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Research Article

Altered epigenetic regulation of homeobox genes in human oral squamous cell carcinoma cells

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ABSTRACT

To gain insight into oral squamous cell carcinogenesis, we performed deep sequencing (RNAseq) of non-tumorigenic human OKF6-TERT1R and tumorigenic SCC-9 cells. Numerous homeobox genes are differentially expressed between OKF6-TERT1R and SCC-9 cells. Data from Oncomine, a cancer microarray database, also show that homeobox (HOX) genes are dysregulated in oral SCC patients. The activity of Polycomb repressive complexes (PRC), which causes epigenetic modifications, and retinoic acid (RA) signaling can control HOX gene transcription. HOXB7, HOXC10, HOXC13, and HOXD8 transcripts are higher in SCC-9 than in OKF6-TERT1R cells; using ChIP (chromatin immunoprecipitation) we detected PRC2 protein SUZ12 and the epigenetic H3K27me3 mark on histone H3 at these genes in OKF6-TERT1R, but not in SCC-9 cells. In contrast, IRX1, IRX4, SIX2 and TSHZ3 transcripts are lower in SCC-9 than in OKF6-TERT1R cells. We detected SUZ12 and the H3K27me3 mark at these genes in SCC-9, but not in OKF6-TERT1R cells. SUZ12 depletion increased HOXB7, HOXC10, HOXC13, and HOXD8 transcript levels and decreased the proliferation of OKF6-TERT1R cells. Transcriptional responses to RA are attenuated in SCC-9 versus OKF6-TERT1R cells. SUZ12 and H3K27me3 levels were not altered by RA at these HOX genes in SCC-9 and OKF6-TERT1R cells. We conclude that altered activity of PRC2 is associated with dysregulation of homeobox gene expression in human SCC cells, and that this dysregulation potentially plays a role in the neoplastic transformation of oral keratinocytes.

Abbreviations: ChIP, chromatin immunoprecipitation; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GO, gene ontology; H3K27me3, histone 3 lysine 27 trimethyl; HNSCC, head and neck squamous cell carcinoma; HOX, homeobox; HPRT1, hypoxanthine phosphoribosyltransferase 1; OSCC, oral squamous cell carcinoma; PRC, polycomb repressive complexes; qRT-PCR, quantitative real time polymerase chain reaction; RA, retinoic acid; RAR, retinoic acid receptor; RARE, retinoic acid response element; RNAseq, RNA sequencing; RXR, retinoid X receptor; shRNA, short hairpin RNA; SCC, squamous cell carcinoma; TNM, TNM classification of malignant tumours; WCMC, Weill Cornell Medical College

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Introduction

Head and neck cancers are very common worldwide [1]. It is estimated that 41,380 new patients will be diagnosed with cancer of the oral cavity and the pharynx and 7890 will die of these diseases in 2013 in the United States alone [2]. More than 90% of all oral cavity cancers are of the squamous cell carcinoma (SCC) type [1]. Many risk factors are known for oral SCC, including tobacco and alcohol abuse and genetic susceptibility [1].

Vitamin A (retinol), its natural metabolites, and its synthetic analogs constitute a class of chemicals often referred to as retinoids. Retinoids play an important role as regulators of cell proliferation and differentiation in embryonic development [3] and organ homeostasis. All-*trans*-retinoic acid (RA) acts as a ligand for a group of nuclear receptors known as retinoic acid receptors α, β or γ (RARs) [4]. RARs function as heterodimers with members of the retinoid X receptor family (RXR α, β or γ) [4]. RAR/RXR heterodimers bind to specific DNA sequences, known as retinoic acid response elements (RAREs), and regulate the transcription of downstream genes [4,5]. Normal epithelial cells require retinoid signaling to differentiate and function properly [6]. The expression of RARs and the metabolism of retinoids are aberrant in oral cancer [7–9]. Retinoids in combination with other drugs have been used to treat oral leukoplakia and squamous cell carcinoma [10].

