Jenni Peltonen

**TP53 AS CLINICAL MARKER IN HEAD AND NECK CANCER**

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TP53 AS CLINICAL MARKER IN HEAD AND NECK CANCER

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**Abstract**

The prognosis of patients with head and neck squamous cell carcinoma has improved only little during the last decades. Clinical markers for the biological aggressiveness of the cancer are few. The most reliable prognostic indicator is the stage of the disease.

Research of the significance of the *TP53* tumor suppressor gene as a predictive marker for prognosis and response to treatment in head and neck cancer has given discrepant results. One reason is probably the attempt to use p53 immunohistochemistry as a surrogate for *TP53* mutations. However, in immunohistochemistry the protein is analyzed and thus the result does not usually correlate with *TP53* mutations. The marker for prognosis of the response to treatment has to be reliable, and the analytical method needs to be both sensitive and specific. In addition to a sensitive method for *TP53* mutation analysis the localization and quality of the mutations have to be analyzed to reveal the significance of the mutation on the function of the p53 protein.

In this study, *TP53* mutations were analyzed, using a sensitive PCR-SSCP method, in the tumors of patients with head and neck squamous cell carcinoma. The quality and localization of mutations were analyzed by sequencing. The frequency of the *TP53* mutations and the effect on the function of the p53 protein were studied using IARC *TP53* mutation database and literature. Correlation of *TP53* mutations with chemical exposure and their significance on prognosis and response to radiation treatment was studied. In addition, the significance of cell cycle regulators cyclin D1, p16, p21 as potential markers of biological aggressiveness of tumors was studied.

The results of this study showed that the patients carrying in their tumor *TP53* mutations in the DNA binding region of the gene had been exposed to chemicals more than patients with no mutation or other types of mutations. These mutations also correlated with biological aggressiveness and prognosis and the response to radiation treatment. It was also shown that a combination of cyclin D1 and p16 analyzed by immunohistochemistry correlated with worse prognosis in head and neck cancer.

**Keywords:** head and neck cancer, prognostic marker, survival, TP53 mutation
Peltonen, Jenni, *TP53* kliinisenä merkkiaineena pään ja kaulan alueen syövissä. 
Oulun yliopisto, Lääketieteellinen tiedekunta, Kliinisen lääketieteen laitos, Syöpätaudit ja sädehoito, Biollääketieteen laitos, Farmakologia ja toksikologia, PL 5000, 90014 Oulun yliopisto; Itä-Suomen yliopisto, Terveystieteiden tiedekunta, PL 1627, 70211 Kuopio

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Oulu

**Tiivistelmä**

Pään ja kaulan alueen levypeiteleyysöpää sairastavien potilaiden ennuste ei ole juurikaan paranut viime vuosikymmeninä. Syövän biologista aggressiivisuutta kuvaavia ennustetekijöitä on vähän, luotettavimpina niistä taudin levinneisyys.

Tutkimustulokset


Väitöskirjatutkimuksen tulokset osoittivat, että potilaat, joiden kasvaimissa oli *TP53*-mutaa tio DNA-sitoutumisalueella, olivat altistuneet kemikaaleille enemmän kuin potilaat, joiden kasvaimissa oli toisenlaista mutaaatioita tai ei mutaaatioa olennaan. Tutkimuksessa havaitut mutaa tiot liittyivät taudin biologiseen aggressiivisuuteen ja huonoon ennusteeseen sekä heikompaan sädehoitovasteeseen. Lisäksi havaittiin, että sykliini D1- ja p16-proteiinin yhdistelmä immunohistokemiallisesti analyysoituna korreloi huonoon ennusteeseen pään ja kaulan syövissä.

**Asiasanat:** ennuste, merkkiaine, pään ja kaulan alueen syöpä, *TP53*-mutaatio
To Eevi, Severi and Tuomas
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Oulu, September, 2011

Jenni Peltonen
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>A</td>
<td>adenine</td>
</tr>
<tr>
<td>BP</td>
<td>benzo(a)pyrene</td>
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<tr>
<td>C</td>
<td>cytokine</td>
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<tr>
<td>CCND1</td>
<td>cyclin D1</td>
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<td>CDK</td>
<td>cyclin dependent kinase</td>
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<td>CDKI</td>
<td>cyclin dependent kinase inhibitor</td>
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<tr>
<td>CR</td>
<td>complete response</td>
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<td>CT</td>
<td>computed tomography</td>
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<td>CYP</td>
<td>cytochrome P450</td>
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<tr>
<td>DBD</td>
<td>DNA-binding domain</td>
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<td>DFS</td>
<td>disease-free survival</td>
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<tr>
<td>DGGE</td>
<td>denaturant gradient gel electrophoresis</td>
</tr>
<tr>
<td>DNE</td>
<td>dominant negative effect</td>
</tr>
<tr>
<td>DM</td>
<td>distant metastasis</td>
</tr>
<tr>
<td>DSS</td>
<td>disease-specific survival</td>
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<tr>
<td>EBV</td>
<td>Ebstein-Barr virus</td>
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<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>ESMO</td>
<td>European Society for Medical Oncology</td>
</tr>
<tr>
<td>G</td>
<td>guanine</td>
</tr>
<tr>
<td>GOF</td>
<td>gain of function</td>
</tr>
<tr>
<td>Gy</td>
<td>Gray (J/kg)</td>
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<tr>
<td>HNSCC</td>
<td>head and neck squamous cell carcinoma</td>
</tr>
<tr>
<td>HPV</td>
<td>human papilloma virus</td>
</tr>
<tr>
<td>IARC</td>
<td>International Agency for Research on Cancer</td>
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<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
</tr>
<tr>
<td>IR</td>
<td>ionizing radiation</td>
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<tr>
<td>LOF</td>
<td>loss of function</td>
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<tr>
<td>LOH</td>
<td>loss of heterozygosity</td>
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<td>LSCC</td>
<td>laryngeal squamous cell carcinoma</td>
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<td>LSH</td>
<td>loop-sheet-helix</td>
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<tr>
<td>M</td>
<td>metastasis</td>
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<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
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<td>MDM2</td>
<td>murine double minute-2</td>
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<td>M phase</td>
<td>mitosis phase</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
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<tr>
<td>N</td>
<td>node</td>
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<tr>
<td>NGS</td>
<td>next-generation sequencing</td>
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<tr>
<td>NLS</td>
<td>nuclear localization signal</td>
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<tr>
<td>OD</td>
<td>oligomerization domain</td>
</tr>
<tr>
<td>OS</td>
<td>overall survival</td>
</tr>
<tr>
<td>p16/INK4A</td>
<td>CDKN2A, cyclin dependent kinase inhibitor 2A</td>
</tr>
<tr>
<td>p21/WAF1/Cip1</td>
<td>CDKN1A, cyclin dependent kinase inhibitor 1A</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PFS</td>
<td>progression free survival</td>
</tr>
<tr>
<td>pTEN</td>
<td>phosphatase and tensin homologue</td>
</tr>
<tr>
<td>Rb</td>
<td>retinoblastoma</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<tr>
<td>RNS</td>
<td>reactive nitrogen species</td>
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<tr>
<td>RT</td>
<td>radiotherapy</td>
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<tr>
<td>SCC</td>
<td>squamous cell carcinoma</td>
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<td>S phase</td>
<td>synthesis phase</td>
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<td>SSCP</td>
<td>single-strand conformation polymorphism</td>
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<td>SV40</td>
<td>Simian virus 40</td>
</tr>
<tr>
<td>T</td>
<td>thymine</td>
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<tr>
<td>TP53</td>
<td>TP53 tumor suppressor</td>
</tr>
<tr>
<td>TA</td>
<td>transactivation activity</td>
</tr>
<tr>
<td>TADI</td>
<td>transactivation domain I</td>
</tr>
<tr>
<td>TADII</td>
<td>transactivation domain II</td>
</tr>
<tr>
<td>TNM</td>
<td>tumor, node, metastasis</td>
</tr>
<tr>
<td>TS</td>
<td>temperature sensitivity</td>
</tr>
<tr>
<td>UICC</td>
<td>International Union Against Cancer</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
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<tr>
<td>WT</td>
<td>wild type</td>
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List of original articles

