p63 and potential p63 targets in squamous cell carcinoma of the head and neck

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Umeå 2008
In memory of my grandfather
# CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ABBREVIATIONS</strong></td>
<td>6</td>
</tr>
<tr>
<td><strong>ABSTRACT</strong></td>
<td>7</td>
</tr>
<tr>
<td><strong>POPULÄRVETENSKAPLIG SAMMANFATTNING</strong></td>
<td>9</td>
</tr>
<tr>
<td><strong>ORIGINAL ARTICLES</strong></td>
<td>10</td>
</tr>
<tr>
<td><strong>INTRODUCTION</strong></td>
<td>11</td>
</tr>
<tr>
<td><strong>Squamous cell carcinoma of the head and neck</strong></td>
<td>11</td>
</tr>
<tr>
<td>Diagnosis and treatment</td>
<td>12</td>
</tr>
<tr>
<td>Molecular mechanisms behind SCCHN</td>
<td>12</td>
</tr>
<tr>
<td><strong>The p53 family</strong></td>
<td>13</td>
</tr>
<tr>
<td>Structure and regulation of p53</td>
<td>13</td>
</tr>
<tr>
<td>p53 in cancer</td>
<td>14</td>
</tr>
<tr>
<td>Structure and regulation of p63</td>
<td>15</td>
</tr>
<tr>
<td>p63 in epithelial development</td>
<td>16</td>
</tr>
<tr>
<td>Mutations in the human p63 gene</td>
<td>18</td>
</tr>
<tr>
<td>p63 in cancer</td>
<td>19</td>
</tr>
<tr>
<td><strong>p73</strong></td>
<td>19</td>
</tr>
<tr>
<td><strong>Proteins connected to p63 and/or SCCHN</strong></td>
<td>20</td>
</tr>
<tr>
<td>β-catenin and PP2A</td>
<td>20</td>
</tr>
<tr>
<td>CD44</td>
<td>21</td>
</tr>
<tr>
<td>Cyclooxygenase 2 (Cox-2)</td>
<td>22</td>
</tr>
<tr>
<td>Epidermal growth factor receptor (EGFR)</td>
<td>23</td>
</tr>
<tr>
<td>Keratins</td>
<td>23</td>
</tr>
<tr>
<td><strong>AIMS</strong></td>
<td>25</td>
</tr>
<tr>
<td><strong>MATERIALS AND METHODS</strong></td>
<td>26</td>
</tr>
<tr>
<td><strong>Patients and specimens</strong></td>
<td>26</td>
</tr>
<tr>
<td><strong>Protein extraction</strong></td>
<td>26</td>
</tr>
<tr>
<td><strong>Immunoblot analysis and quantification</strong></td>
<td>28</td>
</tr>
<tr>
<td><strong>Cell transfections</strong></td>
<td>29</td>
</tr>
<tr>
<td>Stable transfections</td>
<td>29</td>
</tr>
<tr>
<td>Transient transfections</td>
<td>30</td>
</tr>
<tr>
<td><strong>RNA extraction</strong></td>
<td>30</td>
</tr>
</tbody>
</table>
Expression of p63, Cox-2, EGFR, β-catenin and PP2A in SCCHN patients and in oral mucosa from smokers (Paper I)  
Expression of Cox-2, EGFR, β-catenin and PP2A in SCCHN patients and smokers/non-smokers  
p63 expression in smokers/non-smokers and SCCHN patients  
Protein expression in normal tissue adjacent to the tumours compared to normal tissue from non-smoking individuals  
Expression of p53 isoforms in SCCHN (Paper II)  
Various p53 isoforms at RNA level in SCCHN  
Detection of in vitro translated and endogenously expressed p53 proteins  
p53 isoform expression in FaDu cells  
Over-expression of p63 in FaDu cells (Paper III)  
Expression of p63 isoforms  
Establishment of cell lines stably expressing p63  
Potential p63 targets (Paper III and IV)  
Cox-2 a potential target of p63  
Expression and regulation of CD44 in FaDu cells  
p63 regulates the expression of keratins
ABBREVIATIONS

ADULT  Acro-dermato-ungual-lacrimal-tooth syndrome
AEC    Ankyloblepharon-ectodermal defects-cleft lip/palate syndrome
APC    Adenomatous polyposis coli
AR     Amphiregulin
cDNA   Complementary DNA
ChIP   Chromatin immunoprecipitation
Cox    Cyclooxygenase
DBD    DNA-binding domain
EEC    Ectrodactyly, ectodermal dysplasia and cleft lip/palate syndrome
EGF    Epidermal growth factor
EGFR   Epidermal growth factor receptor
GSK3β  Glycogen synthase kinase- 3β
HER-1  Human epidermal factor 1
HPV    Human papilloma virus
LMS    Limb mammary syndrome
mdm    Murine double minute
miRNA  MicroRNA
NF-κB  Nuclear factor- kappa b
NLS    Nuclear localization signal
PCR    Polymerase chain reaction
PP2A   Protein phosphatase 2A
RE     Response element
RHS    Rapp-Hodgkin syndrome
RT-PCR Reverse transcriptase polymerase chain reaction
SAM    Sterile alpha motif
SCC    Squamous cell carcinoma
SCCHN  Squamous cell carcinoma of the head and neck
SHFM   Split hand/foot malformation
STAT3  Signal transducer and activator of transcription
SUMO-1 Small ubiquitin-like Modulator 1
TA     Transactivation
TGF-α  Transforming growth factor- alpha
TID    Terminal inhibitory domain
TNM    Tumour-Node-Metastasis
ABSTRACT

Squamous cell carcinoma of the head and neck (SCCHN), the 6th most common cancer worldwide, has a low 5-year survival. Disease as well as treatment often causes patients severe functional and aesthetic problems. In order to improve treatment and diagnosis at earlier stages of tumour development it is important to learn more about the molecular mechanisms behind the disease. p63, an important regulator of epithelial formation, has been suggested to play a role in the development of SCCHN. Six different isoforms of p63 have been found and shown to have various functions. The aim of the studies in this thesis was to learn more about the role of p63 and proteins connected to p63 in SCCHN. Expression of p63, Cox-2, EGFR, β-catenin, PP2A and p53 isoforms was mapped in tumours and normal tumour adjacent tissue from patients with SCCHN using western blot or RT-PCR. Results showed no significant difference between tumours and normal tumour adjacent tissue concerning expression of EGFR and β-catenin. Cox-2 and PP2A showed significantly higher expression in tumours while p63 was more expressed in normal tumour adjacent tissue. However, expression of all these proteins in normal tumour adjacent tissue differed from tissue from disease-free non-smoking individuals. Smoking in itself did not affect expression of these proteins. The p53 isoforms p53, p53β, p53γ, Δ133p53, Δ133p53β and Δ133p53γ were expressed at RNA level in samples both from tumours and normal tumour adjacent tissue, though most of them at fairly low levels. The functional properties of the different p63 isoforms have not been fully mapped. By establishing stable cell lines over-expressing the different p63 isoforms we investigated their specific effect on tumour cells from SCCHN. Only the ΔNp63 isoforms could be stably over-expressed, whereas no clones over-expressing TAp63 could be established. Using microarray technique, cell lines stably expressing the ΔNp63 isoforms were studied and CD44, Keratins 4, 6, 14, 19 and Cox-2 were found to be regulated by p63. In conclusion, the present project adds new data to the field of p63 and SCCHN. For example, we have shown that clinically normal tumour adjacent tissue is altered compared to normal oral mucosa in non tumour patients, and that smoking does not change expression of p63, Cox-2, EGFR, β-catenin or PP2A in oral mucosa. Novel p53 isoforms are expressed in SCCHN, and even though levels are very low they should not be overlooked. Furthermore, CD44, keratins 4, 6, 14, 19 and Cox-2 were identified as p63 targets in SCCHN.

Projekten sammanfattade i denna avhandling har genom olika metoder och modellsystem ökat vår kunskap om mekanismerna bakom SCCHN. Många av kroppens funktioner styrs med hjälp av proteiner. Proteinet p63 är viktigt för att bilda ytskiktet, det så kallade skivepitelet, vilket bygger upp bl.a. vår hud och munslömmarna. En orsak till defekter som gomspalt och harmynthet är att p63 inte fungerar normalt. p63 har också föreslagits vara inblandat i utvecklingen av SCCHN och studier av detta protein har varit centrala i avhandlingsarbetet. Några viktiga iakttagelser som gjorts är bland annat att vävnad som ligger nära SCCHN-tumörer och kliniskt ser normal ut inte är normal i fråga om innehåll av olika proteiner. Detta är en viktig upptäckt då denna typ av vävnad ofta används som kontrollvävnad i olika försök. Vidare verkar rökning, som är en riskfaktor för att drabbas av SCCHN, inte påverka produktionen av en del proteiner som man vet är inblandade i utvecklingen av SCCHN och studier av detta protein har varit centrala i avhandlingsarbetet. Några viktiga proteiner som p63 är viktiga för att upptäcka om när det finns scchn.
ORIGINAL ARTICLES

This thesis is based on the following articles referred to in the text by their roman numerals.


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INTRODUCTION

SQUAMOUS CELL CARCINOMA OF THE HEAD AND NECK

Squamous cell carcinoma of the head and neck (SCCHN) is the sixth most common cancer in the world, with about 500,000 new cases annually (Shirai and O’Brien, 2007). In Sweden, about 500 new cases of SCCHN are diagnosed every year (Socialstyrelsen: Cancerregistret). Of tumours arising in the head and neck area more than 90% are squamous cell carcinoma, a tumour of epithelial origin. SCCHN is an umbrella term including cancers at several sites in the head and neck area, for example nasal cavity, oral cavity, nasopharynx, hypopharynx, oropharynx and larynx (Figure 1) with different aetiologies and prognosis. SCCHN develops in the lining of the mouth, nose and throat consisting of squamous cells. The location of the tumours can lead to severe problems relating to disfigurement and dysfunction caused by the disease as well as treatment. The mean age at diagnosis is around 60 years, however, an increased incidence of patients under the age of 40 has been seen, especially concerning SCCHN of the tongue (Annertz et al., 2002; Funk et al., 2002; Shiboski et al., 2005).

