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Original Research Article

# Plasma proteins as prognostic biomarkers in radiotherapy treated head and neck cancer patients

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ABSTRACT

*Background:* Blood-based protein biomarkers can be a useful tool as pre-treatment prognostic markers, as they can reflect both variations in the tumor microenvironment and the host immune response. We investigated the influence of a panel of plasma proteins for the development of any failure defined as recurrent disease in the T-, N-, or M-site in HNSCC.
*Methods:* We used a multiplex bead-based approach to analyze 19 proteins in 86 HNSCC patients and 15 healthy controls. We evaluated the associations between the biomarkers, loco-regional failure, failure in the T-, N-, or M-site, overall survival (OS), p16 status, and hypoxia.
*Results:* In 41 p16 positive oropharynx cancer patients we identified a profile of biomarkers consisting of upregulation of IL-2, IL-4, IL-6, IL-8, eotaxin, GRO-a, and VEGF and downregulation of VEGFR-1 and VEGFR-2 with a significantly reduced risk of failure ($p < 0.01$). None of the individual proteins were associated with outcome.
*Conclusion:* The identified plasma profile potentially reflects an activated immune response in a subgroup of the p16 positive patients.


## Introduction

Traditionally, the predominant etiological factors for head and neck squamous cell carcinoma (HNSCC) have been tobacco smoking and alcohol consumption [1]. In the past 20 years, it has become increasingly clear that there is an etiological linkage to human papilloma virus (HPV) infections, and a subgroup of HPV-positive HNSCC has been established [2]. Overall, HPV-positive HNSCC constitute an entity of patients with a different molecular biology [3], a different clinical profile, and a more favorable prognosis [4].

Besides HPV status, a number of other prognostic factors are relevant for HNSCC, including tumor stage, nodal stage, a history of tobacco smoking [5], as well as hypoxia [6]. Furthermore, biopsy-based biologically distinct subtypes that are independent of HPV status have been introduced, and the subtype with the most advantageous prognosis shows a prominent immune and mesenchymal phenotype [7].

Blood-based biomarkers can be useful as pre-treatment prognostic markers, as they can reflect variations in tumor microenvironment and host immune response and can complement biopsy-based biomarkers that evaluate tumor cells directly. Although several studies have investigated the prognostic value of various circulating proteins in HNSCC [8–16], there is no consensus as to which are the most promising prognostic biomarkers, or whether biomarkers should be analyzed individually or combined into profiles. We hypothesize that a panel of circulating endogenous markers in HNSCC is associated with outcome after primary radiotherapy and that these markers are influenced by HPV-status and tumor hypoxia.

In this study, we aimed to investigate the influence of a panel of proteins in the blood for the development of failure defined as recurrent disease in the T-, N-, or M-site in HNSCC. We used a multiplex bead-based approach to analyze 19 previously described proteins (cytokines, chemokines, angiogenic factors, and receptors) [8–16]. We evaluated the associations between the circulating

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biomarkers, HPV status, smoking history, and hypoxia evaluated by a 15-gene hypoxia profile [17].

## Materials and methods

### *Patients and samples*

The prospectively collected study-population comprised of 86 previously untreated HNSCC patients treated at the Department of Oncology at Aarhus University Hospital, Denmark, between July 2005 and September 2011 (treated according to the DAHANCA 18 protocol) [18,19]. Additionally, 15 healthy controls from the Danish blood bank at Aarhus University Hospital were enrolled between March 2012 and November 2014. The patients received primary radiotherapy (RT) according to the DAHANCA guidelines (http://www.dahanca.dk). The prescribed dose was 66–68 Gy, two Gy/fraction, six fractions per week. All patients were prescribed orally administered hypoxic radiosensitizer, with Nimorazole 1200 mg/m$^2$, 90 min before each fraction of RT. Patients with locally advanced disease were given Cisplatin intravenously concomitant with RT once a week for a maximum of six cycles (40 mg/m$^2$, maximum dose 70 mg). The patient, tumor and control characteristics are presented in Table 1.

### *Blood sample processing and multiplex analysis of circulating proteins*

Plasma samples were obtained by venipuncture and taken in lithium heparin vials, kept on ice until separation within three hours of collection, and stored at −80 °C until further processing. Procedures on sample processing and analyses have previously been described in detail [20]. Briefly, multiplex analysis was performed of 19 proteins (Table 2), in three different pre-mixed bead-based antibody assays (Bio-Plex Pro™ human Reagent Kit, Bio-Rad), according to the manufacturer's protocol using the Luminex 100 (BIO-PLEX 200 SYSTEM) and Bio-Plex manager software (version 6.1). For measured values out of range (OOR) the values above the upper limit of quantification were replaced by the highest recorded value of the standard curve. For values below the lower limit of quantification the OOR values were replaced by the lowest recorded value of the standard curve divided by two. For two patients the amount of available plasma was insufficient to perform the analysis in 14 of the investigated proteins.

**Table 1**
Patient, tumor and control characteristics.

| | All patients ($n$ = 86) | | Blood bank controls ($n$ = 15) | |
|---|---|---|---|---|
| | $n$ | (%) | $n$ | (%) |
| Age (years) | | | | |
| Median | 58 | | 55 | |
| Range | (34–77) | | (51–63) | |
| ⩽60 years | 51 | 59 | 13 | 87 |
| >60 years | 35 | 41 | 2 | 13 |
| Sex | | | | |
| Female | 16 | 19 | 7 | 47 |
| Male | 70 | 81 | 8 | 53 |
| Smoking status | | | | |
| >10 pack years | 56 | 65 | | |
| ⩽10 pack years | 30 | 35 | | |
| Tumor site | | | | |
| Sinonasal carcinoma | 3 | 3 | | |
| Rhinopharynx | 5 | 6 | | |
| Oral cavity | 5 | 6 | | |
| Oropharynx | 56 | 65 | | |
| Hypopharynx | 5 | 6 | | |
| Supraglottic larynx | 8 | 9 | | |
| Glottis | 2 | 2 | | |
| Subglottis | 2 | 2 | | |
| Tumor stage | | | | |
| T1-2 | 58 | 67 | | |
| T3-4 | 28 | 33 | | |
| Nodal stage | | | | |
| N0 | 8 | 9 | | |
| N1-3 | 78 | 91 | | |
| Disease stage | | | | |
| I-II | 4 | 5 | | |
| III-VI | 81 | 94 | | |
| Unknown | 1 | 1 | | |
| HPV/p16 status | | | | |
| Positive and oropharynx | 41 | 48 | | |
| Negative or non-oropharynx | 42 | 49 | | |
| Unknown | 3 | 3 | | |
| Hypoxia by gene classifier | | | | |
| More hypoxic | 25 | 29 | | |
| Less hypoxic | 57 | 66 | | |
| Unknown | 4 | 5 | | |
| Chemotherapy | | | | |
| Yes | 79 | 92 | | |
| No | 7 | 8 | | |

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