This thesis is based on the following articles, which are referred to in the text by their Roman numerals:


*Peltonen J née Hakkarainen J
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1 Introduction

Head and neck cancers represent a heterogeneous group of tumors (Psyrri et al. 2010). The vast majority (more than 90%) are squamous cell carcinomas (HNSCCs), so that the term head and neck cancer is often used to describe all carcinomas arising from the epithelial lining of the sinonasal tract, oral cavity, pharynx, and larynx, and showing microscopic evidence of squamous differentiation (Ferlay et al. 2010, for a review see Pai & Westra 2009). Globally, almost 600,000 new cases are diagnosed every year. Although patients presenting with early-stage disease have rather high cure rates, those diagnosed with a locoregionally advanced disease have had relatively poor prognosis and limited therapeutic options. Their 5-year survival is approximately 50%. These tumors are commonly diagnosed at advanced stages and mortality rates remain high. (Psyrri et al. 2010, Schaaij-Visser et al. 2010) The last decade has seen the integration of chemoradiotherapy with some improvements in the survival in HNSCC patients. The major cause of death among these patients is a local-regional recurrence. At present, the prognosis for patients with HNSCC is largely determined by the stage of the disease, particularly the presence of lymph node metastases (for a recent review see Leemans et al. 2011).

It is estimated that around 43% of cancer deaths are due to tobacco use, unhealthy diets, alcohol consumption, inactive lifestyles and infection, and synergistic effect between exposures is likely (Petersen 2009). Synergism has been demonstrated between smoking and radon or asbestos in lung and esophageal cancers (IARC 2004, Nelson & Kelsey 2002). The most important risk factors for HNSCC are tobacco and excessive alcohol consumption. Alcohol potentiates the tobacco-related carcinogenesis and is an independent risk factor as well. However, the role of environmental exposures, including occupational factors, in head and neck carcinogenesis has not been fully revealed. In addition, human papilloma virus (HPV) infection has been associated with HNSCC, mostly cancer in the oropharynx (Chung & Gillison 2009).

TP53 mutations are common in HNSCC. TP53 gene encodes the p53 protein involved in many key events in the cell, like regulation of cell cycle, and glucose metabolism in cancer cells, DNA-repair, apoptosis and senescence (Bensaad & Vousden 2007). The potential existence of a molecular linkage between carcinogen exposure and cancer is illustrated by the TP53 mutation spectrum in different human cancers (Vähäkangas 2003). Although large amounts of studies have investigated the value of TP53 status as a prognostic marker in various types
of cancer, results have often been contradictory (Olivier et al. 2007). The main reason certainly resides in the fact that the majority of studies have used immunohistochemistry (IHC) to assess the TP53 status. However, IHC detects protein with antibodies and is not a method for analyzing TP53 gene mutations (Hainaut & Vähäkangas 1997, Robles & Harris 2010). Certainly, when mutation detection is used for clinical purposes, it is important to use a proper optimized and validated method, such as PCR-SSCP and sequencing, which are currently commonly used in carcinogenesis research. The diversity of the type and functional consequences of TP53 gene mutations is another issue that needs to be taken into account when analyzing the prognostic value of mutations.

Advancing understanding of molecular biology of HNSCC has significant potential for the development of clinical markers for prognosis and treatment selection in patients with HNSCC. The ultimate goal in studying the molecular biology of HNSCC is to apply this information to everyday clinical practice in an effort to improve patient outcome.

The present study was designed to study TP53 alterations as a clinical marker of aggressiveness in head and neck squamous cell carcinoma. Furthermore, the expression of some other cell cycle-regulating proteins such as cyclin D1, p16 and p21 in tumor tissue was explored, with specific focus on whether they correlate with patient outcome.
2 Review of the literature

2.1 Head and neck squamous cell carcinoma

2.1.1 HNSCC epidemiology

Head and neck squamous cell carcinoma (HNSCC) is a significant health concern worldwide with a prevalence of more than 1.6 million (Ries et al. 2006). The incidence of HNSCC is higher in men than in women. (Psyrri et al. 2010) According to the Finnish Cancer Registry, approximately 650 new cases are diagnosed each year in Finland (Finnish Cancer Registry, Cancer Statistics at www.cancerregistry.fi, accessed 12 May 2011). Despite rapid advances in the treatment of HNSCC, the 5-year survival has improved only marginally, from 54.4% to 59.4%, over the past two decades (Forastiere et al. 2001, reviewed by Psyrri et al. 2010, Schaaij-Visser et al. 2010). Survivors suffer serious and devastating morbidities including speech and swallowing problems, disfigurement and exorbitant healthcare costs (Psyrri et al. 2010, Vissink et al. 2003). Although the age-adjusted incidence of laryngeal, oral cavity, and hypopharyngeal cancers has been in decline, the incidence of oropharyngeal cancer has been on the rise, particularly among individuals under 45 years of age (reviewed by Pai & Westra 2009, Toner & O’Regan 2009).