Figure 1. The term SCCHN includes tumours located at different sites.

The most well-known risk factors for development of SCCHN are smoking and alcohol abuse (Jefferies and Foulkes, 2001) which in combination have a synergistic effect. Other known risk factors are smokeless tobacco such as gutkha, masala and betel quid. In Sweden a type of smokeless tobacco, moist snuff (snus), is used by about 12% of the adult population (Folkhälsoinstitutet). Snuff as a risk factor is heavily discussed despite the fact that several studies have shown no increased risk for development of SCCHN in Swedish snuff users (Lewin et al., 1998; Luo et al., 2007). In vitro snuff extract has been shown to cause morphological changes in epithelial cells and long-term exposure causes disturbances in the differentiation process (Merne et al., 2004).
Another risk factor discussed for development of SCCHN is human papilloma viruses (HPV), and about 20-30% of head and neck cancers contain HPV viruses (Schlecht et al, 2007; Syrjanen, 2005). There are more than 110 different HPV types, with HPV type 16 being the predominant type found in SCCHN (Schlecht et al, 2007; Syrjanen, 2005). Other risk factors that have been considered are familial and environmental issues.

**Diagnosis and treatment**

Diagnosis is often made at a late stage of SCCHN development. Staging of SCCHN is performed using the Tumour-Node-Metastasis (TNM) classification system which describes the anatomical extent of the disease based on three components:

- **T** – extent of the primary tumour
- **N** – absence or presence and extent of regional lymph node metastasis
- **M** – absence or presence of distant metastasis

Based on the TNM system tumours can be classified into different stages, I-IV. Staging can be seen as a summary of the TNM classification and also provides some prognostic information, lower stages indicate a better prognosis and higher chance to survive. The treatment strategies vary between different hospitals and countries. In Umeå, Sweden, the common treatment includes surgery and/or radiation therapy, whereas chemotherapy is not used normally. In other countries chemotherapy is more commonly used, and at later stages of disease a combination of radiotherapy, surgery and chemotherapy has been suggested to increase disease free survival (Lefebvre, 2005). An issue discussed in the treatment of cancer is the role and existence of cancer stem cells (Blagosklonny, 2007; Mackenzie, 2006). Different markers for distinguishing stem cells from somatic cells have been suggested. For example, p63 has been suggested as a stem cell marker in keratinocytes (Pellegrini et al, 2001). However, the question of how to distinguish the different types of stem cell-like cells and what role they may play in cancer and in cancer treatment still remains.

The five-year survival for SCCHN patients is about 50% including all SCCHN subgroups, however, prognosis varies between the different subtypes.

**Molecular mechanism behind SCCHN**

In order to improve treatment and enable diagnosis at an early stage, the understanding of the molecular mechanisms behind SCCHN is of importance and intense research is ongoing. Technologies such as microarrays, screening of gene changes in various samples easily generate very much data and hundreds of genes can be found to be differentially expressed between tumour and normal samples. These genes then need further analysis in order to evaluate the impact of each of them.
A few examples of genes often discussed in connection with development of SCCHN are p53, p16/p21/p27, EGFR, STAT3 and cyclin D1 (Gleich and Salamone, 2002). This thesis focuses on a few of the genes that have been suggested to be involved in SCCHN development in particular p63 and genes connected to p63.

THE P53 FAMILY

In 1979 the tumour suppressor p53 was first described in cells transfected with the SV40 virus interacting with the large T-antigen (Lane and Crawford, 1979; Linzer and Levine, 1979). Two decades later, in 1997, two homologues to p53 were discovered, p63 and p73 (Kaghad et al., 1997; Schmale and Bamberger, 1997; Yang et al., 1998). p53 has been intensively studied regarding its function in tumour development, and p63 and p73 have also been suggested to be involved in tumourigenesis. However, it has turned out that both p63 and p73 seem essential in development; p63 plays an important role in development of epithelia (Mills et al., 1999; Yang et al., 1998; Yang et al., 1999) and p73 is involved in neurogenesis and natural immune responses (Pozniak et al., 2000; Yang et al., 2000). Intense research is ongoing in order to evaluate the role of the p53 family members in tumourigenesis as well as the cross-talk between them. The focus of this thesis is mainly on p63, but a small part also deals with p53.

Structure and regulation of p53

The TP53 gene is located on chromosome 17p13 and encodes a protein of 393 amino acids consisting of several functional domains. The transactivation (TA) domain is located in the N-terminus containing both a transcriptional activating region and a proline rich domain. The middle part of the protein comprises the DNA-binding domain (DBD) which also is the major part of the protein. p53 forms dimers and tetramers and the oligomerization domain is located in the C-terminus. Adjacent to the oligomerization domain the basic domain is located, suggested as an additional DBD (Ahn and Prives, 2001). Initially p53 was suggested to be an oncogene but over the last decade it has been proposed to function as a tumour-suppressor in its wild-type conformation while when mutated it does not protect against tumour development. p53 is suggested to be involved in regulation of a wide variety of cellular processes such as cell-cycle arrest, DNA repair mechanisms, apoptosis and senescence. The short half-life (<20 minutes) gives low steady-state levels in the absence of cellular stress, while upon cellular stress p53 is activated leading to different cellular outcomes.
p53 is tightly regulated by murine double minute 2 (mdm) which binds and ubiquitinates p53, targeting it for proteasomal degradation (Haupt et al., 1997; Kubbutat et al., 1997). p53 is also regulated through posttranslational modifications such as phosphorylation, acetylation, ubiquitination and sumoylation. For many years only one variant of p53 was acknowledged even if reports suggested the existence of different variants of p53 (Courtois et al., 2002; Flaman et al., 1996; Ghosh et al., 2004; Wolf et al., 1985; Yin et al., 2002). Recently several p53 variants were described in detail and at least nine different isoforms suggested to exist (Bourdon et al., 2005). In addition to full-length p53, alternative splicing of intron 9 produces p53β (also called p53i9) and p53γ (Bourdon et al., 2005; Chow et al., 1993; Flaman et al., 1996). Alternative splicing of intron 2 or alternative initiation of translation produces the Δ40p53 (also called p47 and Δp53) isoforms (Δ40p53, Δ40p53β and Δ40p53γ) which lack the 40 first amino acids in the N-terminus (Courtois et al., 2002; Ghosh et al., 2004; Yin et al., 2002). Furthermore, an internal promoter in intron 4 was found, producing the Δ133p53 isoforms (Δ133p53, Δ133p53β and Δ133p53γ) (Bourdon et al., 2005). The same year another study proposed an additional p53 isoform lacking 198 nucleotides in exons 7, 8 and 9 (Rohaly et al., 2005), Δp53. Consequently p53 can exist in at least ten different variants (Figure 2). Investigations of the roles of the individual p53 isoforms has just started, however, they seem to be expressed in a wide range of normal tissues but in a tissue dependent manner, suggesting specific regulation mechanisms of the internal promoter and splicing event (Bourdon et al., 2005).

p53 in cancer

TP53 is one of the most commonly mutated genes in human cancer, with about 50% of all human tumours studied showing a mutation. It was first thought to function as an oncogene because of its wide expression in tumours. However, later it was discovered that mutated p53 functions as an oncogene and wild type p53 as a tumour suppressor. Most of the mutations occur in the DBD of the TP53 gene (Greenblatt et al., 1994). In accordance with other human tumours, SCCHN shows mutations in approximately 50% of the cases (Boyle et al., 1993; Brachman et al., 1992). The prognostic significance of TP53 mutations in SCCHN varies between different studies (Nylander et al., 2000). The reason for this can be variable sensitivity and accuracy between different methodologies employed for studying p53 as well as the presence of different p53 isoforms with different characteristics (Munro et al., 2005). Recent research focuses on microRNA (miRNA) which are small RNA molecules, about 22 nucleotides, functioning as negative gene regulators. Several hundreds of different human miRNAs have been found and especially one, miR-34, has been suggested to function in a network with p53 (He et al., 2007). Further studies will reveal the impact of miRNAs in tumour biology.
### Introduction

**Figure 2.** Structure of the ten p53 isoforms and the six p63 isoforms. α, β and γ isoforms are formed by alternative splicing, Δ40p53 isoforms are formed by alternative splicing of intron 2 or by alternative initiation of translation (previously called p47 or Δp53). Δ133p53 isoforms are produced by an internal promoter in intron 4. ΔNp63 isoforms are formed by an alternative promoter in intron 3. NLS: Nuclear localization signal, SAM: sterile-alpha-motif, TID: Terminal inhibitor domain. Figure adapted from Paper II; Mills, 2006.

**Structure and regulation of p63**

p63 is located on chromosome 3q27-29 and several variants of the p63 protein have been identified (Yang et al, 1998). Alternative promoter usage and differential mRNA splicing leads to formation of at least six different isoforms (Figure 2). The N-terminal promoter gives rise to the full-length proteins, TAp63, which contain the TA1 domain.
An internal promoter gives rise to the N-terminal truncated isoforms, ΔNp63, with a second TA domain, TA2, within exons 11-12 (Ghioni et al, 2002) and a C-terminal inhibitory domain (TID) (Serber et al, 2002). Alternative splicing of the C-terminus forms three variants designated: α, β and γ. The structure of p63 resembles that of p53 with a TA domain, a DBD with 60% homology to the DBD of p53 and an oligomerization domain. p63 is able to bind the canonical p53-responsive element whereas the amino acid differences between the DBDs may alter the specificity of p63 DNA binding. In addition, specific response elements through which p63 can regulate target genes have been found (Ortt and Sinha, 2006; Osada et al, 2005). Both p53 and p63 contain the oligomerization domain in the C-terminal part of the protein. p53 has a basic domain suggested to have a regulatory function whereas the α isoforms of p63 contain a sterile alpha motif (SAM) domain thought to be involved in protein-protein interactions, no SAM domain is present in p53. Furthermore, an inhibitory domain adjacent to the SAM domain causes decreased activity and the p63β isoforms lacking both the SAM and inhibitory domain but containing the TA2 domain consequently are efficient transactivators (Ghioni et al, 2002) whilst the p63γ isoforms lack the SAM domain, the TID domain as well as the TA2 domain.