Homeodomain proteins are transcription factors [11]. In vertebrates, groups of homeodomain genes known as Hox genes are located on chromosomes in clusters [11]. Recent reports suggest that HOX genes are often dysregulated in human oral SCC (OSCC). HOXA1, HOXA10, and HOXB7 are expressed at higher levels in OSCC as compared to healthy mucosas and may be prognostic markers for OSCC [12–14]. Expression of HOX genes during embryogenesis is regulated by RA [15]. RA induces expression of genes in the 3′ ends of Hox clusters, resulting in an increase in these transcripts in the anterior part of the body, while transcripts of 5′ Hox cluster genes that are not induced by RA are enriched posteriorly [15]. We and others have identified retinoic acid response elements (RAREs) in the enhancers of Hoxa1, Hoxd1, Hoxa4, and Hoxb1 [5,16–18]. Silencing of Hox gene expression during development and cell differentiation is mediated by the activity of Polycomb complexes [15]. Core components of the human Polycomb repressive complex 2 (PRC2) include: E(Z) homolog 2 (EZH2), SUZ12, embryonic ectoderm development (EED) and retinoblastoma-binding protein p48 (RBAP48, or RBBP4) [19]. EZH2 is enzymatically active, catalyzing the mono-, di- and tri- methylation of lysines 9 and 27 of histone 3; this results in transcriptional silencing of the underlying genes. In addition to EZH2, both SUZ12 and EED are indispensable for PRC2 enzymatic activity. EZH2 can also act independently of other PRC2 proteins [20]. Several recent reports suggest that high expression and activity of EZH2 might be associated with tumor proliferation and poor prognosis in oral SCC patients [21–23].

Our hypothesis is that neoplastic transformation of oral keratinocytes involves changes in the epigenetic regulation of transcription. We have tested this hypothesis by assessing the differences in RNA transcripts between cultured immortalized, non-tumorigenic and neoplastically transformed human oral keratinocytes by RNAseq and qRT-PCR. We have also determined the roles of SUZ12 and RA in the regulation of differentially expressed genes by chromatin immunoprecipitation and by shRNA mediated depletion of SUZ12 in immortalized non-tumorigenic oral keratinocytes and in SCC lines.

Materials and methods

Cell culture and chemicals

Immortalized non-transformed human oral keratinocytes cell lines OKF4-TERT1, OKF6-TERT1 and OKF6-TERT1R [24], were kindly provided by Dr. James G. Rheinwald, Harvard Medical School. These cells were cultured in Keratinocyte-SFM medium (#10744019, Gibco, CA, USA), supplemented with 0.3 mM $CaCl_2$, 0.2 ng/ml EGF, penicillin/streptomycin/L-glutamine (#10378-016, Gibco, CA, USA) and bovine pituitary extract (BPE), as previously described [24]. Human OSCC lines SCC-9, SCC-15 and SCC-25 were cultured in Dulbecco's Modified Eagle's Medium: Nutrient Mixture F-12 (DMEM/F-12; Gibco, CA, USA), supplemented with 10% fetal calf serum and 400 ng/ml hydrocortisone. HEK293 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, CA, USA), supplemented with 10% fetal calf serum. Stock solutions of all-trans retinoic acid (RA; Sigma-Aldrich, St. Louis, MO) were prepared in 100% ethanol at the beginning of each experiment and diluted in growth medium to a concentration of 1 μM. For mRNA analyses, cells were plated in 10 cm^2 tissue culture plates at the density of 2×10^6 cells/plate and treated with 1 μM RA or vehicle (0.1% ethanol) for 48 h. For chromatin immunoprecipitation, the cells were plated in 15 cm^2 tissue culture plates at a density of 3×10^6 cells/ plate and treated with 1 μM RA or vehicle. For cell proliferation assays, cells were plated in 12-well plates at a density of 2×10^4 cells per well, 3 wells for each cell line in each of three biological repeats of the experiment. The cells were treated with 1 μM RA or vehicle in the growth medium for 6 days and counted using a cell and particle counter (Coulter Z; Beckman Coulter, Inc., Fullerton, CA). Stable Suz12 knockdown (shRNA sequence TRCN0000038728) and control cell populations were established as described [25].

Deep sequencing and data analysis

For deep sequencing, total RNA was isolated from cells using an RNeasy mini kit (Quiagen Inc. Valencia, CA, USA), including an in-column DNAse treatment. RNA quality check, cDNA library preparation, and deep sequencing were performed by the Weill Cornell Medical College Genomics Resources Core Facility. Data analysis was performed with guidance and help from Dr. Fabienne Campagne (Institute for Computational Biomedicine, Weill Cornell Medical College (WCMC)). Sequencing data were uploaded into the GobyWeb application (Campagne lab) for alignment to the genome. Differential expression analysis was performed initially using GobyWeb [26]. Differential expression analysis was repeated using EdgeR, an RNAseq analysis package already cited in published reports [27–29]. To focus on potentially the most relevant genes, we generated lists of transcripts with at least a threefold difference between the groups. Further analysis was performed using software available publicly online from DAVID (The **D**atabase for **A**nnotation, **V**isualization and **I**ntegrated **D**iscovery [30]).