Etiology of head and neck squamous cell cancer

The most important risk factors for HNSCC are tobacco and excessive drinking of alcohol. Alcohol potentiates the tobacco-related carcinogenesis and is also an independent risk factor (Altieri et al. 2004, Rodriguez et al. 2004, reviewed by Boffetta & Hashibe 2006, Argiris et al. 2008, La Vecchia et al. 2008). However, the respective contributions of these risk factors are difficulty to study because these two habits are strongly associated with each other (Waddell & Levy 2000). The relative risk for head and neck carcinoma among tobacco and alcohol abusers is 20 times that of non-smokers and non-drinkers (Hashibe et al. 2009). However, for both cessation of drinking and cessation of smoking, the risk was reduced to the level of never users after 20 years of quitting these habits (Marron et al. 2010). Epidemiologic studies also suggest a strong association between most types of smokeless tobacco products (e.g., chewing tobacco, khaini, snuff) and oral
carcinogenesis (Cogliano et al. 2004, Chen et al. 2006, Petersen 2009, for a review see Boffetta et al. 2008). Smokeless tobacco consumption is emerging as a major risk factor for HNSCC. (IARC 2007, Petersen 2009, Secretan et al. 2009) Although the combination of tobacco and alcohol account for more than 75% of the population-attributable risk, other factors have been recognized as contributing to the etiology of head and neck squamous cell cancer (for a recent review see Dasgupta et al. 2011). These include gastroesophageal reflux, poor dental hygiene, occupation, infectious agents, diet and various inherited syndromes (Sturgis et al. 2004, Toner & O’Regan 2009). Occupational risk factors include exposure to hard wood dust, concrete dust, cement dust, metal dust and textile fibers (Muscat & Wynder 1992, Dietz et al. 2004, for a review see Wünsch Filho 2004). In addition to the factors mentioned above, heavy metals such as cadmium, nickel and lead can cause cancer of the upper aerodigestive tract (for a recent review see Khlifi & Hamza-Chaffai 2010). Moreover, most studies suggest that oral cancer patients have a history of diet low in fruit and vegetables (Boeing et al. 2006, Pavia et al. 2006, Peters et al. 2008, Edefonti et al. 2010). In a quite recent meta-analysis, a combined estimate based on 16 studies showed that each serving of fruit consumed per day reduced the risk of oral cancer by approximately 50% (Pavia et al. 2006). Based on a number of epidemiologic studies, low dietary folate intake is recognized as an independent risk factor for HNSCC (Almadori et al. 2002, Pelucchi et al. 2003, Kane 2005, Bossi et al. 2008, Kawakita et al. 2011). In addition, reactive oxygen species (ROS), reactive nitrogen species (RNS), diabetes and a family history of HNSCC are also potential risk factors for HNSCC (Bahar et al. 2007, Rasheed et al. 2007).

A growing amount of evidence suggests that some viruses contribute to the cause of head and neck cancer. Human papilloma virus (HPV) has also been identified as an etiologic agent in a subset of HNSCCs, mostly cancer in the oropharynx. (Chung & Gillison 2009, Lajer & von Buchwald 2010, Snow & Laudalio 2010) The HPV types (most often HPV16 and occasionally HPV18) identified in HPV-positive tonsillar SCC are similar to those found in cervical cancers. (for a recent review see Lajer & von Buchwald 2010) HPV infection may even act synergistically with carcinogenic agents such as tobacco and alcohol. (Snow & Laudalio 2010) However, there is strong correlation between history of nonsmoking and HPV infection (Weinberger et al. 2006, D’Souza et al. 2010, Jung et al. 2010), and a correlation between non-drinking and HPV-positive status has also been found (Gillison et al. 2000, Weinberger et al. 2006, D’Souza et al. 2010), although some studies found contradicting results (Braakhuis et al. 2004, 22
Jung et al. 2010). Epstein-Barr virus (EBV) has been strongly linked to the development of nasopharyngeal carcinoma (Chien et al. 2001, Yoshizaki et al. 2011).

Fanconi anemia and dyskeratosis congenital are rare inherited syndromes that cause head and neck squamous cell cancer. Several other well characterized but rare inherited syndromes have been to some extent associated with HNSCC, e.g. laryngeal cancer in Bloom and Li Fraumeni syndromes, and oral cancer in Bloom syndrome, ataxia telangiectasia and xeroderma pigmentosa (Toner & O’Regan 2009). Li-Fraumeni syndrome is a classic cancer predisposition disorder that is commonly associated with germline mutations of the TP53 tumor suppressor gene (Malkin 2011).

### 2.1.2 Diagnosis and staging

The symptoms of HNSCC patients depend on the location of the primary anatomical site and stage of the tumor. Staging is based on tumor, node and metastasis (TNM) classification and usually done according to the International Union Against Cancer’s (UICC) classification system (Sobin et al. 2009). (Table 1) After clinical assessment and imaging studies (ultrasound, head and neck computed tomography (CT) scan or magnetic resonance imaging (MRI), chest x-ray, positron emission computed tomography (PET/CT)), eventually including fine needle aspiration cytology, malignant head and neck tumors are staged clinically according to the TNM classification system (Sobin et al. 2009). Prognostic information related to the size of the primary tumor (T), extent of regional metastatic disease (N), and presence/absence of distant metastasis (DM). (M) is combined according to an internationally standardized system and the disease is categorized as Stage I-IV. TNM staging is the most important tool for assessing prognosis in patients with HNSCC (see a recent review Leemans et al. 2011). However, it must be noted that there are important prognostic features (e.g. depth of infiltration, extracapsular spread) that are not included in the TNM staging system and need to be considered separately.
Table 1. TNM classification for oral cavity squamous cell carcinoma.

<table>
<thead>
<tr>
<th>TNM class definition</th>
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<tr>
<td><strong>T- Primary Tumor</strong></td>
</tr>
<tr>
<td>Tis: Carcinoma in situ</td>
</tr>
<tr>
<td>T1: ≤2cm</td>
</tr>
<tr>
<td>T2: &gt;2cm to 4cm</td>
</tr>
<tr>
<td>T3: &gt;4cm</td>
</tr>
<tr>
<td>T4a: Invades through cortical bone, deep/extrinsic muscle of tongue, maxillary sinus, skin</td>
</tr>
<tr>
<td>T4b: Invades masticator space, pterygoid plates, skull base, internal carotid artery</td>
</tr>
<tr>
<td><strong>N – Regional Lymph Nodes</strong></td>
</tr>
<tr>
<td>NX Regional lymph nodes can not be assessed</td>
</tr>
<tr>
<td>N0 No regional lymph node metastasis</td>
</tr>
<tr>
<td>N1 Ipsilateral single ≤3 cm</td>
</tr>
<tr>
<td>N2 N2a Ipsilateral single &gt;3cm to 6 cm</td>
</tr>
<tr>
<td>N2b Ipsilateral multiple ≥6 cm</td>
</tr>
<tr>
<td>N2c Bilateral or contralateral ≥6 cm</td>
</tr>
<tr>
<td>N3 &gt;6 cm</td>
</tr>
<tr>
<td><strong>M – Distant Metastasis</strong></td>
</tr>
<tr>
<td>MX Distant metastasis can not be assessed</td>
</tr>
<tr>
<td>M0 No distant metastasis</td>
</tr>
<tr>
<td>M1 Distant metastasis</td>
</tr>
</tbody>
</table>


2.1.3 Treatment modalities in HNSCC

The current primary management of HNSCC includes radiation therapy and surgery, either alone or in combination with chemotherapy (reviewed by Forastiere et al. 2001, Langendijk et al. 2010). Modern surgical and chemoradiation techniques seem effective in improving local control and providing reduction in patient morbidity, thus offering better quality of life (Nutting et al. 2011). Radical management of HNSCC, especially laryngeal squamous cell carcinoma (LSCC), can still dramatically affect the quality of life of those patients who survive (for reviews see Vissink et al. 2003, Chin et al. 2006).