Degradation of proteins occurs via lysosomal and proteasomal pathways. Several pathways for p63 degradation have been suggested, for example through the E3 ligase Itch which interacts with and ubiquitinates p63 promoting p63 proteasomal degradation (Rossi et al, 2006a; Rossi et al, 2006b). Moreover, sumoylation has been implied to be involved in degradation, and small ubiquitin-like modulator 1 (SUMO-1) attaches to ΔNp63α and targets it for proteasome mediated degradation (Ghioni et al, 2005). Additional stress signals such as ultraviolet radiation and cisplatin treatment have been observed to induce phosphorylation and ubiquitin-mediated degradation of p63 (Fomenkov et al, 2004; Westfall et al, 2005). Mdm2 plays an important role in degradation of p53, however, the effect of the interaction between mdm2 and p63 is not clear as one study indicates mdm2 to enhance the transcriptional activity of p63 (Calabro et al, 2002) whereas another study shows mdm2 to repress p63-mediated transcription (Kadakia et al, 2001).

p63 in epithelial development
Two main theories about p63’s function in development of stratified epithelia are discussed, agreeing upon the important role of p63 in this process. The various isoforms complicate the situation further and give different results. The two main hypotheses are that:

- p63 regulates the differentiation program of squamous epithelial cells (Mills et al, 1999)
- p63 maintains the proliferative potential of stem cells (Yang et al, 1999)
These hypotheses are based on two separate knockout mice models with different genetic backgrounds developed by two separate labs (Mills et al, 1999; Yang et al, 1999). The overall phenotype is similar and the knockout mice have severe development defects for example lack of squamous epithelia such as oral mucosa, mammary and salivary glands, hair follicles and skin, and die soon after birth due to dehydration (Mills et al, 1999; Yang et al, 1998; Yang et al, 1999). Defects in limb and craniofacial development are also seen. The craniofacial defects include cleft lip and palate as well as lack of teeth. On the other hand, the different knockout mice differ in development phenotypes, for example Yang et al, 1999, found clumps of differentiated keratinocytes that were thought to be remnants of epidermis. From this they draw the conclusion that p63 is required for epidermal stem cell maintenance. In accordance with this another study also showed enrichment of p63 in epithelial stem cells (Pellegrini et al, 2001). However, several studies have also failed to show the p63 enrichment in stem cells (Larderet et al, 2006; Morris et al, 2004) thus the subject is still an open question. In contrast, the other mouse knockout model by Mills showed no development of differentiated epidermis and concluded that p63 plays a role in differentiation of keratinocytes (Mills et al, 1999). p63 has also been suggested to be required in regulation of cell death during neural development (Jacobs et al, 2005).

Expression of the different isoforms also seems to differ in various backgrounds. Koster et al observed TAp63 to be expressed already at E7.5, earlier than ΔNp63 which is expressed from E9.5 (Koster et al, 2004). Furthermore, ectopically expressed TAp63 in mice induced stratification of the single layered lung epithelium as well as blocked skin differentiation. In vitro TAp63 but not ΔNp63 was able to block keratinocyte differentiation and induce expression of K14. This lead to the conclusion that TAp63 is the molecular switch responsible for epithelial stratification whereas the main function for ΔNp63 is to counterbalance TAp63 enabling cell differentiation (Koster et al, 2004). Other studies have, however, shown ΔNp63 to be the main isoforms expressed even in early development of epithelial tissue (Laurikkala et al, 2006). ΔNp63 is also the main isoform detected in mature epidermis, expressed mainly in the basal layers (Nylander et al, 2002; Thurfjell et al, 2004). Its expression is down-regulated in suprabasal layers, a down-regulation suggested to be caused by signalling through Notch (Nguyen et al, 2006). In vitro the ΔNp63α isoform has been suggested to have a dominant-negative effect on the TAp63 isoforms (Yang et al, 1998).
Mutations in the human p63 gene

The developmental role of p63 in animal models can be connected to human developmental disorders. Mutations in the human TP63 gene cause dominantly inherited syndromes with developmental defects with the hallmarks of ectodermal dysplasia, facial clefting and limb abnormalities. Ectodermal dysplasia is characterized by abnormal development or growth of tissues derived from the embryonic ectoderm. Facial clefting is the result of incomplete fusion of embryonic facial processes. Limb abnormalities are often split hand/foot malformations. Five different syndromes and one non-syndromic single malformation are known to be the result of mutations in different domains of the TP63 gene and are characterized by various combinations of the p63 syndrome phenotypes.

**Ectrodactyly, ectodermal dysplasia and cleft lip/palate syndrome (EEC):** mainly caused by mutations in the DNA-binding domain (Celli 	extit{et al}, 1999). Characterized by ectodermal dysplasia and facial clefts.

**Ankyloblepharon-ectodermal defects-cleft lip/palate syndrome (AEC),** also known as Hay-Wells syndrome: Mutations in the SAM domain has been found in these patients (McGrath 	extit{et al}, 2001). A typical hallmark of AEC syndrome is the fusion of eyelids.

**Limb mammary syndrome (LMS):** Mutations in the N and C-terminals have been found (van Bokhoven 	extit{et al}, 2001). The phenotype of LMS includes malformation of the hands and/or feet and hypoplastic nipples and/or mammary glands.

**Acro-dermato-ungual-lacrimal-tooth syndrome (ADULT):** One mutation has been found in the DNA-binding domain (Propping 	extit{et al}, 2000) and exons 3 and 4 (Amiel 	extit{et al}, 2001; Slavotinek 	extit{et al}, 2005). Clinically the syndrome is very similar to LMS, however, orofacial clefting has not been observed in ADULT while nails, skin and teeth are very often affected.

**Rapp-Hodgkin syndrome (RHS):** Mutations have been localized to the C-terminus of the p63 protein (Bougeard 	extit{et al}, 2003; Kantaputra 	extit{et al}, 2003). Disease features include orofacial clefting and sparse fine hair.

**Split hand/foot malformation (SHFM):** Caused by several mutations along the whole TP63 gene (van Bokhoven 	extit{et al}, 2001) and is characterized by limb malformations.

For a summary of all syndromes caused by p63 mutation see Rinne 	extit{et al}, 2007.
Introduction

p63 in Cancer
The homology between p53 and p63 led to the speculation that p63 also plays an important role as tumour suppressor. However, p63 is rarely mutated in human tumours indicating that it is not a tumour suppressor. In many different tumour types an over-expression of p63 has been seen, for example breast cancer, bladder cancer, lung cancer and SCCHN, indicating that p63 may function as an oncogene. There is an ongoing debate whether p63 is an oncogene or a tumour suppressor. It has been suggested that TAp63 isoforms are p53-like and work as tumour suppressors, activating known p53 targets such as p21, bax and mdm2 (Shimada et al, 1999). TAp63α induces apoptosis in several cell systems by triggering signals via death receptors and mitochondria (Gressner et al, 2005). On the other hand, ΔNp63 seems to be anti-p53 like and works as an oncogene. Recently two different p63 +/- strains showed different results regarding p63’s role in tumourigenesis, with one indicating that p63 heterozygous mice are more tumour prone than p63 homozygous mice (Flores et al, 2005) while the other study showed that p63 +/- are less tumour prone (Keyes et al, 2006).

One strategy to investigate the role of p63 in tumour development is to identify p63 targets. Regulation of downstream targets occurs by binding to sequence-specific response elements (REs). p63 can bind p53 REs and activate p53 targets such as bax and p21 involved in apoptosis and cell cycle arrest. p63-specific REs have, however, also been identified differing from canonical p53 REs (Osada et al, 2005; Trink et al, 2007).

p73
p73 is located on chromosome 1p36 in a region frequently demonstrating loss of heterozygosity in human cancers (Kaghad et al, 1997). Like p53 and p63, p73 exists in several variants, and two promoters and/or differential splicing provide four different ways to produce p73 N-terminal variants. Furthermore alternative splicing of downstream exons can give rise to nine different C-terminal ends (α, β, γ, δ, ζ, ε, θ, η and η′) (Murray-Zmijewski et al, 2006). Altogether 14 different p73 proteins have been described even though theoretically 35 mRNA variants can be expressed encoding 29 different proteins. From studies using knockout mice lacking both TAp73 and ΔNp73 showing developmental defects in their nervous systems it was suggested that p73 contributes to neural development (Yang et al, 2000) and in the developing mouse brain ΔNp73 isoforms are highly expressed (Pozniak et al, 2000). In cancer the p73 gene is rarely mutated, however, both over-expression and reduced expression has been detected in different tumour types. For example lymphomas and leukaemias show reduced expression of p73 while tumours such as breast, ovary, lung and colorectal cancer show over-expression of p73 (Chen et al, 2000; Sun, 2002; Tokuchi et al, 1999; Zaika et al, 1999).
PROTEINS CONNECTED TO P63 AND/OR SCCHN

Several genes/proteins are involved in the development of tumours and mapping this network is important in understanding the cause of the disease. One suggested player in the development of SCCHN is p63 although not as a single player but as a part of numerous different genes/proteins interacting with or in some way connected to p63 and cooperating in tumourigenesis. In this thesis some of the proteins with a suggested connection to p63 or SCCHN have been studied and discussed.

