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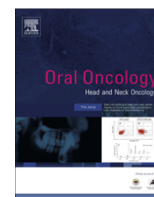

The gene expression profile of inflammatory, hypoxic and metabolic genes predicts the metastatic spread of human head and neck squamous cell carcinoma

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SUMMARY

Objectives: To assess the prognostic value of the expression profile of the main genes implicated in hypoxia, glucose and lactate metabolism, inflammation, angiogenesis and extracellular matrix interactions for the metastatic spread of head and neck squamous cell carcinoma.
Patients and methods: Using a high-throughput qRT-PCR, we performed an unsupervised clustering analysis based on the expression of 42 genes for 61 patients. Usual prognostic factors and clustering analysis results were related to metastasis free survival.
Results: With a median follow-up of 48 months, 19 patients died from a metastatic evolution of their head and neck squamous cell carcinoma and one from a local recurrence. The unsupervised clustering analysis distinguished two groups of genes that were related to metastatic evolution. A capsular rupture ($p = 0.005$) and the "cluster CXCL12 low" ($p = 0.002$) were found to be independent prognostic factors for metastasis free survival. Using a Linear Predictive Score methodology, we established a 9-gene model (VHL, PTGER4, HK1, SLC16A4, DLL4, CXCL12, CXCR4, PTGER3 and CA9) that was capable of classifying the samples into the 2 clusters with 90% accuracy.
Conclusion: In this cohort, our clustering analysis underlined the independent prognostic value of the expression of a panel of genes involved in hypoxia and tumor environment. It allowed us to define a 9-gene model which can be applied routinely to classify newly diagnosed head and neck squamous cell carcinoma. If confirmed by an independent prospective study, this approach may help future clinical management of these aggressive tumors.

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Introduction

With an evaluated incidence of 550 000 cases and 300 000 deaths world-wide in 2008, head and neck squamous cell carcinoma (HNSCC) remains a common disease [1]. Locally advanced HNSCCs are frequent at diagnosis and are associated with a high risk of metastatic spread. Loco-regional control of these tumors is frequently achieved by a combination of aggressive surgery and radiation therapy, with concomitant chemotherapy in cases with a high risk of recurrence. Nevertheless, metastatic dissemination remains difficult to predict and occurs in at least 20% of cases with a great impact on prognosis [2,3]. Today, treatment decisions are based on clinical and histological risk factors (TNM staging, node involvement, extracapsular spread, and positive margins after surgery) (NCCN guidelines Version 1.2012) that do not, however, reflect the true biological heterogeneity of these tumors and incompletely predict survival. Thus, beyond clinical staging, the biological assessment of HNSCC is the next step toward prognosis evaluation. For example, human papilloma virus (HPV) infection

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has been identified as an independent risk factor for HNSCC [4] and is associated with increased survival, even though, to date, there is no specific treatment for HNSCC that is related to the HPV status of the tumor. In the upcoming era of personalized medicine, many other biological factors may be associated with a greater resistance to treatment or higher risk of dissemination.

Intratumoral hypoxia and necrosis are frequently observed in locally advanced cases of HNSCC. Hypoxia has been reported to increase radiation therapy [5] and chemotherapy resistance [6], which worsens the prognoses of these patients [7]. In this context, we have recently reported the favorable prognostic value of a high intratumoral level of CXCL12 assessed by a quantitative RT-PCR assay (qRT-PCR) [8]. This pro-inflammatory cytokine and its receptor, CXCR4, are implicated in the metastatic spread and cell migration in most of malignant diseases [9–13], including HNSCCs [14,15], and both are up-regulated by the hypoxia-induced transcription factor HIF1A [16]. Nevertheless, many other genes induced by hypoxia are related to the prognosis or metastatic dissemination of this disease, even if no consensual signature has yet been defined.

Proteins associated with the lactate and glucose metabolism (like SLC16A4 or SLC2A1) were shown to co-localize with hypoxic areas of HNSCC biopsies [17], and elevated tumor lactate concentrations were related to an increased risk of metastatic spread in HNSCC [18]. Proteins associated with the extracellular matrix such as metalloproteinase (MMP2,7,9 [19,20]) or PLAU-SERPINE-1 [21,22] have also been reported to correlate with prognostic in HNSCC. Angiogenesis is also induced by HIF1A [23] and may contribute to metastatic spread of HNSCC. Finally, many cross-talks exist between those pathways, and defining the best set of markers implies to assess the expression of many genes in the same time. Even if some studies have analyzed the gene expression profile between tumor and metastatic nodes at diagnosis, very few have correlated tumor biology to metastatic evolution. Furthermore, analysis by RNA-array are associated with pitfalls such as the choice of the better marker among the many probes used for each gene; or the difficulty of transposition to daily practice.