The cornerstones in the treatment of HNSCC are surgery and radiotherapy (for recent reviews see Mehra et al. 2008, Langendijk et al. 2010, Pyrró et al. 2010). For early stage (I-II), either of these modalities can be used alone (Licita & Felip 2009, Langendijk et al. 2010). For locally advanced stage III and IV
combined therapy (surgery plus RT) is preferable. Postoperative radiotherapy is generally considered standard in the case of high-risk factors after surgery alone, including positive (<1 mm) or close (1–5 mm) surgical margins, lymph node metastases with extranodal spread, 2 or more positive lymph nodes, invasion of the soft tissues and/or skin of the neck, more than 5 mm subglottic extension and perineural growth (Langendijk et al. 2010).

A change in the treatment protocol has taken place over the past decade, however. Concomitant chemoradiotherapy is increasingly used as primary treatment for advanced tumors instead of surgery as it allows organ preservation, while surgery is preserved for salvage treatment (Forastiere et al. 2001, Brockstein & Vokes 2004). It seems that because of organ preservation, patients may achieve better long-term quality of life after chemoradiotherapy compared with the use of conventional surgery and postoperative radiation (Nguyen et al. 2002). Chemoradiotherapy is generally recommended as an adjuvant therapy in the postoperative setting for patients with high risk for recurrence instead of RT alone (Brockstein & Vokes, 2004, for ESMO clinical recommendations see Licitra & Felip 2009).

The preferred treatment depends not only on primary tumor location and extension, but also on the expertise of the institute where the treatment is given. In addition, a multidisciplinary treatment schedule should be established in all cases. Many patient-related aspects need to be considered when choosing the most appropriate treatment for an individual patient. Overall, despite the progresses in treatment, only modest improvement in the prognosis of patients diagnosed with HNSCC has been reported in the last decades. In Finland, the most favorable improvement in survival rates has been reported in the treatment of pharynx cancer. The prognosis of larynx cancer patients has improved only very slightly in Finland. The prognosis of oral cavity cancer has remained about the same (Parkin et al. 2005, Jemal et al. 2011).

Monoclonal antibody against epidermal growth factor receptor (cetuximab) used in combination with radiation therapy has recently been established as an effective regimen for improving regional control and survival in patients with locoregionally advanced HNSCC (Bonner et al. 2006, Curran et al. 2007).

Cisplatin plus fluorouracil has been widely accepted as a reference regimen in patients with recurrent or metastatic HNSCC (for ESMO clinical recommendations see Licitra & Felip 2009). Vermorken et al. (2008) have noticed that combining cetuximab with platinum-based chemotherapy with fluorouracil (platinum-fluorouracil) significantly prolongs the median overall survival.
However, single-agent cisplatin or methotrexate are acceptable alternatives (Argiris et al. 2008). Among the new drugs, taxanes have substantial activity as monotherapy (Colevas 2006). At present, there is no standard second-line chemotherapy regimen for the treatment of recurrent or metastatic HNSCC (Argiris et al. 2008).

2.1.4 Prognostic factors

A major problem with most types of head and neck cancer is that more than half of the patients present with locally advanced cancer at the diagnosis phase although distant metastasis are uncommon (Seiwert & Cohen 2005). Early detection of this type of cancer would therefore dramatically increase the survival rate (Seiwert & Cohen 2005, for a recent review see Dasgupta et al. 2011). A second important reason for this poor survival is the relatively high recurrence rates observed in these patients (Morris et al. 2011a,b). Carcinomas recur at the primary site in about 30–50% of the cases with advanced tumors, even after complete resection of the primary carcinoma. (Argiris et al. 2008) Complete resection implies that the margins of the surgical specimen have been thoroughly investigated by a pathologist and are reported to be free of tumor. A local recurrent tumor is assumed to be the result of growth of tumor tissue left in place after surgery of the primary tumor and is presently defined according to clinical criteria as occurring within two-three years after the first tumor at a distance less than 2 cm away from that tumor. (Argiris et al. 2008, for a recent review see Leemans et al. 2011) Another reason for poor survival is that second primary tumors often develop in at least 2 cm between primary tumors (Leemans et al. 2011). The time interval between the occurrence of the primary and secondary carcinoma is also considered as a criterion. Tumors that arise after three years are usually considered second primary tumors. (Leemans et al. 2011) These second primary tumors develop on average with a constant rate of 3–4% per year (Di Martino et al. 2000). The low rate of survival of patients with locoregional and distant recurrences has highlighted the need for new approaches for diagnosis and treatment.

The established prognostic factors are primary tumor site, TNM stage and comorbidity, including performance status (Mehanna et al. 2010b). The prognosis for patients with HNSCC is largely determined by the stage at presentation (Leemans et al. 2011). The extent of the tumor, as well as the presence of lymph-node metastases and distant metastases, determines the stage. Staging of HNSCC
is assessed by clinical examination, imaging, cytology of lymph nodes and
definite histopathology after surgery (such as radicality and extranodal spread).
Histologically, HNSCC is usually classified as well, moderately or poorly
differentiated SCCs. The histologic tumor grade is based on degree of squamous
differentiation (keratinization, pearl formation and intercellular bridges), degree
of cellular pleomorphism and mitotic index (number of visible mitotic figures) in
tumor cells. Some studies have found tumor grade to have prognostic significance,
but determinations of grade using traditional histopathologic criteria often vary
among different observers. Therefore, tumor grade is of limited prognostic value
compared with clinical stage, as many or most HNSCCs are graded as moderately
differentiated, and no clear correlation has been found between grade and tumor
size. (Thomas et al. 2005). Useful markers associated with biological aggressive
behavior are needed to predict the outcome of the HNSCC patients. However,
none of potential prognostic factors has been accepted as a routine clinical marker.

2.2 Carcinogenesis

2.2.1 Chemical carcinogenesis

Carcinogenesis is a multistep process driven by carcinogen-induced genetic and
epigenetic damage in susceptible cells that gain a selective growth advantage
(Olivier et al. 2004). Cigarette smoking (Lubin et al. 1984, Peto et al. 1996) and
dietary factors (IARC 1983) are among the most important external chemical risk
factors of cancer. Other well-known risk factors include drugs and exposure to
environmental and occupational agents. Today, about 200 different compounds
and mixtures are known or anticipated to be human carcinogens (Luch 2005,
http://www.monographs.iarc.fr). Chemical carcinogens can be classified by their
function into two categories, genotoxic and non-genotoxic carcinogens. Most
genotoxic carcinogens require metabolic activation into forms that bind to cellular
macromolecules and thereafter exert their carcinogenic effects. Whereas some
complete carcinogens have the capacity to induce the whole process of
carcinogenesis, many others need to act together to generate cancer because they
act specifically at a certain stage of carcinogenesis (for a review see Irigaray &
Belpomme 2010). When confronted with carcinogen exposures, human cells
develop responses similar to those for any other foreign compound or drug. A
great majority of human chemical carcinogens require metabolic activation to
elicit detrimental effects (reviewed by Luch 2005). Chemical carcinogens are metabolized by a wide variety of carcinogen-metabolizing enzymes, of which the cytochrome P450 (CYP) family of enzymes are the most important (Pelkonen & Raunio 1997, Shi et al. 2007).