β-catenin and PP2A
Glycoproteins that act as adhesion molecules can be divided into five major families: cadherins, the immunoglobulin superfamily, the integrins, the selectins and CD44 (El-Bahrawy and Pignatelli, 1998). Catenins belong to the cadherin family and three forms of catenins, α-catenin, β-catenin and γ-catenin have been identified. β-catenin is a 92 kDa protein with homology to the Drosophila segment polarity gene Armadillo (McCrea et al., 1991). Several studies have demonstrated E-cadherin/catenin complexes to play a key role in cell adhesion. β-catenin is a target of the Wnt signalling cascade, which contributes to the control of cell proliferation during development and tumorigenesis. In the absence of Wnt signals, a complex containing GSK3β, axin and APC promotes phosphorylation of β-catenin leading to ubiquitination of the protein and degradation via the proteasomal pathway giving low levels of β-catenin. On the other hand, when the cell is stimulated, soluble Wnt factors bind to receptors called frizzleds whereby the GSK3β, axin and APC complex is inhibited leading to accumulation of β-catenin in the nucleus. In the nucleus β-catenin forms a complex with Tcf family members and works as a transcription factor. Another factor involved in the regulation of β-catenin is protein phosphatase 2A (PP2A) a serine-threonine protein phosphatase (Janssens and Goris, 2001; Kikuchi, 2000). Simplified, it can be said that PP2A associates with APC, axin and GSK3β and contributes to β-catenin destruction (Figure 3). p63 can in turn interact with PP2A leading to accumulation of β-catenin in the nucleus (Patturajan et al., 2002). Alterations in the β-catenin signalling pathway is discussed in cancer and in SCCHN effects of such alterations have been suggested to promote cell invasion (Yang et al., 2006) and also impact survival, where low expression of β-catenin indicates decreased patient survival (Rodriguez-Pinilla et al., 2005; Yu et al., 2005).
**Introduction**

**Figure 3.** Activation of β-catenin. A. In the absence of signals through the wnt pathway β-catenin is degraded. B. Wnt activates the Frizzled receptor leading to transport of β-catenin into the nucleus. Adapted from Barker and Clevers, 2000; Kikuchi, 2000.

**CD44**

The type 1 transmembrane glycoprotein CD44 is a cell surface receptor for hyaluronate (Aruffo et al, 1990). CD44 contains at least 19 exons and alternative splicing of exons 5a to 14, called exons v1-v10, causes huge heterogeneity of mRNA transcribed from the gene (Screaton et al, 1992). Several hundreds of different combinations of sequences can theoretically be created. Two groups of transcripts are formed with the smaller group known as CD44 standard transcripts (CD44s) lacking all of the 10 variant exons. The other group comprises the larger CD44 variants (CD44v) encoding transcripts containing sequences from exons v1-v10. CD44 is important in cell-cell interactions, cell adhesion and migration, processes critical in normal immune system development and function, and control of cell proliferation (Kaya et al, 1997). Its involvement in cancer includes interaction between hyaluronate and CD44, a signalling pathway influencing tumour cell progression and resistance to chemotherapy.
The different variants of CD44 have been reported to be involved in tumour progression (Franzmann et al., 2001; Gunthert et al., 1991) and CD44v6 is highly expressed in SCCHN and squamous cell carcinoma (SCC) of the lung, cervix and skin (Heider et al., 2004; Schrijvers et al., 1993). CD44v3 is associated with tumour cell growth and migration in SCCHN (Wang et al., 2007). CD44 also physically associates with EGFR in cancer cells and promotes EGFR-mediated signalling and tumour progression (Wang and Bourguignon, 2006). CD44 has recently received attention as a marker of cancer stem cells in SCCHN. The CD44+ population of SCCHN cells possess stem cell properties of self-renewal and differentiation, whereas CD44- cells do not show self-renewal properties (Prince et al., 2007).

Cyclooxygenase 2 (Cox-2)
Cyclooxygenase, or prostaglandin-endoperoxidase, is an enzyme catalysing the production of prostaglandin from arachidonic acid. The existence of two variants of the enzyme is well known, Cox-1 and Cox-2. Cox-1 is constitutively expressed in many tissues and involved in the production of prostaglandins that control normal physiological functions. While in contrast Cox-2 is not detected in normal tissue but is rapidly induced by growth factors, oncogenes, carcinogens and tumour-promoting phorbol esters (inflammatory and mitogenic stimuli), the constitutively expressed Cox-1 is essentially unaffected by these factors (Herschman et al., 2003). Cox-2 is important in for example neonatal survival (Loftin et al., 2001) and in inflammatory processes induction of Cox-2 can both promote inflammation via prostaglandins but also have an anti-inflammatory role (Lawrence et al., 2002). In several types of cancer including SCCHN Cox-2 has been observed to be up-regulated (Chan et al., 1999; Gallo et al., 2001; Terakado et al., 2004).

The up-regulation of Cox-2 in cancer is suggested to decrease apoptosis (Tsujii and DuBois, 1995) and up-regulate VEGF leading to increased angiogenesis which is important for tumour growth (Gallo et al., 2001). In addition, Cox-2 up-regulation has been connected with increased invasiveness (Tsujii et al., 1997). SCCHN patients expressing high levels of Cox-2 have been seen to have a shorter disease-free as well as overall survival and also respond more poorly to radiotherapy (Chen et al., 2005; Itoh et al., 2003; Nix et al., 2004; Terakado et al., 2004). A supposed connection between p53 and Cox-2 is somewhat contradictory with results on the one hand indicating that p53 induces Cox-2 and that absence of Cox-2 can enhance p53-mediated apoptosis (Benoit et al., 2006; Han et al., 2002). On the other hand, p53 has been reported to decrease the expression of Cox-2 in SCCHN cell line systems (Lee et al., 2004). In accordance with this wild-type p53 was shown to repress expression of Cox-2 while mutated p53 had no effect (Subbaramaiah et al., 1999). p63 has been found to be correlated with Cox-2 in skin tumours (Wu et al., 2007).
**Introduction**

**Epidermal growth factor receptor (EGFR)**
The transmembrane glycoprotein, EGFR also called human epidermal factor 1 (HER-1) or c-erbB-1 is a member of the c-erbB family of receptor tyrosine kinases. EGFR is a receptor containing an extracellular receptor domain, a transmembrane domain and an intracellular domain with tyrosine kinase function. Several ligands have been suggested to activate the receptor, for example epidermal growth factor (EGF), transforming growth factor alpha (TGF-α) and amphiregulin (AR). Ligand binding to the receptor results in homo- or heterodimerization leading to autophosphorylation of the receptor. This activation leads to downstream signalling through different pathways, for example Ras/MAPK, PI-3 kinase, akt and STAT, pathways that regulate gene expression, proliferation, angiogenesis and inhibition of apoptosis. EGFR is expressed on all normal cells except hematopoietic cells and is over-expressed in a wide variety of tumours including breast cancer, non-small-cell lung cancer and SCCHN (Hynes and Lane, 2005). The most common mutation in EGFR in human cancers is known as EGFRvIII, a deletion of a part of the extracellular domain leading to a constitutively active receptor (Lorimer, 2002). Different strategies for inhibition of EGFR signalling in therapy are the use of antibodies or small molecule tyrosine kinase inhibitors. However, EGFR inhibitors have only shown limited success as monotherapy in SCCHN (Choong and Cohen, 2006). p63 is suggested to be downstream of EGFR (Matheny et al, 2003), however, in contrast another study have shown TAp63γ to repress EGFR expression (Nishi et al, 2001). A connection between EGFR and Cox-2 has been found showing that EGFR inhibition in combination with Cox-2 inhibitors gives effective inhibition of angiogenesis and tumour growth (Chen et al, 2004). Tobacco smoke increases the amount of EGFR ligands in oral epithelia (Du et al, 2005; Moraitis et al, 2005).

**Keratins**
The function of epithelial tissue is to protect the organism from physical, chemical and microbial damage. Epithelial tissue can be divided into three different classes
- Keratinized stratified squamous epithelium – such as epidermis, palate, and gingiva
- Stratified non-keratinized epithelia – such as buccal mucosa and esophagus
- Simple non-stratified epithelia – such as kidney and liver
A network of proteins forms the structure of the epithelial tissue, the cytoskeleton, with one of the main components being keratins. In humans keratins consist of a large family of numerous members (Presland and Dale, 2000) divided into type I (acidic) and type II (neutral or basic) keratins depending on their sequence.
Introduction

Usually keratins form pairs comprising one type I keratin and one type II keratin. The expression and function of different keratins is complex and varies at different differentiation stages and in different epithelial tissues. Overall it can be said that keratins regulate migration of normal and transformed epithelial cells and several keratins are used as epithelial differentiation markers. For example:

**Keratin 4**: Expressed in non-keratinized epithelia such as buccal mucosa, esophagus and vagina, often in pair with K13. Has been shown to be lower expressed in oral SCC compared to leukoplakia (Ohkura et al., 2005).

**Keratin 6**: Two isoforms exist with keratin 6A being the main form expressed (Takahashi et al., 1995). It is essential for proliferative and migratory responses and often expressed in squamous cancers.

**Keratin 14**: Expressed in dividing keratinocytes of the skin epidermis and in oral stratified squamous epithelia, in pair with keratin 5. K14 is down-regulated when cells start to differentiate and is significantly higher expressed in oral SCC compared to premalignant lesions (Ohkura et al., 2005). p63 can regulate the expression of K14 (Romano et al., 2007).

**Keratin 19**: As K14, K19 is expressed in the basal layer of oral stratified epithelia but down-regulated in cells lines established from invasive oral SCC (Crowe et al., 1999).

![Keratin expression in keratinized and non-keratinized epithelium](image)

**Figure 4.** Keratin expression in keratinized and non-keratinized epithelium. Adapted from Presland and Dale, 2000.
AIMS

The goal of this thesis was to learn more about the molecular mechanisms behind SCCHN and in particular the involvement of p63 and p63 connected proteins.

SPECIFIC AIMS

- Map protein expression profiles of p63, Cox-2, EGFR, β-catenin and PP2A in normal and tumour tissue from patients with SCCHN
- Investigate if there is variation in expression of p63, Cox-2, EGFR, β-catenin and PP2A in heavy smokers compared to non-smokers
- Map the expression of p53 isoforms in normal and tumour tissue from patients with SCCHN
- Establish and investigate cell lines of SCCHN origin stably expressing the different isoforms of p63
- Identify potential targets of p63 using cell lines stably over-expressing p63 isoforms
MATERIALS AND METHODS

PATIENTS AND SPECIMENS

Tissue samples comprised punch biopsies (4 mm in diameter) from the buccal mucosa of heavy smokers and age- and sex- matched controls, together with tumour and corresponding normal tissue from patients with SCCHN. A person who had smoked for at least ten years and currently smoked at least ten cigarettes per day was considered a heavy smoker. None of the smokers/non-smokers showed any clinical signs of inflammation and all samples were histologically normal with no signs of neoplastic changes. From the SCCHN patients, both tumour material and corresponding clinically normal tissue was collected. Tissue samples were divided into two and immediately frozen in liquid nitrogen and stored at -80°C. All samples were collected after informed consent and permission for the project had been granted by the Ethics Committee at Umeå University (dnr 01-057 and dnr 01-210). Information about all participants is summarised in table 1.