Thus, the aim of this study was to assess the prognostic value of the expression profile of a literature-based panel of genes implicated in hypoxia, glucose and lactate metabolism, inflammation, angiogenesis and extracellular matrix interactions for the metastatic spread of HNSCC.

Patients and methods

Patients

All patients treated for HNSCC between January 2004 and December 2010 at the Centre Henri Becquerel and the Centre Hospitalo-Universitaire of Rouen were screened for inclusion in this study. This study was approved by our institutional scientific committee and is in accordance with the Declaration of Helsinki. A written approval for tissue preservation and molecular analysis was obtained for every patient at the time of diagnosis.

At the initial diagnosis, patients received a clinical examination, fibroscopy of the upper aerodigestive tract and a computed tomographic (CT) scan of the neck and chest. The clinical or pathological stage was defined according to the TNM classification of malignant tumors [24]. Only the patients with initial curative treatment were included in this study.

Patients who had metastatic disease, those who had been treated for another cancer within the 3 years preceding the diagnosis of HNSCC and those who did not complete the planned treatment were excluded. Treatment modalities were chosen by the practitioner according to the recommendations of our head and neck tumor board.

Histological features

Diagnosis of HNSCC was based on the initial tumor biopsy. Histological analysis of the tumor was completed after surgical excision and included the pathological size of the tumor, the state of the margins (invaded or free), the histological grade, the presence of vascular or lymphatic involvement, the presence of invaded lymph-nodes and the presence (or absence) of an extracapsular spread.

Immunohistochemistry for p16 analysis and HPV status

A tissue micro-array (TMA) was performed for 59 of the 61 paraffin-embedded tumor specimens. The p16 expression levels of the tumors were evaluated with the CINtec_ Histology kit (MTM Laboratories, Germany). Positive p16 expression was defined as strong and diffuse nuclear and cytoplasmic staining in 80% or more of the tumor cells. In the cases of positive p16 expression, an in situ hybridization was performed to confirm the HPV status using a Benchmark XT system, with the Inform HPV III Family 16 probe (B) and Inform HPV II Family 6 probes (Ventana Medical Systems, USA).

Gene selection

The selection of the 42 genes of interest has been based on an analysis of the literature for the main genes implicated in hypoxia, glucose and lactate metabolism, inflammation, angiogenesis and extracellular matrix interactions for the metastatic spread of HNSCC (Supplementary Table I).

Gene expression profiling

Frozen tissue was obtained from a tumor fragment of the surgical excision specimen of the tumor (not from metastatic nodes) before any exposure to radiation therapy or chemotherapy. Samples were collected prospectively and stored at −80 °C. Molecular analysis was performed at the same time for every sample. Snap-frozen tumor samples were sectioned for histological analysis: at least 50% of each sample was required to be composed of non-necrotic SCC. RNA extraction was performed using Tripure Isolation Reagent (Roche, USA) and concentration was adjusted to between 300 and 500 ng/μL and then stored at −80 °C. The RNA quality was assessed by an Agilent 2100 bio-analyzer. Low quality samples were excluded from subsequent analyses after an independent evaluation (MB) based on their electropherogram and RNA Integrity Number (RIN).

Two micrograms of total RNA was reverse transcribed into cDNA using random primers and the M-MLV reverse transcriptase kit (Invitrogen, USA). RNA copy numbers for the 42 genes of interest and 3 housekeeping genes (GAPDH, ACTB and B2 M) were simultaneously quantified in duplicate by real-time quantitative PCR using the 1536 Light Cycler hardware and software (Roche). Gene probes were selected from the Roche Universal Probe Library and details about the qPCR protocol can be found on the dedicated site of the manufacturer (www.roche-applied-science.com).

Data processing

The reproducibility of the data was assessed by computing the average inter-replicate distance for each gene to detect and discard unreliable gene expression values. The one dimensional partitioning around medoids (PAM) algorithm, as implemented in the R

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