2.2.2 Molecular mechanisms of carcinogenesis

Cancer is a leading cause of death worldwide. Cancer is regarded as a genetic disease that occurs due to sequential accumulation of genetic alterations especially in oncogenes and tumor suppressor genes (Vähäkangas 2003, Vogelstein & Kinzler 2004). These alterations cause abnormal activation or inactivation of a number of critical pathways and signaling cascades, resulting in uncontrolled cellular growth (reviewed by Vogelstein & Kinzler 2004). The hallmarks of cancer comprise six biological capabilities acquired during the multistep development of human tumors. They include sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis (reviewed by Hanahan & Weinberg 2000, 2011). Furthermore, an increasing body of research suggests that some enabling hallmarks of cancer are involved in the pathogenesis of cancer. Most prominent is the development of genomic instability in cancer cells. A second enabling characteristic involves the inflammatory state of premalignant and malignant lesions that is driven by cells of the immune system, some of which serve to promote tumor progression through various means (Hanahan & Weinberg 2011).

Environmental, viral and chemical agents, ionizing radiation, as well as physical substances can promote carcinogenesis (reviewed by Peto 2001, Wogan et al. 2004), increasing the rate of cell proliferation (Hanahan & Weinberg 2011). After the exposure to the carcinogen, 20–40 years can pass until the clinical detection of a solid tumor (reviewed by Wogan et al. 2004). The risk of cancer can therefore be associated with lifestyle and environmental factors, even though hereditary factors also play a role (reviewed by Peto 2001, Ponder 2001, Balmain et al. 2003).

The carcinogenesis process can be described as a series of consecutive genetic changes, analogous to evolution, leading to the conversion of normal cells into cancer cells (Hanahan & Weinberg 2000). Carcinogenesis can be modeled in three stages: initiation, promotion and progression (Foulds 1954, for a recent review see Irigaray & Belpomme 2010). Tumor initiators can be defined as
carcinogens capable of inducing the first driver mutation in a dividing cell, through direct or indirect mutagenesis, so that an initial clone of mutated cells can emerge. Tumor promoters can be defined as non-genotoxic carcinogens capable of causing clonal expansion of initiated cells, i.e. able to induce proliferation of mutated cells and to prevent these cells from undergoing apoptotic loss, so the possibility of additional genetic and/or epigenetic changes is preserved.

The majority of tumors are monoclonal since they derive from a single progenitor cell. In a multistep tumorigenesis process, clonal expansions involving genetic and epigenetic alterations follow each other (reviewed by Ponder 2001, Balmain et al. 2003) (Figure 1). Within a tumor, different subclones can have distinct alterations caused by simultaneous clonal expansion of different clones as a result of instability in a tumor genome (reviewed by Hanahan & Weinberg 2011, Leemans et al. 2011). Moreover, the accelerated cell proliferation in cancer allows mutations to occur at an increased rate (Hanahan & Weinberg 2011). Furthermore, communication of different cell types in a tumor microenvironment is important in cancer development and progression. Tumor-surrounding stromal cells, for instance, can contribute to angiogenesis and invasion. Communication between cancerous epithelial cells and stromal cells can also cause changes in stromal cells, differentiating them from the normal state (reviewed by Tlsty & Hein 2001).

2.2.3 Molecular biology of head and neck cancers

It is known that HNSCC as well as other cancers emerge after the accumulation of genetic changes in epithelial cells exposed to carcinogenesis. This hypothesis consists of the following basic principles: neoplasms are the result of inactivation of tumor-suppressor genes and/or activation of proto-oncogenes, and there could be variations in the order of genetic events, but it is eventually the net accumulation of genetic alterations that determines the malignant phenotype (Kim & Califano 2004). The opportunity of a genetic predisposition to cause head and neck cancer is suggested by its sporadic occurrence in some cases in young adults, non-smokers and non-drinkers.
Tumors often develop within preneoplastic fields of genetically altered cells. Head and neck squamous cell carcinoma develops in the mucosal linings of the upper respiratory and digestive tract (for a recent review see Leemans et al. 2011). Common regions of chromosomal loss reported in HNSCC are at 1p, 3p, 4p, 5q, 8p, 10p, 11q, 13q, and 18q, with gains at 1q, 3q, 5p, 7q, 8q, 9q, 11q, 12p, 14q, and 15q (Glazer et al. 2009, Leemans et al. 2011). One of the most studied oncogenes in HNSCC is the epidermal growth factor receptor (EGFR). It has been shown to be overexpressed in a majority of HNSCC tumors (Kalyankrishna & Grandis 2006, Leemans et al. 2011). This receptor tyrosine kinase belongs to the ErbB family of cell surface receptors and has many downstream signaling targets associated with carcinogenesis. Once phosphorylated, the receptor can signal via the MAPK, Akt, ERK, and Jak/STAT pathways. These pathways are related to cellular proliferation, apoptosis, invasion, angiogenesis, and metastasis (Kalyankrishna & Grandis 2006, Leemans et al. 2011). Molecular changes include amplification and overexpression of amplification of cyclin D1 and other oncogenes (reviewed by Perez-Ordonez et al. 2006, Leemans et al. 2011). In addition, TP53 tumor suppressor gene mutations and p16 alterations have been described and may have a role in HNSCC molecular carcinogenesis (Brennan et al. 1995, for reviews see Perez-Ordonez et al. 2006, Pai & Westra 2009, Molinolo et al. 2009, Leemans et al. 2011).
2.3 Cell cycle regulators

2.3.1 Cell cycle

Disruption of the physiological balance between cell proliferation and death is a universal feature of all cancers. The cellular programs of proliferation, differentiation, senescence and apoptosis are closely linked to the cell cycle regulatory machinery. Many of the molecular alterations causing abnormal biological behavior of cancer cells are based on aberrations of cell cycle regulation (reviewed by Todd et al. 2002). The mammalian cell division cycle is divided into four phases. In the S phase (synthesis phase) cells generate a single copy of their own genetic material, which is then divided between two identical daughter cells (mitosis or M phase). The gap phases of the cell cycle, G1 and G2, are stages during which cells prepare for the orderly execution of the S and M phases. Non-dividing cells can exit the cell cycle and enter a quiescent state (G0) (Figure 2).