Cell lines used were FaDu (ATCC), a cell line derived from a human squamous cell carcinoma of the oropharynx, and Saos-2, a cell line derived from human osteosarcoma. Both cell lines were cultured in DMEM (Gibco, Invitrogen) with 10% foetal calf serum (Invitrogen) at 37°C with 10% CO₂. p53, p63 and p73 status for these cell lines is given in table 2.

PROTEIN EXTRACTION

One piece of each tissue sample was used for protein extraction through pulverisation using a Microdismembrator (B. braun biotech international) thereafter ~100µl lysis buffer (0.5% NP-40, 0.5% Na-dodecyl, 0.1% SDS, 150 mM NaCl, 50 mM Tris pH 7.5, 1 mM EDTA, 1 mM NaF and protease inhibitor cocktail (Sigma-Aldrich Chemie, Steinheim, Germany)) was added to the sample. Protein concentrations were determined using BCA Protein Assay Kit (Pierce, Rockford, IL, USA). The same procedure was used for extracting protein from cell lines except that the cell pellet was washed with PBS, and lysis buffer then directly added to the cell pellet. When analysing the established stable clones these were directly lysed in wells using 100 µl of another lysis buffer (2% SDS, 30% glycerol, 300 mM β-mercaptoethanol and 100 mM Tris-HCL (pH 6.8)) (Ghioni et al, 2002). Protein concentration was not measured for these samples.
### Materials and methods

#### Table 1. Summary of participants

<table>
<thead>
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<table>
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M – male, F – female, S – smoker, NS – non-smoker, and PS – previous smoker
Materials and methods

Table 2. p53 family status of cell lines used

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<th>Saos-2</th>
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Protein status

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p53 RNA detected by nested RT-PCR, p63 and p73 RNA detected by light cycler with a cut-off value of 31 cycles or * 36 cycles. Proteins were detected by antibodies indicated within parenthesis using western blot.

IMMUNOBLOT ANALYSIS AND QUANTIFICATION

Bio-Rad PROTEAN III mini-gels and PROTEAN II xi/XL vertical electrophoresis systems were used to separate and analyse proteins. Thirty µg of total protein extract was mixed with loading buffer, boiled for 10 minutes and electrophoresed on gels of appropriate percentage acrylamide. From stable clones 20µl of the extracted protein was loaded on the gel. Proteins were transferred to nitrocellulose membranes (Hybond ECL;GE Healthcare, Buckinghamshire, UK) and stained with ponceau red for evaluation of transfer efficiency and loading. Membranes were blocked in 5% milk with 0,4% Tween in PBS thereafter incubated with antibodies diluted in the same blocking solution. An overview of the antibodies used is given in table 3. For chemiluminescence detection, Chemidoc XRS (Bio-Rad) was used in combination with ECL Advance (GE Healthcare, Buckinghamshire, UK). For quantification in paper I a standard curve of known protein concentration samples was created and relative protein concentrations calculated. In paper III a relative quantification between samples was performed. All experiments were repeated at least two times.
### Materials and methods

#### Table 3. Antibodies used in the different studies

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#### Secondary antibodies

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### CELL TRANSFECTIONS

#### Stable transfections

In order to establish cell lines stably expressing the different p63 isoforms FaDu cells were seeded in 90 mm Petri dishes and at 90-95 % confluence transfected with 12.21 µg DNA, either empty pcDNA3 vector or pcDNA3 vector with insert of myc-tagged ΔNp63α, ΔNp63β or ΔNp63γ cDNA (Yang et al, 1998), using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Forty eight hours after transfection cells were plated at low concentration (20 000 cells/ml) on new plates and selected with G418, 800µg/ml (VWR international). Colonies formed and after approximately two weeks clones were picked and transferred to 12-well plates where they grew until confluence when they were divided into two wells, of which one was used for analysis of p63 protein expression. Clones expressing ΔNp63α, ΔNp63β and ΔNp63γ were saved and expanded for further analysis. Clones containing empty vector were screened by RT-PCR.
Materials and methods

Transient transfections
For transient transfections FaDu or Saos-2 cells in 90 mm petri dishes were transfected at 90-95% confluence with 12.21µg DNA, empty pcDNA3 vector or pcDNA3 vector with insert of myc-tagged TAp63α, TAp63β, TAp63γ, ΔNp63α, ΔNp63β or ΔNp63γ cDNA using Lipofectamine 2000 (Invitrogen) under conditions suggested by the manufacturer. Cells were harvested at 24, 48, 72, 96 and 120 hours after transfection. At least two independent transfections were performed.

RNA EXTRACTION

The other half of the biopsy was used for extraction of RNA. Tissue was cut into pieces in a petri dish on dry ice, covered with 250 µl TRIzol reagent (Invitrogen) and transferred to a tube and further homogenized with a pellet mixer (MERCK Eurolab), and another 250 µl TRIzol then added. For extraction from cell pellets TRIzol was added directly to the tube. The following procedure was the same for tissue and cell samples using 500 µl TRIzol, volume adjusted for small pieces of tissue and small cell pellets. All incubations were carried out at room temperature if nothing else is stated. After 5 minutes incubation with TRIzol, 100 µl chloroform was added and incubated for 3 minutes thereafter samples were centrifuged for 15 minutes at 13 000 rpm at 4°C. The upper aqueous phase was transferred to a new tube, diluted with equal amount of isopropyl alcohol, incubated for 10 minutes and centrifuged for 10 minutes at 13 000 rpm at 4°C. The supernatant was carefully removed and the pellet washed in 70% ethanol, air dried and dissolved in DEPC-\(\text{H}_2\text{O}\). Concentration was measured using a Nano-drop (ND-1000 spectrophotometer) and samples then stored at -80°C.

CDNA SYNTHESIS

Two different kits for cDNA synthesis were used. In paper II the Cloned AMV First-Strand cDNA Synthesis kit (Invitrogen) was used according to the manufacturer’s instructions, with 300 ng of total RNA. In the other experiments the 1st strand cDNA synthesis kit for RT-PCR (AMV) (Roche, Penzberg, Germany) was used according to the manufacturer’s instructions, with 500 – 1000 ng of total RNA in a reaction volume of 20µl.
Materials and methods

PCR

Nested PCR
In paper II nested PCR was used to analyse different p53 isoforms in tumour and normal samples from SCCHN patients. cDNA synthesised using the kit from Invitrogen was amplified in one round or two rounds, comprising 35 cycles each, of PCR (nested PCR). In nested PCR two different primer pairs were used, the pair in the first round covering a longer part of cDNA while the second pair was located inside the first pair. For primer combinations and primer sequences see Tables 4 and 5.

Table 4. Primer pairs used in nested PCR

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<th>1st PCR</th>
<th>2nd PCR</th>
<th>Fragment size (bp)</th>
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<tbody>
<tr>
<td>P53</td>
<td>e2.1/RT1</td>
<td>e2/RT2</td>
<td>~1250</td>
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<tr>
<td>P53β</td>
<td>e2.1/RT1</td>
<td>e2/p53b</td>
<td>~1050</td>
</tr>
<tr>
<td>P53γ</td>
<td>e2.1/RT1</td>
<td>e2/p53g</td>
<td>~1000</td>
</tr>
<tr>
<td>Δ133p53</td>
<td>i4f1/RT2</td>
<td>i4f2/RDNp53</td>
<td>~750</td>
</tr>
<tr>
<td>Δ133p53β</td>
<td>i4f1/RT2</td>
<td>i4f2/p53b</td>
<td>~750</td>
</tr>
<tr>
<td>Δ133p53γ</td>
<td>i4f1/RT2</td>
<td>i4f2/p53g</td>
<td>~750</td>
</tr>
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</table>

Quantitative PCR
Cox-2 expression was analysed using quantitative PCR and a human Cox-2 kit (Search LC, Heidelberg, Germany) on a Light-cycler™ from Roche. cDNA was synthesised with the kit from Roche, using 500 ng total RNA, and cDNA diluted 12 times before use in PCR. As internal control for cDNA integrity and relative cDNA quantity in the different tissue samples, β-actin was used. All reactions were run in duplicate and a mean value of the two samples calculated. Results were expressed as the relative amount of product adjusted to the level of internal control amplification obtained for the same sample.

Semi-quantitative PCR
For confirmation of differentially expressed genes in the microarray, semi-quantitative PCR was used with Fast Start taq DNA polymerase (Roche diagnostics). After optimization samples were subjected to 25-30 cycles and products analysed by gel electrophoresis.
### Table 5. Sequences of primers used.

