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Phosphoproteome analysis reveals differences in phosphosite profiles between tumorigenic and non-tumorigenic epithelial cells

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ABSTRACT

Oral cancer disease represents a significant fraction of all human cancer types and its poor early diagnosis contributes to reduced individual survival rate. The identification of proteins modulated in tumorigenic cells and its post-translational modifications may improve our understanding of tumor development in epithelial cells. We have analyzed the phosphoproteome of tumorigenic (SCC-9) and non-tumorigenic (HaCaT) cell lines using MS-based approach in order to identify phosphopeptides with differing patterns of modifications and/or abundance. Our results revealed the identity of 4,206 protein phosphorylation sites with sixty-two sites showing to be significantly modulated between the two cell lines. The phosphoproteome data showed an overrepresentation of proteins with a possible role in nuclear regulatory functions. Pathway analysis was further performed on the phosphoproteome dataset and differences and commonalities of the functional pathways present in tumorigenic and non-tumorigenic cells were identified. Phosphopeptides that belong to the proteins lamina-associated polypeptide 2 isoform alpha and serine–arginine repetitive matrix protein 2 were identified with differential abundance and they appear as promising tumor-related phosphopeptides. These two proteins may be related to the structural alterations generally found in the nucleus of tumorigenic cells. The identification of phosphorylation sites in tumorigenic cells may contribute to disclose novel signaling mechanisms associated with OSCC.

Significance
Oral Squamous Cell Carcinoma (OSCC) is an important cancer disease affecting thousands of people worldwide. Many cellular processes related to the development of oral cancer remain unknown; however, the studies performed in vitro with cancer cells have contributed to guide more specific research which may be further performed by using in vivo approaches or clinical samples. To our knowledge, only few studies have been published showing the results of phosphoproteome profiling of squamous cell carcinoma models, and many signaling proteins must be identified and functionally characterized in order to increase the knowledge available about the complexity of the signaling networks responsible for oral cancer development and its progression. Furthermore, our knowledge

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regarding proteins exclusive or very low abundant in cancer cells remains limited. A better understanding of the differences between signaling pathways present in epithelial cell lines may contribute to reveal the processes underlying the OSCC.

1. Introduction

Oral cancer is a disease which affects thousands of people worldwide every year and represents up to 2% of all types of human cancer, being the Oral Squamous Cell Carcinoma (OSCC) the most frequent type of oral cancer [1]. Due to the difficulty in the early diagnosis, the percentage of patients who will survive for more than five years is quite low compared to other types of cancer. The development of laboratory tests to improve diagnosis may contribute to the early detection of cancer in the oral cavity and may contribute to increase effectiveness of the medical treatments thus reducing mortality [1]. The identification of proteins involved in tumorigenesis and the characterization of their possible role in regulating tumor development have been recognized for many years as an important approach to broadening our knowledge about the cellular processes underlying cancer disease [2]. It has been shown that many proteins involved in the development and proliferation of tumor cells are part of complex signaling cascades and they function as regulators of cellular responses [3,4]. Within these signaling cascades, many proteins have shown to play a role in phosphorylation and autophosphorylation events, regulating signaling cascades at several different levels [4].

Several signaling pathways have been described so far including cancer-related proteins, such as ERK, EGFR and BCR-ABL1, which were described through proteomics approach [4]. These findings lead to a more holistic and dynamic view of the tumor development, showing that post-translational modifications (PTMs) may play an essential role in the regulation of cellular responses. Nevertheless, from the overall number of phosphosites predicted in a cell, suggested to be around 100,000 sites, most of them have probably not been described so far even with the recent advances on proteomic techniques [5]. Therefore, it is important to identify the phosphorylation events in the proteome of a cell or tissue in order to disclose the whole protein phosphorylation profiles of tumor cells. Furthermore, the identification of the phosphorylated proteins in several differing tumorigenic and non-tumorigenic cell lines may not only assist in the elucidation of the variations in signaling pathways responsible for tissue-specificity tumor development but may also contribute to the identification of phosphorylation events commonly occurring in different cell lines. Recently, by comparing the phosphoproteome of developmental stages of skin cancer, proteins associated with early and late cellular responses were identified in mice, providing new insights into the progression of the disease [6]. However, to our knowledge, no study has described the phosphoproteome of human epithelial cell lines used for in vitro studies in oral cancer research. We have recently demonstrated that HaCaT and SCC-9 cell lines have differing proteome profiles of its secretome. Changes in cellular migration and adhesion were observed in these two cell lines. Extracellular matrix, membrane-bound and secreted proteins were identified to be differentially expressed in tumorigenic (SCC-9) [7] and non-tumorigenic (HaCaT) [8] cells. Candidate proteins related to migration of SCC-9 cells were verified [9]. The comparative proteome analysis of secretome from HaCaT and SCC-9 cell lines had nicely illustrated that there are cellular mechanisms, which may be particularly more evident in tumorigenic cells. However, changes in protein post-translation modifications in cell lines have not been clearly demonstrated. HaCaT cells are spontaneously immortalized cells and retain their capacity to reconstitute a structured and stable epidermis in vivo, also showing non-invasive behavior when transplanted to epidermis in vivo [8,10]. Therefore, these cells have an epidermal origin and even with the differentiation suffered through the immortalization process their non-invasive behavior was retained. In contrast, the SCC-9 cell line which also has an epidermal origin, retained its invasiveness and capacity to develop progressive tumors after transplantation in vivo [11]. By comparing the phosphoproteome of HaCaT and SCC-9 cells we may then disclose candidate proteins which play a role in signaling mechanisms related to the invasiveness of tumorigenic epithelial cells which should further contribute to a better understanding of tumor progression in OSCC. In order to deeply investigate the signaling mechanisms that may explain the differences between tumorigenic and non-tumorigenic cell lines, we performed a phosphoproteome analysis. Phosphopeptide enrichment and identification analysis were performed with titanium dioxide (TiO_2) beads followed by high resolution mass spectrometry (MS), respectively. We identified 4,206 high confident phosphosites, of which 4,115 have been found deposited at Phosphosite annotation databases. Gene ontology (GO) term enrichment analysis was performed and revealed overrepresented functional GO categories related to RNA metabolism in SCC-9. Phosphoproteins identified in both cell lines have shown to possess general GO term enrichment for nuclear or translational regulatory functions. Besides, pathways and networks related to phosphoproteins identified in our study were retrieved through the use of Ingenuity knowledge base, showing important variations in the components of pathways regulated in tumorigenic and non-tumorigenic cells. Moreover, phosphosites with differential abundance were identified and the possible functional role of these phosphorylated proteins in tumorigenic cells is discussed.

2. Material and methods

2.1. *Cell culture*

The human Oral Squamous Cell Carcinoma (OSCC) cell line SCC-9 [7] was obtained from American Type Culture Collection