Fig. 2. Cell cycle regulators and cell cycle.
Cyclin-dependent kinases (Cdks) are the driving force behind the cell cycle transitions and are regulated by cyclins (for a review see Kim & Diehl 2009). Indeed, there are at least nine Cdks (Cdk1 to Cdk9) in mammalian cells. Noteworthy, Cdk4, Cdk6, Cdk2 and Cdk1 are involved in cell cycle regulation (Schafer 1998). The cyclins of the D-type family (D1, D2, and D3) bind and activate Cdk4 and Cdk6 (Bates et al. 1994). Binding to a cyclin partner changes the structure of Cdks and is necessary for the activation of Cdks (Jeffrey et al. 1995). Cdks also have a role in DNA replication. There are several ways available when preventing constant activation of the Cdks. Cyclin-dependent kinase inhibitors (CDKIs) are able to inhibit the kinase activity of Cdks by mimicking ATP-binding and preventing formation of an active conformation (Vidal & Koff 2000). Two families of CDKI have been identified. The INK4 (inhibitors of Cdk4) family members p15\textsubscript{INK4B}, p16\textsubscript{INK4A}, p18\textsubscript{INK4C} and p19\textsubscript{INK4D} specifically inhibit cyclin D-associated kinases. The Cip/Kip family currently has three members: p21\textsubscript{Cip/Waf1/Sdi1}, p27\textsubscript{Kip1} and p57\textsubscript{Kip2}, which bind and inhibit cyclin A and cyclin B-associated Cdks (reviewed by Tsihlias et al. 1999). An interesting variation to the traditional inhibitory activities of CDKIs has emerged from studies showing the Cip/Kip family members promoting the assembly and activation of cyclin D-Cdk complexes (Cheng et al. 1999, Sherr & Roberts 1999, for a recent review see Kim & Diehl 2009).

### 2.3.2 Cyclin D1

The \textit{CCND1} gene is a proto-oncogene located on chromosome 11q13 encoding cyclin D1, being a key regulator of the G1 phase of the cell cycle (Lukas et al. 1995). There is increasing evidence for a role of cyclin D1 in G1 phase progression in the cell cycle (reviewed by Kim & Diehl 2009). Overexpression of cyclin D1 accelerates progression through the G1 phase of the cell cycle and reduces the requirement of the cell for mitogens (Quelle et al. 1993, for review see Diehl 2002). Overexpression of cyclin D1 has been reported in a variety of human tumors, including head and neck squamous cell carcinomas, and has been associated with aggressive behavior in HNSCC including higher local recurrence, higher metastatic potential and diminished survival (Jares et al. 1994, El-Naggar et al. 1995, Akervall et al. 1997, Michalides et al. 1997, Fracchiolla et al. 1997, Namazie et al. 2002, Higuchi et al. 2007). The mechanisms underlying cyclin D1 overexpression in cancer include gene amplification, chromosomal translocation and mitogenic stimulation of gene transcription (Kim & Diehl 2009). Cyclin D1
is an important cell cycle protein binding cyclin-dependent kinase (CDK) 4 or 6 to phosphorylating and inactivating the RB1 tumor suppressor protein, thus driving the cell from G1 into S-phase. This is suggested to promote cancer cell growth (Ragin et al. 2006).

2.3.3 p16

p16NK4A is a member of the Ink4 family of CDK inhibitors. It is encoded by a gene localized on chromosome 9p21 within the INK4A/ARF locus, which encodes for two different proteins with different promoters: p16INK4A and p19ARF. Both proteins have antiproliferative biological activity, and are involved in the retinoblastoma protein (Rb) and p53 pathways, respectively (Serrano 1997, Weber et al. 2003). It is well known that p16INK4A contributes to the regulation of cell cycle progression by inhibiting the S phase (reviewed by Romagosa et al. 2011). In addition to the action of p16 in cell cycle regulation, this protein has also been implicated in other processes, such as apoptosis, cell invasion and angiogenesis, and these activities may be related to its overexpression in cancer. Close to 50% of all human cancers show p16 inactivation ranging from 25 to 70%; these include head and neck squamous cell carcinoma (Gonzalez & Serrano, 2006).

p16 is over-expressed in human papillomavirus (HPV)-related tumors in an unsuccessful attempt to stop cell proliferation (Reuschenbach et al. 2008). An absence of TP53 mutations in addition to expression of p16 is part of the distinct molecular profile identified in the subset of HNSCC because of HPV (Snow & Laudagio 2010). Recent studies have shown that patients with HPV16-positive HNSCC have an improved DFS and better response to radiotherapy, and thus a better prognosis than those with HNSCC unrelated to HPV (Dayyani et al. 2010, for a review see Syrjänen 2005). In this context, p16 overexpression has been suggested to have a major impact on treatment response and survival in patients with head and neck cancer treated with conventional radiotherapy (Gupta et al. 2009, Lassen et al. 2009, Fisher et al. 2010).

2.3.4 p21

The cyclin-dependent kinase (CDK) inhibitor p21 (CDKN1A) mediates the induction of cell-cycle arrest in response to a variety of stimuli, mainly through its ability to inhibit the kinase activity of CDK2 and CDK1 (for a recent review see
Abbas & Dutta 2009). The role of p21 in promoting DNA damage-induced G1 growth arrest relies to a great extent on its well-described transcriptional activation by p53 (Macleod et al. 1995, Abbas & Dutta 2009). Given its crucial role in halting cellular proliferation, it is not surprising that p21 has been found to be frequently misregulated in human cancer (Abbas & Dutta 2009). p21 somatic mutations are extremely rare (Abukhdeir & Park 2008). The expression of p21 is usually reduced or absent in quiescent cells. Thus, the loss of its expression or function has been implicated in the genesis or progression of many human malignancies (Macleod et al. 1995, Abbas & Dutta 2009). However, other studies also suggest that p21 can promote cancer, indicating a paradoxical effect leading to tumor-suppressing or tumor-promoting properties of p21, depending on the cellular context (Rowland & Peeper 2006, Abukhdeir & Park 2008, Abbas & Dutta 2009). Although the significance of p21 has been investigated in several different cancers including head and neck cancers, clinically the association between p21 and prognosis has been controversial (Erber et al. 1997, Jeannon et al. 1999, Pruneri et al. 1999, Fischer et al. 2011).

2.3.5 p53

The protein encoded by the \textit{TP53} tumor suppressor gene, the p53 tumor suppressor, was first identified in 1979 as a protein that was bound by SV40 T-antigen in SV40 induced tumors (Robins et al. 2005). p53 protein becomes activated as a response to stress signals. It is a sequence-specific DNA-binding transcription factor that binds DNA as a tetramer and activates or represses transcription of a large number of target genes, associated with e.g. cell cycle arrest, DNA repair genes and apoptosis-inducing genes (El-Deiry 1998, Vousden & Lu 2002, Sax & El-Deiry 2003, Ho & Benchimol 2003). Moreover, the p53 protein is known to be involved in angiogenesis and regulation of oxidative stress and glucose metabolism (Komarova et al. 2005, Hussain & Harris 2006, Bensaad & Vousden 2007). Gadea et al. (2007) showed that a loss of wild-type p53 function was enough by itself to confer an increased migratory capacity to cells (Gadea et al. 2007).