<table>
<thead>
<tr>
<th>Primer</th>
<th>5’ Primer Sequence</th>
<th>3’ Primer Sequence</th>
<th>T (°C)</th>
<th>Study</th>
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<tr>
<td>CD44</td>
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<td></td>
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</tr>
<tr>
<td></td>
<td>3’ TCC TTG TCTCATCAGCTGTC</td>
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<td>CD44 Var.</td>
<td>5’ GCACAGAGCAATCCCTGCTACC</td>
<td>58 IV</td>
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<td></td>
<td>3’ GGAGTTGGAATGTGCCAGTCTGCT</td>
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<td>CD44 ChIP</td>
<td>5’ AGTTGGATGATAGATGGACC</td>
<td>59 IV</td>
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<td></td>
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<tr>
<td></td>
<td>3’ GGAAAGAGGAGAGAGCTCATT</td>
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<td></td>
<td></td>
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<tr>
<td>Cox-2-ChIP</td>
<td>5’ CTGTTGAAAGCAACTAGCT</td>
<td>58 III</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3’ AGACTGAAAAAGAGGCCAT</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Cox-2-ChIP</td>
<td>5’ ACCAGTATCTCTATAGGGAAG</td>
<td>60 III</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3’ CCTTCTCTCAGGAATCTGA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cox-2-ChIP</td>
<td>5’ GATCCATGGTCACAATC</td>
<td>59 III</td>
<td></td>
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<tr>
<td></td>
<td>3’ GTCCTAAGCATTACCTG</td>
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<td>IGFBP3</td>
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<td>54 III, IV</td>
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</tr>
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<td>Keratin 4</td>
<td>5’ CAGAATGTCCGAGAATGGCC</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>3’ GTC TCC TCTATGTC TCTTGC</td>
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<td>Keratin 6A</td>
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<td></td>
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<td>Keratin 14</td>
<td>5’ CAGATCCAGGAGATGATGG</td>
<td>61 IV</td>
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<td>3’ TTG CCATCG TCACATCCAT</td>
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<tr>
<td></td>
<td>3’ TGAACACGGCTTAGCATCC</td>
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<td>Neomycin</td>
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<td>60 III</td>
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<tr>
<td></td>
<td>3’ TCAATTTCGAAACCCAGATC</td>
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</tr>
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<tr>
<td></td>
<td>3’ AAGCTAGGACGGCCATATGG</td>
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<td>*p53: e2.1</td>
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<td>*p53: p53g</td>
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<td>*p53: RDNp53</td>
<td>5’ TCGTAAAGCTGCTAGCATCTGAG</td>
<td>60 II</td>
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</table>

*For combination of 5’ and 3’ p53 primers see table 4.

**MICROARRAY**

The stable FaDu cells expressing ΔNp63α, ΔNp63β and ΔNp63γ respectively were used for microarray analysis. Total RNA was extracted as described above and 20 µg total RNA taken to the microarray facility at Umeå University for preparation of double-strand cDNA and biotinylated cRNA that was hybridized to HG-U133A chips (Affymetrix). All procedures were done according to the Affymetrix protocol in the department of clinical microbiology at Umeå University.
Materials and methods

Expression values were calculated using dCHIP software (Li and Wong, 2001) and default setting (PM-MM model). Expression values from dCHIP were truncated to 0 and probe sets with high variance within replicates or no change between different conditions were excluded. By comparing triplicates of control cells with duplicate samples of each p63 over-expressing clone, genes were identified that had a fold change of at least 2.0 at 90% confidence and a mean expression level of at least 20 in either treated or control arrays.

CHROMATIN IMMUNOPRECIPITATION (ChIP)

To investigate whether p63 directly binds to the CD44 and Cox-2 gene promoters, chromatin immunoprecipitation assay (ChIP) was used, EZ ChIP™ (Upstate, Lake Placid, NY, USA), according to the manufacturer’s protocol. Potential binding sites were identified using a programme designed to recognize p53 response elements. Antibodies used were Δ2, which recognizes ΔNp63 isoforms (KN-Δ) (Nylander et al, 2002) and pre-immune sera from Δ2. Immunoprecipitated DNA was analysed by PCR using primers for the CD44 promoter region 573bp upstream of the transcription initiation site and primers for the Cox-2 promoter region 1076 bp upstream of the transcription initiation site. As positive control for the assay, TRAF4 (Gu et al, 2007) was used and at least three separate experiments were performed. Primer sequences are given in table 5.

IMMUNOHISTOCHEMISTRY

To confirm keratin expression in SCCHN tumours immunohistochemistry was performed. Paraffin-embedded samples were sectioned, dewaxed and protocols for each antibody optimized. Staining was performed using a Ventana staining machine and reagents according to the supplier’s recommendations. Immunohistochemistry was performed by Kerstin Näslund.
RESULTS AND DISCUSSION

EXPRESSION OF P63, COX-2, EGFR, β-CATENIN AND PP2A IN SCCHN PATIENTS AND IN ORAL MUCOSA FROM SMOKERS (PAPER I)

p63, Cox-2, EGFR, β-catenin and PP2A are proteins suggested to be involved in the development of SCCHN, and smoking is one of the most well known risk factors for progression of SCCHN. Both Cox-2 and EGFR have previously been found to be altered in smokers compared to non-smokers (Moraitis et al., 2005; Takeyama et al., 2001). To investigate if smoking leads to alterations in protein expression we set out to map the expression of p63, Cox-2, EGFR, β-catenin and PP2A in oral mucosa from smokers compared to oral mucosa from non-smoking age- and sex- matched individuals. Furthermore, we investigated the expression of these proteins in squamous cell carcinoma of head and neck patients. Twelve matched pairs of smokers/non-smokers and 14 tumours and corresponding clinically normal tissue were studied using western blot and specific antibodies directed against the different proteins. Expression of the individual proteins was then quantified.

Expression of Cox-2, EGFR, β-catenin and PP2A in SCCHN patients and smokers/non-smokers

Results from western blot quantification showed that expression of Cox-2 and PP2A was significantly higher in samples from SCCHN patients than from normal tumour adjacent tissue, while expression of EGFR and β-catenin was not. Analysis of protein expression in smokers and non-smokers showed no significant difference in the expression of Cox-2, EGFR, β-catenin or PP2A. The reason we could not confirm previous data stating that Cox-2 and EGFR expression is connected to smoking could be that previous studies have mapped RNA levels whereas we studied protein expression. Another factor to take into consideration is the limited size of our material comprising 12 matched pairs of smokers/non-smokers.

p63 expression in smokers/non-smokers and SCCHN patients

Both smokers/non-smokers and tumour/normal tumour adjacent tissue were subjected to analysis with the 4A4 antibody recognizing all p63 isoforms and the long-2 antibody detecting the TAp63 isoforms only. The 4A4 antibody detected a band in 2/12 smokers, 6/12 non-smokers, 13/14 normal and 8/14 tumours whereas the long-2 antibody detected protein in 8/9 smokers, 8/11 non-smokers, 13/14 normal samples and 3/14 tumours. Due to lack of material, analysis with the long-2 antibody could not be done on all samples from smokers/non-smokers.
The statistical analysis of p63 expression based on the 4A4 antibody revealed no significant difference between smokers and non-smokers, however, normal tumour adjacent tissue showed significantly higher levels of p63 compared to tumours. Previous data has indicated ΔNp63 to be up-regulated in tumours compared to normal tissue based on studies of RNA (Thurfjell et al, 2004). This could not be confirmed in the present study. The discordance between the results may be explained by the measurement of protein and RNA respectively. However, results from analysis with the long-2 antibody showed that TAp63 is up-regulated in normal tumour adjacent tissue which is in accordance with previous studies that have revealed TAp63 to be expressed in normal tissue in bladder cancer while levels in tumours decreased (Park et al, 2000). On the other hand, data from transgenic mice showed that in skin, reactivated expression of TAp63 predisposed to tumour development and progression (Koster et al, 2006). Taken together this suggests that TAp63 functions are highly complex and may not always activate tumour suppressor pathways.

Protein expression in normal tissue adjacent to the tumours compared to normal tissue from non-smoking individuals
Quantification of western results showed that clinically normal tissue adjacent to the tumour is not normal regarding expression of proteins involved in tumourigenesis. For example β-catenin, EGFR and PP2A were more expressed in normal tumour adjacent tissue compared to tissue from disease-free non-smokers although not statistically significant. Our data are in accordance with previous data showing that the genetic changes in tumour patients occur within a field of 7 cm in diameter from the tumour and can give rise to a second primary tumour (Tabor et al, 2004; Tabor et al, 2002). This finding is important to take into consideration when collecting control material from patients with neoplastic changes.

EXPRESSION OF P53 ISOFORMS IN SCCHN (PAPER II)

One of the most common genetic alterations in SCCHN is disruption of the p53-pathway. However, the prognostic significance of p53 in SCCHN is reported to vary. One reason may be presence of different isoforms of p53, thus we investigated the expression of p53 isoforms in tumour samples from patients with SCCHN.
Results and discussion

Various p53 isoforms at RNA level in SCCHN
Twenty-one tumour samples (T), sixteen normal tumour adjacent samples (N) and eight non-smokers (NS) were analysed using nested RT-PCR and primers designed previously by our collaborators (Bourdon et al, 2005). Primers used were p53 isoform specific for p53, p53β, p53γ, Δ133p53, Δ133p53β and Δ133p53γ. Results are summarized in table 6.

Table 6. Summary of p53 isoform expression in SCCHN. Number of samples; tumours 21, normal tumour adjacent 16 and non-smokers 8

<table>
<thead>
<tr>
<th></th>
<th>p53</th>
<th>p53β</th>
<th>p53γ</th>
</tr>
</thead>
<tbody>
<tr>
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<td>T 21</td>
<td>N 16</td>
<td>NS 8</td>
</tr>
<tr>
<td>Variable</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Negative</td>
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<table>
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<tr>
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<th>Δ133p53</th>
<th>Δ133p53β</th>
<th>Δ133p53γ</th>
</tr>
</thead>
<tbody>
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<td>N 9</td>
<td>NS 3</td>
</tr>
<tr>
<td>Variable</td>
<td>3</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Negative</td>
<td>7</td>
<td>4</td>
<td>1</td>
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</tbody>
</table>

In a few samples variable results were seen with one replicate showing positive result and one negative result. The reason for the variable results is most likely that levels of the isoforms were very low, and nested-PCR a very sensitive method enabling amplification of only a few molecules. By ordinary RT-PCR we could strengthen this assumption. Using more RNA as starting material could be a plausible way of getting more consistent results. However, our data indicated that the levels of the different p53 isoforms are low in SCCHN.

