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Comparative proteome analysis to explore p53 pathway disruption in head and neck carcinogenesis

Tieneke B.M. Schaaij-Visser[a,b,c], *Ruud H. Brakenhoff*[c], *Jeroen W.A. Jansen*[a,b], *Martina C. O'Flaherty*[a,b], *Serge J. Smeets*[c], *Albert J.R. Heck*[a,b], *Monique Slijper*[a,b,*]

[a]*Biomolecular Mass Spectrometry and Proteomics Group, Bijvoet Center for Biomolecular Research and Utrecht Institute for Pharmaceutical Sciences, Utrecht University, The Netherlands*

[b]*Netherlands Proteomics Centre, The Netherlands*

[c]*Tumorbiology Section, Dept. Otolaryngology/Head-Neck Surgery, VU University Medical Center, Amsterdam, The Netherlands*

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ABSTRACT

The 5-year-survival rate of head and neck squamous cell carcinoma (HNSCC) has been only moderately improved over the last few decades. HNSCC develops in precursor fields of genetically altered mucosal cells, typically characterized by p53 pathway disruption, that mostly do not give any clinical symptoms. Patients present therefore often with invasive carcinomas in an advanced stage. After tumor resection, part of these fields frequently stays behind unnoticed, causing secondary tumors. Identification of these precursor fields would allow screening and early detection of both primary and secondary tumors.

Our aim was to identify differential proteins related to p53 dysfunction. These proteins may serve as valuable biomarkers that can predict the presence of a precursor field. We used a squamous cell model for p53 inactivation, which was analyzed by 2D-DIGE and LC-MS/MS. This approach enabled us to identify a set of 74 proteins that were differentially expressed in cells with normal versus disrupted p53 function. For six proteins the major changes in expression were verified with immunohistochemical staining. The most promising result was the identification of peroxiredoxin-1 which allowed immunohistochemical discrimination between normal epithelium and precursor field tissue with a *TP53* mutation.

1. Introduction

Head and neck cancer develops in the mucosal linings of the upper aero- and digestive tract and is the sixth most common cancer worldwide [1]. More than 90% of head and neck tumors are squamous cell carcinomas. Specific risk factors for developing HNSCC are tobacco smoke, alcohol abuse, and infection with the human papilloma virus (HPV) [2]. Currently, the overall five-year-survival rate of HNSCC is approximately 60% [1]. A large proportion of patients presents with advanced stage disease and despite combined and improved treatment modalities, the prognosis still leaves much to be desired.

Braakhuis et al. [3] have recently described an adapted molecular progression model for the carcinogenesis of HNSCC. This model indicates that head and neck tumors develop in a large precursor field of genetically altered epithelial cells [4–6]. Although these fields can reach dimensions of multiple centimeters in diameter, they mostly do not elicit clinical

Abbreviations: dn, dominant negative; FDR, false discovery rates; FFPE, formalin-fixed paraffin embedded; HNSCC, head and neck squamous cell carcinoma; HPV, human papilloma virus; tsLT, temperature-sensitive allele of SV40 large T antigen gene; IHC, immunohistochemistry; shR, short hairpin RNA.

* *Corresponding author*. Biomolecular Mass Spectrometry and Proteomics Group, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands. Fax: +31 30 2518219.

E-mail address: m.slijper@uu.nl (M. Slijper).

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symptoms. Only approximately 20% of the fields can be detected as leukoplakia or erythroplakia, i.e. as white or red patches on the mucosa, respectively. Since the precursor fields are larger than the primary tumor and mostly invisible, they are not always completely resected when the tumor is excised. The remaining field can lead to the development of secondary tumors, clinically assigned as local recurrences and second primary tumors [6,7]. The availability of biomarkers to identify these precursor fields would thus permit analysis of the surgical margins for fields, and additionally allow more frequent surveillance during follow-up and early diagnosis of second primary tumors. Although precursor fields can be identified by genetic analyses, these methods are laborious, necessitating micro-dissection of tissue biopsies, and of limited sensitivity. Detection of these fields by immunohistochemistry using suitable protein biomarkers would facilitate implementation of this knowledge in clinical management.