The p53 protein is expressed in almost all tissues as a constitutively repressed protein. The main mechanism of repression is protein-protein interaction with the product of the oncogene MDM2, which targets p53 protein to proteasome degradation. This feedback regulatory loop allows cells to recover from G1 arrest. Several classes of signals can lead to the de-repression of p53 and to its
accumulation by posttranslational modifications (for a recent review see Hollstein & Hainaut 2010). These signals include DNA-damaging agents (genotoxic stress), constitutive activation of growth signaling cascades (oncogenic stress) as well as other types of stress, such as depletion in ribonucleotides or hypoxia (Jungmann 2000).

In response to abnormal proliferative signals and many forms of cellular stress, p53 induces cells to undergo a transient arrest in G1 that is believed to allow time for repair of damaged DNA before the initiation of S phase. (Kastan et al. 1991) Loss of the p53 function or loss of the ability to activate a p53 response induces tumors allowing proliferation of the cells with a DNA-damage and promotes neoplasia in transgenic p53 null mice (Donehower et al. 1992, for a review see e.g. Lozano & Zambetti 2005). It appears that several factors, including cell type specificity, the presence or absence of survival factors in the external environment, the extent of DNA damage, the level of p53 and the post-translational modifications, are involved in the choice between cell cycle arrest and apoptosis (for a review see Liebermann et al. 2007).

A large amount of studies have associated abnormal p53 protein expression as well as somatic TP53 mutation with poor survival or lack of response to therapy (for a recent review see Robles & Harris 2010). Nonetheless, the clinical significance of p53 status for patient outcome continues to be one of the most controversial areas of p53 research (Munro et al. 2005).

2.4 TP53 tumor suppressor gene and p53 protein

2.4.1 Structure of TP53 gene and p53 protein

TP53 gene is located on human chromosome 17p13.1. It is 20 kb long and contains 11 exons, with the first exon being non-coding. (Hainaut & Hollstein 2000) The TP53 belongs to a small family of related genes including two other members, p63 and p73. TP53 encodes for a 53kDa phosphoprotein that is expressed at very low levels in the nucleus of normal cells (Soussi et al. 2000, Hainaut & Hollstein 2000). It is active as a homotetramer (Joerger et al. 2006, Wang & El-Deiry 2007). The human p53 has 393 residues. The p53 transcription factor has a classical organization, including an N-terminal transactivation domain (a major sub-domain, TADI, residues 1–40; and a minor one, TADII, residues 43–73); a proline-rich domain (residues 65–97); a central DNA-binding
domain (DBD, residues 102–292); a hinge region containing the main nuclear localization signal (NLS, residues 300–325); a C-terminal oligomerization domain (OD, residues 325–356); and a basic region (residues 363–393) (Marcel & Hainaut 2009). (Figure 3) The p53 DBD is made of an immunoglobulin like b-sandwich of two antiparallel b-sheets, providing a scaffold for a flexible DNA-binding surface (Cho et al. 1994). This surface is formed by two large loops (L2 and L3) stabilized by a zinc atom and a loop–sheet–helix motif (loop L1). Zinc binding (coordinated by His179, Cys176, Cys238, and Cys242) is critical for correct folding, and requires reduction of thiol groups on cysteines (for a review see Olivier et al. 2010). Hotspot mutations are at residues involved either in making contacts with DNA (DNA contact mutations) or in supporting the structure of the DNA-binding surface (structural mutations) (Joerger et al. 2007).

Fig. 3. The TP53 gene and protein. TP53 mutation distribution in human cancer (as described in the IARC database) and mutation hot spots are shown in relation to the exons and functional domains. Highly conserved regions are marked with darker shading. IARC database http://www-p53.iarc.fr Published with permission.

2.4.2 TP53 mutations in human cancer

Somatic mutations in TP53 are found in approximately 40% of all human cancers (http://www-p53.iarc.fr). The frequency varies according to tumor type and ranges from about 10% in leukemia to up to 60% in ovarian and colorectal cancers. Wide variations have been reported between different studies of the same tumor type, probably reflecting methodological and geographic differences. In
cancers with low mutation rates, p53 is often inactivated by alternative mechanisms.

Cancer-associated mutations in TP53 are primarily missense substitutions non-randomly distributed along the molecule, particularly in the central DNA-binding-domain (http://www-p53.iarc.fr). These single amino acid changes affect p53’s transcriptional activity to various degrees. Not all mutations have equal deleterious effect on p53 function, and some missense mutants may even acquire new functions (Martin et al. 2002, Kato et al. 2003, Petitjean et al. 2007, for a recent review see Oren & Rotter 2010). Martin et al. (Martin et al. 2002) have analyzed structural data of different TP53 mutations based on crystal structure of TP53 gene (Cho et al. 1994), changes in the properties of amino acids and hydrogen bonding between different amino acids after amino acid substitution (Baker & Hubbard 1984). There is a growing amount of evidence that not only the TP53 mutation itself but structural and functional effects in the TP53 gene are important to p53 transcriptional activity. Different mutations have very different consequences for the function of the p53 protein.

Most of the TP53 mutations in human cancers are missense mutations (Hainaut & Hollstein 2000) that can either cause a loss of tumor suppressor function (LOF) in various degrees or, in some cases, a gain of oncogenic function (GOF) (Van Oijen & Slootweg 2000, Cadwell & Zambetti 2001, Kato et al. 2003, Petitjean et al. 2007). In addition to various degrees of LOF, some mutant proteins inhibit the functions of the wt allele by a dominant-negative effect (DNE) (see TP53 Function database, http://www-p53.iarc.fr, van Oijen & Slootweg 2000, Cadwell & Zambetti 2001, Petitjean et al. 2007). Recent studies have been carried out in an attempt to provide an explanation for the structural effects of most disease-related TP53 mutations (Cuff & Martin 2004, Martin et al. 2002) and functional impact of TP53 mutations (Petitjean et al. 2007a, Petitjean et al. 2007b, http://www-p53.iarc.fr). TP53 database provides structured data and analysis tools to study mutation patterns in human cancers and cell-lines and to investigate the clinical impact of mutations (http://www-p53.iarc.fr). Experimental assays have been performed in yeast and human cells to measure various properties including: i) transactivation activities (TAs) of mutant proteins on reporter genes placed under the control of various p53 response-elements (p53-RE); ii) capacity of mutant proteins to induce cell-cycle arrest or apoptosis; iii) ability to exert dominant negative effect (DNE) over the wild-type protein; iv) temperature sensitivity (TS) of mutant; and v) activities of mutant proteins that are independent and unrelated to the wild-type protein (gain of function [GOF])
(Petitjean et al. 2007a, b). It contains annotations related to the clinical and pathological characteristics of tumors, as well as the demographics and carcinogen exposure of patients (Olivier et al. 2002).

