | Over-expression       | 4       | 0.4                   | 4       | 0.4                 | 4       | 0.004 |
| Maspin | No change             | Not reached | -                 | 5       | 0.004               | 5       |
|        | Under-expression      | 11      | 0.4                   | 11      | 0.03                | 35      | 0.03 |
|        | Over-expression       | 4       | 0.4                   | 4       | 0.4                 | 4       | 0.004 |
| SCCA   | No change             | Not reached | -                 | 11      | 0.1                 | 11      |
|        | Under-expression      | 11      | 0.3                   | 11      | 0.1                 | 11      |
|        | Over-expression       | 4       | 0.3                   | 4       | 0.3                 | 4       | 0.004 |
| Claudin7 | No change          | Not reached | -                 | 4       | 0.009               | 4       |
|        | Under-expression      | 11      | 0.1                   | 11      | 0.1                 | 11      |
|        | Over-expression       | 4       | 0.1                   | 4       | 0.1                 | 4       | 0.009 |

EGFR: Epidermal growth factor receptor; N: lymph node metastasis; NNT: non-neoplastic tissue; SCCA: squamous cell carcinoma antigen; T: primary tumor; VEGF: vascular endothelial growth factor.
Here we show that antibody panitumumab possibly more effective in HPV-negative and keratinizing tumors, and to our knowledge this is the first time that an association between VEGF with the non-HPV related tumors is reported in the tissue. This is in accordance with the recent finding of augmented plasma VEGF levels in p16-negative tumors (5). Despite all these associations, there was no statistically significant difference with respect to the VEGF status regarding prognosis.

To conclude, we showed for the first time that lymph node metastasis has a different expression profile compared to the parental tumor, and that this has a significant prognostic influence.

**Conflicts of Interest**

The Authors have no conflicts of interest to disclose

**Authors’ Contributions**

JMD and MP received the idea, MLS performed the experiments, GK, MG, MF and JMP collected and analyzed data and interpreted results, GK wrote the manuscript, all authors corrected and approved the final draft.

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References

1 Leemans CR, Braukhuis BJ and Braunkenhoff RH: The molecular biology of head and neck cancer. Nat Rev Cancer 11: 9-22, 2011. PMID: 2160525. DOI: 10.1038/nrc2982

2 Keck MK, Zuo Z, Khattri A, Stricker TP, Brown C, Imanogu M, Rieke D, Endhardt K, Fang P, Bragelman J, DeBoer R, El Diniali M, Aktolga S, Lei Z, Tan P, Rozen SG, Salgla R, Weichselbaum RR, Lingen MW, Story MD, Ang KK, Cohen EE, White KP, Vokes EE and Seiwert TY: Integrative analysis of Head and Neck Cancer identifies two biologically distinct HPV and three non-HPV subtypes. Clin Cancer Res 21: 870-882, 2014. PMID: 25492084. DOI: 10.1158/1078-0432.CCR-14-2481

3 Chen Z, Zhang K, Zhang X, Yuan XH, Yuan Z, Jin L and Xiong M: Comparison of gene expression between metastatic derivatives and their poorly metastatic parental cells implicates crucial tumor-environment interaction in metastasis of head and neck squamous cell carcinoma. Clin Exp Metastasis 20: 335-342, 2003. PMID: 12856721.

4 Riesterer O, Milas L and Ang KK: Use of molecular biomarkers for predicting the response to radiotherapy with or without chemotherapy. J Clin Oncol 25: 4075-4083, 2007. PMID: 17827456.

5 Baruah P, Lee M, Wilson POG, Odutoye T, Williamson P, Hyde N, Kaski JC and Dumitriu IE: Impact of p16 status on pro- and anti-angiogenesis factors in head and neck cancers. Br J Cancer 113: 653-659, 2015. PMID: 26171937. DOI: 10.1038/bjc.2015.251

6 Lioni M, Brafford P, Andl C, Rustgi A, El-Deiry W, Herlyn M and Smalley KSM: Dysregulation of claudin-7 leads to loss of E-cadherin expression and the increased invasion of esophageal squamous cell carcinoma cells. Am J Pathol 170: 709-721, 2007. PMID: 17255337. DOI: 10.2353/ajpath.2007.060343

7 Wang Y, Sheng S, Zhang J, Dziczik SH, Li S, Fang F, Wu N, Zheng Q and Yang Y: Elevated maspin expression is associated with better overall survival in esophageal squamous cell carcinoma (ESCC). PLoS One 8: e63581, 2013. PMID: 23717449. DOI: 10.1371/journal.pone.0063581

8 Malhotra U, Zaidi AH, Kosovec JE, Kasi PM, Komatsu Y, Rotoloni CL, Davison JM, R C, Irvin, Hoppo T, Nason KS, Kelly L a, Gibson MK and Jobe BA: Prognostic value and targeted inhibition of survivin expression in esophageal adenocarcinoma and cancer-adjacent squamous epithelium. PLoS One 8: e78343, 2013. PMID: 24223792. DOI: 10.1371/journal.pone.0078343

9 Deng Z, Hasegawa M, Yamashita Y, Matayoshi S, Kiyuna A, Agena S, Uehara T, Maeda H and Suzuki M: Prognostic value of human papillomavirus and squamous cell carcinoma antigen in head and neck squamous cell carcinoma. Cancer Sci 103: 2127-2134, 2012. PMID: 22937809. DOI: 10.1111/cas.12009

10 Karpathiou G, Casteillo F, Giroult J-B, Forest F, Fournel P, Monaya A, Froudarakis M, Demollard JM, Prades JM and Peoc’h M: Prognostic impact of immune microenvironment in laryngeal and pharyngeal squamous cell carcinoma: Immune cell subtypes, immuno-suppressive pathways and clinicopathologic characteristics. Oncotarget 8: 19310-19322, 2017. PMID: 28038471. DOI: 10.18632/oncotarget.14242

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