Disruption of the p53 pathway is one of the earliest events in the carcinogenesis of HNSCC, and is therefore a typical characteristic of precursor fields [8,9]. The p53 pathway maintains the integrity of the cellular genome and is composed of a network of proteins that responds to a variety of stress signals. This network involves cellular homeostatic mechanisms that monitor DNA replication, chromosome segregation, and cell division [10]. Stress signals induce site-specific post-translational modifications of p53, like protein serine- and threonine phosphorylation, and lysine side chain acetylation, -methylation, -ubiquitination or -sumoylation. This results in activation of p53 as transcription factor, initiating cell cycle arrest, DNA repair, cellular senescence or apoptosis [11]. A number of feed-back loops have been described that act upon the p53 response, of which many act through the MDM-2 protein that targets p53 for proteasome-mediated degradation. A well-known stress signal that activates the p53 pathway is DNA damage, although the different types of DNA damage activate distinctive enzyme activities, for example gamma irradiation activates ATM kinase and CHK-2 kinase, whereas UV radiation activates ATR, CHK-1 and casein kinase-2. More than 4800 genes carry one or more p53 binding motifs [12], and it is thought that specific gene regulation is related to both cell type and stress type. This makes p53 one of the most prominent tumor suppressor proteins.

In HNSCC, the p53 pathway can be abrogated by mutation of the p53 gene, targeted degradation of the p53 protein by the HPV E6 protein, and inactivation of any of the modulators of p53 [13]. Mutation of the p53 gene occurs in 50% of all cancers and this percentage is even higher in head and neck tumors [13,14]. Different types of p53 mutations were found with distinct effects on p53 protein expression. Most p53 mutations found in human cancers are missense mutations that result in over-expression of an altered p53 protein with dominant-negative activity [14]. Over-expression of the aberrant protein allows detection by IHC [9]. In contrast, nonsense or frameshift mutations generate truncated or unstable p53 proteins that subsequently are degraded and cannot be detected by IHC. The same holds true in the case of HPV E6 induced degradation of p53. These various effects of p53 pathway disruption on the protein level hamper the application of p53 as a protein biomarker. Since p53 function loss is typically one of the earliest changes in carcinogenesis [3], proteins that indicate p53 dysfunction may act as valuable biomarkers to identify precursor field presence, however, no such markers are available.

The aim of this study was to detect protein biomarkers that indicate p53 malfunction, by using a quantitative proteomics approach. To investigate the effect of p53 function disruption, we used a conditionally immortalized squamous cell model in which the described modes of p53 inactivation were introduced, i.e. missense and nonsense mutations and inactivation by HPV E6. These cell models for p53 pathway inactivation were compared with wild type p53 control cells by differential proteome analysis through 2D-DIGE and LC-MS/MS, to determine which protein levels and pathways are affected. 2D-DIGE was chosen as it allows relative quantification of protein levels for many samples simultaneously by the use of an internal standard. Moreover, 2D-DIGE permits evaluation of proteins at a wide range of expression levels, due to the 4 log dynamic range of detection the CyDyes. Immunohistochemistry was used to verify the results on the cell model and to assess the value of the detected proteins as potential biomarkers to detect precursor field tissue.

2. Materials and methods

Fig. 1 represents the schematic workflow, showing the preparation steps to create the p53 inactivation model. This was followed by 2D-DIGE analysis of the lysates, protein identification by LC-MS/MS and verification of the results using immunohistochemistry.

2.1. *Conditionally immortalized human squamous cell model*

The generation of this model for p53 inactivation and its characteristics are described elsewhere [15]. In short, primary keratinocytes were harvested from surgically removed uvulas of patients treated for snoring. To create the conditionally immortalized squamous cell model, the temperature-sensitive SV40 large T antigen gene (tsLT) and the catalytic subunit of telomerase (hTERT) were introduced into the primary keratinocytes, as described previously for hepatocytes and fibroblasts [16,17]. The tsLT blocks p53 and pRb at 32 °C, allowing the cells to proliferate at the permissive temperature of 32 °C. At 39 °C, the tsLT is inactive, and the cells immediately enter proliferation arrest in less than one population doubling. The cells die after this proliferation arrest within 1–3 weeks after temperature shift to 39 °C, most likely by apoptosis and similar to normal primary keratinocytes at the end of their lifespan [15].

2.2. *Inactivation of p53 in the squamous cell model*

To mimic p53 inactivation in tumors, the tsLT transfected cells were transduced with either one of the following constructs: 1) dominant negative mutant p53(R175H) (dn p53; mimicking missense mutation), 2) short hairpin p53 RNA (shR p53; mimicking nonsense mutation) [16], and 3) the HPV E6 gene (mimicking