Detection of in vitro translated and endogenously expressed p53 proteins
p53 protein is mainly regulated by post-translational mechanisms and the expression of p53 RNA and protein thus not correlated, emphasising the importance of studying protein expression as well. By producing in vitro translated p53 isoform proteins we analysed different antibodies directed against p53, to see if they could recognize the different isoforms. Results showed that the DO-12 antibody directed against an epitope in the DNA-binding domain and the polyclonal antibody Sapu recognized all the different in vitro translated isoforms of p53.
Results and discussion

DO-1, recognizing an epitope in the N-terminal, only identified p53, p53β and p53γ, and the 1801 antibody, distinguishing an epitope located within residues 32-79, recognised all isoforms except Δ133p53. When tumours and normal tumour adjacent tissue were analysed using DO-1 a few samples with several bands could be identified where a few of these bands showed a shift in molecular weight indicating that different isoforms were expressed. However, that could not be confirmed with the DO-12 antibody. Results showed p53 isoforms to be expressed at too low levels for detection with the antibodies used. A limiting factor is that antibodies are not specific for the individual isoforms and maybe also not sensitive enough. In order to determine the impact of the different isoforms in clinical samples we thus need better isoform specific antibodies.

p53 isoform expression in FaDu cells

In our control SCCHN cell line, FaDu, all isoforms except Δp53 were expressed at consistent RNA level. However, at protein level we could not detect any isoform except the full-length p53 using four different antibodies, indicating either low levels of these or lack of translation.

OVER-EXPRESSION OF P63 IN FADU CELLS (PAPER III)

The specific regulation of different p63 isoforms is not fully known and also hard to study in vivo. By transfecting cells with cDNA for the different isoforms and using a selective agent, the different isoforms can be constitutively expressed. The cell line used, FaDu, was derived from SCCHN of oropharynx.

Expression of p63 isoforms

Cells with different p63 status, FaDu and Saos-2 cells were transiently transfected with the six p63 variants in order to investigate protein expression and maintenance of the isoforms. Both FaDu and Saos-2 cells showed similar patterns using western blot analysis of protein extracts from cells harvested at 24, 48, 72, 96, and 120 hours after transfection. TAp63 isoforms were only detectable up to 48 hours after transfection and TAp63β more unstable than TAp63α and TAp63γ. ΔNp63α and ΔNp63γ were expressed up to 96 hours after transfection while ΔNp63β was lost after 72 hours. These data indicated that cells poorly tolerate TAp63 and expression decreases more rapidly than ΔNp63 that is more tolerated by cells. Alternatively it could be that the half-life of TAp63 is shorter than for ΔNp63.
Establishment of cell lines stably expressing p63

Due to an antibiotic resistance gene in the vector containing the gene of interest, transfected FaDu cells constitutively expressing the gene can be selected by using an antibiotic as selection “medium”. We used geneticin (G418) to select clones expressing p63. Established clones were screened by western blot except clones transfected with empty vector that were screened by RT-PCR. Among 115 G418-resistant clones tested, not a single clone expressed detectable levels of the TA\textsubscript{p}63 isoforms. The inability to express TA\textsubscript{p}63 isoforms may not be so strange due to its indicated ability to induce growth arrest and apoptosis (Ghioni \textit{et al}, 2002; Gressner \textit{et al}, 2005). On the other hand, a few clones expressing Δ\textsubscript{N}p63 could be established, one expressing Δ\textsubscript{N}p63\textsubscript{α}, one expressing Δ\textsubscript{N}p63\textsubscript{β} and two expressing Δ\textsubscript{N}p63\textsubscript{γ}. In total, 122 Δ\textsubscript{N}p63 transfected clones were tested. All clones selected for expression of the empty vector showed positive result by PCR. In accordance with previous data, Δ\textsubscript{N}p63\textsubscript{β} showed lower expression compared to Δ\textsubscript{N}p63\textsubscript{α} and Δ\textsubscript{N}p63\textsubscript{γ} (Ghioni \textit{et al}, 2002). Data indicated that cells do not tolerate high levels of Δ\textsubscript{N}p63\textsubscript{β} which is the most efficient transactivator of the isoforms.

Potential p63 Targets (Paper III and IV)

The regulatory network of p63 is complex and incompletely mapped, however, it is known that p63 can activate or repress transcription of p53 target genes but also has unique transcriptional targets. p63 plays a key role in development of normal squamous epithelium and in squamous cancers alterations in p63 expression has been observed. To further investigate the p63 network intense research is ongoing trying to find potential regulators of p63 as well as potential targets. In these studies we used the microarray technology in the search for potential targets of p63 in the SCCHN derived cell lines over-expressing p63.

Cox-2 a potential target of p63

Up-regulation of Cox-2 in various cancers including SCCHN has been previously seen. When analysing the clones stably over-expressing the different Δ\textsubscript{N}p63 isoforms using the microarray technique we identified Cox-2 up-regulation compared to control cells. Δ\textsubscript{N}p63\textsubscript{α} caused a 3.5 fold up-regulation, Δ\textsubscript{N}p63\textsubscript{β} an 11.6 fold up-regulation and Δ\textsubscript{N}p63\textsubscript{γ} a 2.7 fold up-regulation. Results were confirmed by quantitative RT-PCR showing similar data as the microarray. Further analysis of Cox-2 protein expression in p63 over-expressing cells showed an increase in all three clones compared to cells transfected with empty vector. In addition, transient transfections also revealed an up-regulation caused by p63 expression although a lower induction compared to stable transfectants.
As the transfection procedure itself induces Cox-2 expression, interpretation of transient transfections is more complicated. Chromatin immunoprecipitation of a potential p63 binding site in the Cox-2 promoter did not show any direct binding of p63 to the Cox-2 promoter indicating that the activation of Cox-2 by p63 occurs via an indirect mechanism. One way of regulating Cox-2 is via NF-κB, which is activated by IKKα, which in turn can be activated by p63. An association between accumulation of NFκB and ΔNp63 with SCCHN has previously been shown (King and Weinberg, 2007). Our data did, however, not support this theory, instead indicated activation through some other alternative pathway.

Expression and regulation of CD44 in FaDu cells
CD44 is a transmembrane glycoprotein cell receptor involved in cell migration and cell adhesion. RNA from the stable clones established was used for microarray analysis revealing CD44 as a potential target of p63. Microarray data showed a 2.0-fold up-regulation with both ΔNp63α and ΔNp63γ, and a 3.9-fold up-regulation with ΔNp63β. Microarray data was confirmed using semi-quantitative RT-PCR, where all three ΔNp63 isoforms showed induced expression of CD44 compared to cells transfected with empty vector. As CD44 has been suggested to be a cancer stem cell marker, our data indicated ΔNp63 as a regulator of stem cell phenotypes in SCCHN.

We also investigated if p63 directly binds to the CD44 gene promoter by chromatin immunoprecipitation. Results could not confirm any direct binding of p63 to the CD44 promoter region used in this study. However, based on these results we cannot exclude that p63 binds to other sites in the CD44 promoter, or that CD44 regulation by p63 is indirect.

In cancer, different CD44 isoforms are predominantly expressed and the importance of combinations of different variants of exons has been discussed. Using primers located in the exons before and after the region comprising the variant exons we could identify expression of several variants in FaDu cells. Especially one of the isoforms was induced by ΔNp63, the 1100 bp isoform. Sequencing of this isoform revealed it to be the isoform containing all variant exons except exon two. These data indicate that p63 has a role in regulation of CD44 mRNA splicing.

p63 regulates the expression of keratins
Keratins are important differentiation markers and their expression in SCCHN has been discussed and analysed. Our microarray data showed four keratins to be potentially regulated by the Δp63 isoforms compared to control cells:
Results and discussion

<table>
<thead>
<tr>
<th>ΔNp63α</th>
<th>ΔNp63β</th>
<th>ΔNp63γ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Keratin 6A: Up-regulated</td>
<td>12.0</td>
<td>15.7</td>
</tr>
<tr>
<td>Keratin 14: Up-regulated</td>
<td>3.4</td>
<td>4.2</td>
</tr>
<tr>
<td>Keratin 4: Down-regulated</td>
<td>37</td>
<td>52</td>
</tr>
<tr>
<td>Keratin 19: Down-regulated</td>
<td>2.5</td>
<td>3.0</td>
</tr>
</tbody>
</table>

Data was confirmed using semi-quantitative RT-PCR and western blots from protein extracts of the clones stably expressing the ΔNp63 isoforms. Results from these analyses were consistent with the microarray data. In addition, immunohistochemistry of normal buccal mucosa and SCCHN tissue identified ΔNp63 in the basal layer up to approximately two-thirds of the epithelial thickness in the normal buccal mucosa and strong expression all through the SCCHN tumours. Keratin 14 was highly expressed in the basal layer of normal oral epithelium and also strongly expressed in tumours while keratin 6A was not expressed in normal oral epithelium but expressed in tumour cells. Keratin 19 could not be detected and keratin 4 was not expressed in the most basal cells and cells at the surface of normal oral epithelium but highly expressed in the other cell layers. Keratin 4 was not expressed in tumours. Keratin 14 was significantly induced by p63 and expressed at high levels in SCCHN, and has previously been shown to have a binding site in the enhancer to which ΔNp63 binds and transactivates the gene (Romano et al., 2007). Combining these and our data indicated that increased expression of keratin 14 in SCCHN at least in part is due to transcriptional activation by increased amounts of the ΔNp63 isoforms.
GENERAL DISCUSSION

Novel findings add valuable information to the understanding of the mechanisms behind SCCHN although the picture also becomes more complex. Work included in this thesis focuses on a known player in SCCHN, p63, as well as potential targets of p63. Looking at the literature, controversies concerning results from different studies of these proteins are found, possibly due to the use of different model systems, different tissues as well as different techniques.

