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p63 contributes to cell invasion and migration in squamous cell carcinoma of the head and neck

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Abstract

The transcription factor p63 is commonly over-expressed in squamous cell carcinomas of the head and neck (SCCHN). By microarray analysis of p63-siRNA-treated SCCHN cells we identified 127 genes whose expression relies on over-expression of p63. More than 20% of these genes are involved in cell motility. Chromatin immunoprecipitation and reporter assay revealed PAI-1 and AQP3 as direct p63 transcriptional targets. In addition to PAI-1, most of the key cell motility-related molecules are up-regulated by p63, such as MMP14 and LGALS1. Our findings indicate a contribution by p63 in cell invasion and migration, supporting an oncogenic role for p63 in SCCHN.

Keywords: p63; Invasion; Migration; SCCHN

1. Introduction

Squamous cell carcinoma of the head and neck (SCCHN) is the sixth most common cancer worldwide. SCCHN arises within basal cells of squamous epithelium and has complex epidemiology and pathogenesis [1]. One common finding in SCCHN and some other squamous carcinomas is over-expression of the p63 protein, [2,3] which associates with amplification of the p63 locus at chromosome 3q27-29 [4,5]. The *p63* gene is a member of the p53 transcription factor family and can produce six different proteins from two promoters and the use of differential splicing. The TAp63 isoforms are similar to p53 in transactivation capacity, whilst the ΔNp63 isoforms act as inhibitors of transcription by occupying DNA binding sites without transactivating [6]. These original observations were, however, an over-simplification, as a second TA domain exists in the N-terminal region and an inhibitory domain has been identified in the C-terminally extended p63α proteins [7,8]. Expression of p63 is required for the proper formation of limbs, epidermis and other epithelial tissues including breast and prostate, as evidenced by the phenotypes of p63-null mice [9,10] and the malformations seen in people that inherit mutations in the *p63* gene [11].

The predominant p63 isoform expressed in normal squamous epithelia and squamous carcinomas including SCCHN is ΔNp63α [2–4,12–14]. Various

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functions have been ascribed to ΔNp63α in squamous epithelia and cancers and it is now well accepted that p63 plays an important role in cell fate specification, cell proliferation, differentiation, senescence and adhesion [15,16]. It has been reported that high p63 expression is associated with a more aggressive phenotype and poor prognosis in oral squamous cell carcinoma (OSCC) [17]. However, it has also been shown that high ΔNp63 protein levels in primary tumours accurately predict response to platinum based chemotherapy and a favorable outcome in head and neck cancer patients [18]. Similarly, Takahashi et al. found that lower p63 expression was associated with poor prognosis in esophageal squamous cell carcinoma [19]. In a recent report, Oliveira et al. found no significant association between p63 protein expression and survival, recurrence or metastasis by examining 106 OSCC patients [20]. In summary, the role(s) of p63 in tumourigenesis is still not clear.

Here, we investigated the role(s) of p63 in SCCHN by using siRNA-mediated inhibition of p63 to identify genes that rely on endogenous p63 expression in FaDu cells. This cell line is derived from a primary human SCCHN, has a similar expression profile of p63 to primary human tumours [3,13] and relies on elevated p63 expression for survival, [21] similar to other human SCCHN cell lines [22,23]. By microarray analysis we could show that p63 modulates multiple aspects of cell adhesion and migration in SCCHN.

2. Materials and methods

2.1. Cell culture and siRNA transfection

The human cell line FaDu originating from a human SCCHN of the hypopharynx (American Tissue Culture Collection, ATCC) was cultured in DMEM containing 10% FCS (Invitrogen, Grand Island, NY, USA). These cells express mutant p53 protein and high levels of p63 similar to primary human SCCHN tumours. At RNA level both N-terminal variants (TA and ΔN) as well as the three C-terminal splice variants (α, β and γ) can be detected, whereas only the ΔN isoform is detected at protein level (own unpublished data). Very low levels of the TAp63 isoforms were detected at mRNA level only. As reported previously, four complementary siRNA oligonucleotides targeting p63 (Dharmacon, Lafayette, CO, USA) were assessed. The p63siRNA3 duplex (5′-CACACAUGGUAUCCAGA UGTT-3′; 5′-CAUCUGGAUACCAUGUGUGTT-3′) targeting all p63 isoforms provided the most efficient inhibition and was used for all experiments. A control siRNA targeting luciferase served as a negative control [21]. Cells were transfected with the oligonucleotide duplexes (100 nM) premixed with Oligofectamine (Invitrogen) in serum-free media for 4 h and the efficiency of inhibition was determined by collecting cells 48 h later for p63 protein and mRNA expression.

2.2. Microarray sample preparation

1.5×10^6 FaDu cells plated 24 h previously were transfected with p63siRNA3 or control siRNA. Total RNA was extracted with Trizol (Invitrogen) 48 h after transfection. Three independent transfections were carried out with p63siRNA3 and two with control siRNA. For each culture, 20 μg total RNA was used for preparation of double-stranded cDNAs and biotinylated cRNAs were hybridized to HG-U133A chips (Affymetrix, Santa Clara, CA, USA) according to standard protocols in the Department of Clinical Microbiology at Umeå University.

2.3. Microarray data analysis

Expression values were calculated with either the RMA method available with the Affy package of BioConductor, [24] or with dCHIP software [25] using the default setting (PM-MM model). Similar results were obtained with both analyses and data presented is derived from dCHIP analysis. Arrays were quality assessed by array images, RNA degradation profiles, histograms and box plots pre- and post-normalisation in BioConductor, and calculation of percentage outliers in dCHIP. Expression values from dCHIP were truncated to 0 and probe sets that had high variance or were not changed were removed. By comparing the two controls with three independent p63 siRNA-treated samples, genes were identified that had a fold change of at least 1.3-fold at 90% confidence, a mean expression level of at least 30 in either treated or control arrays and a difference of at least 10.

2.4. Semi-quantitative and auantitative RT-PCR analysis

The same cDNA for microarray analysis was used for microarry data confirmation. Semi-quantitative RT-PCR analysis was performed for p63 using AmpliTag Gold DNA polymerase (Applied Biosystems, Branchburg, NJ, USA) in a GeneAmp 9600 thermal cycler (Perkin-Elmer). Quantitative RT-PCR was then performed for 14 selected genes using the Light Cycler (Roche Diagnostics Corp) and the LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics Cort) according to the manufacturer's instructions. Primer sequences are listed in Table 1.

2.5. Western blotting

Total proteins extracted from FaDu cells were separated on 10% or 15% SDS–polyacrylamide gels. The following antibodies were used: 4A4 mouse monoclonal