Previous data from our lab has shown ΔNp63 to be significantly higher expressed in SCCHN tumours compared to normal tumour adjacent tissue at RNA level (Thurfjell et al., 2004). However, when analysing protein expression using western blot on samples from SCCHN patients, some of them also included in the previous study of RNA, no increased expression of the ΔNp63 isoforms could be seen. When comparing these western blot data with immunohistochemical staining of paraffin embedded samples of the same tumours it seemed that by immunohistochemistry proteins were highly expressed whereas when using western blot they were not recognized at all times. As no paraffin embedded samples from normal tumour adjacent tissue was available we could not compare the two methods on these samples, which were the ones showing highest TAp63 expression using western blot. However, it can be concluded that results from immunohistochemical and western blot analysis do not correlate, indicating that the antibodies we used for detection of p63 were not ideal for western blot analysis. Another reason for the difference between western blot and immunohistochemistry is the different levels of contamination by for example blood, lymphocytes and connective tissue that may vary in western blot samples whereas in immunohistochemistry you look at tumour cells only. Furthermore, it should be taken into consideration when comparing results from RNA and protein analysis that RNA and protein expression do not always correlate. It has previously been shown that p53 is mainly regulated at the post-translational level and p63 RNA and protein expression do not always correlate either (Gu et al., 2006; Katoh et al., 2000; Okada et al., 2002), which may reflect post-translational modifications of p63 that influence protein stability.
Crosstalk between proteins

Development of cancer is a complex event of genetic and epigenetic alterations and like a big puzzle all pieces must fit together in order to get the whole picture. The present work has investigated several players in the signalling pathways of SCCHN tumour formation, however, the crosstalk between the different molecules are only partly investigated. In addition, other studies have also found connections between the different players. The p53 family itself is complex with several isoforms and crosstalk between these are important to consider. For example it has been suggested that ΔNp73 and mutant p53 associate with the ΔNp63 promoter and induce its transcription (Lanza et al., 2006). Furthermore, p73 expression has been shown to affect the DNA-binding capabilities of p63 isoforms (Johnson et al., 2007). Previous studies have shown a physical interaction between EGFR and CD44 leading to phosphorylation of EGFR in a cell line derived from oral squamous cell carcinoma (Wang and Bourguignon, 2006). In addition, EGFR is connected to Cox-2 that in turn is connected with β-catenin as well as p63. Activation of EGFR stimulates Cox-2 production in colon cancer cell lines (Coffey et al., 1997) and accumulation of β-catenin can lead to up-regulation of Cox-2 (Araki et al., 2003). The link between Cox-2 and CD44 is somewhat contradicting, with studies that have found Cox-2 to suppress CD44 as well as studies showing activation of CD44 to induce Cox-2 (Kinugasa et al., 2004; Murphy et al., 2005). These results may depend on different model systems or the fact that different CD44 variants have different functions. The known correlations between p63 and these proteins show that ΔNp63 can induce β-catenin accumulation in the nucleus (Patturajan et al., 2002) and EGFR has been shown to regulate p63 (Matheny et al., 2003) but in contrast TAp63γ has been shown to repress EGFR expression (Nishi et al., 2001). Our data further indicate that p63 can regulate Cox-2, CD44 and keratins. Figure 5 shows a proposed model of the protein network in SCCHN.

In summary, our data show that Cox-2 and PP2A are up-regulated and that several isoforms of p53 are expressed in SCCHN. Furthermore we show that p63 may be a regulator of Cox-2, CD44 and keratins, whereas no direct connection to β-catenin or EGFR could be identified. Our microarray data were confirmed using several methods, and in retrospect, additional immunohistochemical analysis as a complement to western blot analysis would have been valuable. The importance of confirming findings from cell lines in clinical material must also be stressed.
Figure 5. Network of p63 and potential p63 targets. p63 has previously been shown to interact with PP2A leading to β-catenin accumulation in the nucleus. Furthermore β-catenin has been suggested to induce Cox-2 expression. In our studies we saw a clear upregulation of Cox-2 by over-expression of p63. Another player suggested to interact with Cox-2 is CD44 which we in our studies has seen to be regulated by p63. EGFR can interact both with Cox-2 and CD44. Finally we also found that keratins can be regulated by p63 that has previously been observed to interact with CD44. Black arrows represent previously found connections and red arrows represent connections found in the present thesis. Double arrow represents the interaction in both directions.
CONCLUSIONS

- SCCHN tumours expressed significantly higher levels of Cox-2 and PP2A compared to clinically normal tissue adjacent to SCCHN, however no significant difference was seen in expression of EGFR or β-catenin. p63 was significantly higher expressed in normal tumour adjacent tissue compared to tumours. Furthermore a difference in protein expression of p63, Cox-2, EGFR, β-catenin, and PP2A could be observed between normal tumour adjacent tissue and normal tissue from disease-free non-smokers.

- Smoking did not influence the expression of p63, Cox-2, EGFR, β-catenin and PP2A proteins in oral mucosa.

- p53 isoforms were expressed in SCCHN patients at RNA level, in particular the p53β isoform, but could not be detected at protein level.

- Cell lines stably over-expressing the ΔNp63 isoforms could be established but not cell lines over-expressing the TAp63 isoforms.

- Cox-2 was found to be a potential p63 target, showing increased expression at both RNA and protein levels in cells over-expressing p63 compared to control cells. p63 up-regulates expression of CD44 and specifically regulated expression of the CD44 variant lacking exon 2 in p63 over-expressing FaDu cells. Keratins 6A and 14 were up-regulated and keratins 4 and 19 down-regulated by p63.

The molecular signalling behind SCCHN is a complicated process. Data from this thesis add to the field that p53 and p63 isoforms, CD44, keratins and Cox-2 may be potential players in this complicated network.
ACKNOWLEDGMENTS

Till sist skulle jag vilja tacka alla som funnits där för mig under mina doktorandår och hjälpit mig på olika sätt och gjort resan så mycket trevligare. Speciellt TACK till:

Min handledare Karin Nylander, det har varit otroligt lärorikt, roligt och spännande att få jobba med dig, tack vare att du gett mig så mycket frihet och ansvar har jag utvecklats och lärt mig så mycket om ”vårat” område, forskning och mig själv. Jag hade inte kunnat haft en bättre handledare än du.

My co-supervisor Philip Coates, even though you have been in Dundee you have always been close by. With e-mails and skype we have solved a lot of scientific issues. I am grateful for all your help, valuable discussions about science and my projects. Your reviews have been invaluable.

Göran Roos, min bihandledare, du har alltid funnits som ett tryggt stöd i rummet bredvid när jag behövt kloka råd. Tack för att du hjälppte mig att få ihop min första föreläsning.

Our colleagues, Jean-Christophe Bourdon in Dundee and Borek Vojtesek in Brno, for fruitful collaborations and help with all kinds of questions and methods. Big thanks to you and your respective co-workers for all your hospitality when I was working in your labs.

Bodil Bäcklund, vad skulle jag ha gjort utan dig. Din erfarenhet i labbet och cellodlingen är enorm och det har varit en ära att få lära mig smarta knep av dig. Tack för att du introducerat opera i mitt liv, perfekt att ha en egen biljettförsäljare så nära.

Åke Dahlqvist, som tyvärr lämnade oss i förtid, var den drivande kraften i insamlandet av viktigt patientmaterial.


Magnus Walhgren, för att du tog dig tid med en frågestund angående klinikbiten.

Ulla-Britt Westman, en levande uppslagsbok när det gäller alla möjliga metoder på lab, det har varit mycket värdefullt att få protokoll och tips från dig. Du har ju även med intresse följt mina stora händelser utanför jobbet.

Åsa Skytt, du har alltid något smart knep när maskiner strular eller någon logisk förklaring till varför antikroppar inte fungerar.

Veronica Janson, för att du har funnits som ett värdefullt bollplank när det gäller metoder, manuskriptskrivande och alla möjliga saker.
Acknowledgments

Ylva Hedberg, du lärde mig vikten av optimering av antikroppar vid western körning, hur man kvantifierar proteinband och kom med ovärderliga knep om manusriptskrivande.

Kerstin Näslund och Britta Lindgren, för hjälpen med immunohistofärgningar.

Bethany van Guelpen, Anna Dahlin och Vincy Eklöf, mina rumskamrater, Bethany du hann ju flytta ut innan jag blev klar men tack för trevliga pratstunder om träning och allt annat viktigt i livet under våra år i rummet. Anna och Vincy, trevligt med nytt sällskap även om jag smet till annat rum på slutet.

Karin Bodén, Terry Persson, Åsa Lundstå, Anette Lindström och Clara Olsson, för hjälpen med all pappersexercis och diskning.

Hela fikagänget i 6M huset, för den trevliga stämningen här i 6M-huset, ni förgyller vardagen med små pratstunder och gott fika.

KN gruppen: Niklas Thurfjell min gamla rumskamrat och doktorandkollega, för intressanta p63- diskussioner och trevligt sällskap. Xiaolian Gu, thanks for all your help with methods we use, enjoyable company at conferences and for spreading so much happiness around you. Majid Ebrahimi, det var roligt att få guida dig på labbet och då du hann före mig med disputation kunde jag ju åka lite snålskjuts på ditt avhandlingskunskande. Matilda Rentoft, du har redan introducerat nya spännande metoder och tankesätt för oss, jag ser fram emot p63-mötet och fortsatt gott samarbete.

Elisabet Berglöf, tack min vän för allt under åren. Tänk att det är snart 10 år sedan vi satt på universum och åt lunch för första gången, vi har hunnit med en del sen dess, tänk att vi snart är doktorer båda två! Vad ska vi nu hitta på?

Mina vänner, det är så härligt att ni finns och förgyller min fritid. Det är toppen med ett avbrott från jobbet slit ibland, med en långlunch, filmkväll, middag, fest, en sväng till fjällen eller något annat roligt.

Familjen Gustafsson, min svärfamilj, för att ni så hjärtligt välkomnat mig i er familj. Det är skönt och trevligt att ha en extrafamilj i stan när mamma och pappa bor en bit bort.

Min underbara familj, Mamma, Pappa, Johan, Karin, Mormor och Farmor, för att ni alltid tror på mig och stöttar mig i allt jag gör. Det är alltid lika härligt att tillbringa tid tillsammans, jag älskar er!

Mikael, min käre man, för att du alltid finns där för mig och får mig att inse att världen inte går under för att försöken på jobbet inte fungerat. Det är sagolikt att få dela livets äventyr med dig. Jag älskar dig av hela mitt hjärta!
This project was supported by Lion’s Cancer Research Foundation, Umeå University, The Swedish Cancer Society, Grant number 4569-B05-05XAC and Västerbotten County Council.
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Reference list updated 2008-01-07