The TP53 mutational spectrum can be a molecular link to etiological causes of cancer (Hollstein et al. 1991, Levine et al. 1991, Bennett et al. 1999, Hussain & Harris 2000, Petitjean et al. 2007). The TP53 mutational pattern has proved to be a clinically relevant “molecular sensor” of genotoxic exposure to environmental carcinogens and endogenous mutagens (Hussain & Harris 2000, for a review see Pfeifer & Besaratinia 2009). Molecular analysis of TP53 mutations has revealed specific types of mutational patterns linked to carcinogen exposure in some cases, e.g. lung cancer and tobacco (for reviews see Pfeifer et al. 2002, Vähäkangas 2003, Hussain & Harris 2006, Jackson et al. 2006). Tobacco smoke contains many agents that potentially induce G to T transversions, such as nitrosamines, aromatic amines and polycyclic aromatic hydrocarbons as well as other agents causing oxidative stress. The dose-dependent association between cigarette smoking and G-to-T transversions in TP53 in lung cancer subjects has been recognized since the early nineties (reviewed by Shi et al. 2007). According to the literature, G-to-T transversion at codon 157 of TP53 (GTC to TTC leading to an amino acid change Val to Phe) is frequent in lung cancers among smokers, but not in other types of cancer, including lung cancers among never-smokers (Hainaut & Pfeifer 2001, Vähäkangas et al. 2001). On the other hand, in lung tumors GC→AT transitions have been associated with never-smoking status (Husgafvel-Pursiainen et al. 2000, Vähäkangas et al. 2001, Le Calvez et al. 2005, see also http://www-p53.iarc.fr/). The most prevalent mutations described in the literature in HNSCC have been GC→AT transitions (see e.g. a review de Moura Gallo et al. 2005, http://www-p53.iarc.fr/) and GC→TA transversions (see e.g. a review Blons & Laurent-Puig 2003, http://www-p53.iarc.fr/).

2.4.3 Clinical significance of p53 alterations

Analysis of TP53 mutations has been studied as a clinical marker in cancer (for a recent review see Robles & Harris 2010). TP53 mutations, but not p53 positive immunohistochemistry (IHC), have been consistently associated with poor prognosis in cancers such as breast, colorectal, head and neck cancer and leukemia (Petitjean et al. 2007a). TP53 mutations may also serve as biomarkers for targeted therapy (Olivier et al. 2009) A multitude of retrospective studies have associated abnormal p53 protein expression as well as somatic mutations with
poor survival or lack of response to therapy (Robles & Harris 2010). The association of poor prognosis has been reported in head and neck cancers (http://www-p53.iarc.fr). When taking into account laryngeal carcinoma, a total of seven studies found an association with poor prognosis (Erber et al. 1998, Cabelguenne et al. 2000, Obata et al. 2000, Temam et al. 2000, Alsner et al. 2001b, Yamazaki et al. 2003, Poeta et al. 2007, and in three studies TP53 mutation status was not related to prognosis (Ronchetti et al. 2004, Gunduz et al. 2008, Eriksen et al. 2005). It has been shown in a prospective, multicenter trial analyzing TP53 mutation status in squamous-cell carcinoma of the head and neck through p53 GeneChip that presence of any TP53 mutation had an adverse effect on survival, but those mutations that resulted in disrupted DNA binding led to an even worse prognosis (Poeta et al. 2007). In agreement with this, TP53 mutations in direct DNA contact areas have been shown to associate with accelerated tumor progression and reduced therapeutic responsiveness in head and neck squamous cell carcinoma (Erber et al. 1998, Cabelguenne et al. 2000, Temam et al. 2000).

**p53 and radiotherapy**

p53 plays an important role in the response of ionizing radiation (IR). p53 is stabilized in vitro and in vivo after IR. The level of p53 protein accumulation in response to IR primarily results from the intensity of DNA damage. (Fei & El-Deiry 2003) Posttranslational modifications of p53 and its interacting proteins determine its stability and transactivation activity. This stabilization and activation is important for p53’s roles in inducing growth arrest when reversible, which is coupled with DNA damage repair to protect cells from IR damage (El-Deiry 2003). Apoptosis occurs in response to a high dose of IR when the damage is irreparable, in order to eliminate the damaged cells (Zhan et al. 1994, Fei & El-Deiry 2003). Loss of p53 function from mutation or deletion has been shown to influence radiation response and progressive growth of tumor cells in most human cancers (Soussi et al. 2005).

p53 is a central mediator of responses to DNA damage and cellular stress and would therefore be expected to play major roles in determining not only the level of tumor aggressiveness but also of chemosensitivity and radiosensitivity (Vogelstein et al. 2000, Burdelya et al. 2006). Sensitivity to radiation therapy is partly mediated by p53 function. (Zhang et al. 1994, Burdelya et al. 2006) Esophageal squamous cell carcinoma patients with a tumor showing p53 overexpression have been associated with poorer response to radiotherapy
(Miyazaki et al. 2005). In head and neck squamous cell carcinoma conflicting results have been reported concerning radiation response and p53 overexpression (Bradford et al. 1997, Raybaud-Diogene et al. 1997, Koelbel et al. 2001, Osman et al. 2002). However, in rectal carcinoma patients no correlation has been seen between p53 alterations and response to radiotherapy (Lopez-Crapez et al. 2005). Gamma radiation in mice with normal p53 status induces rapid apoptosis while the same does not happen in p53-deficient animals (Komarova et al. 2000). Increased sensitivity to radiation has been seen in human esophageal carcinoma cells that originally bore a p53 mutation, but have been retrovirally transduced with wild-type p53 gene (Matsubara et al. 1999). Moreover, HNSCC cells with mutant p53 and over-expression of normal EGFR did not respond to radiation, anti-EGFR monoclonal antibody or their combination therapy (Laytragoon-Lewin et al. 2009). Furthermore, based on the literature it seemed that no single factor in p53 pathway or missense mutations alone had a correlation to the radiosensitivity in head and neck tumor cell lines (Farnebo et al. 2008). There have been a limited number of inconclusive studies on TP53 mutations in patients treated with primary radiotherapy (for a review, see Silva et al. 2007).

2.5 Analysis of p53 alterations

2.5.1 TP53 mutation analysis

Methods for the analysis of mutations in the TP53 gene are widely used in carcinogenesis research (Szymanska & Hainaut 2003, Vähäkangas 2003) and actively studied in clinical medicine, e.g., as a tool for prognosis and treatment (Lane & Lain 2002, Seemann et al. 2004).

Of the mutation analysis methods for TP53, PCR-SSCP (single-strand conformation polymorphism), denaturant gradient gel electrophoresis (DGGE), and sequencing have taken their place among those most frequently used for this purpose and serve as important reference methods in the validation of new and emerging high-throughput mutation analysis methodologies. Because the SSCP screening method gives an indication of a mutation without specifics about the changed nucleotides, positive results have to be analyzed further by sequencing. (Figure 4) Microarray-based high-throughput methods have replaced the more traditional sequencing methods (Tennis et al. 2006). However, one clear trend in cancer genome sequencing is that the continuing advance of cost-effective NGS
(next-generation sequencing) has almost completely replaced the traditional Sanger sequencer in genome sequencing and re-sequencing for discovery of genetic variation (Huang et al. 2011, for a review see Mardis & Wilson 2009).

Fig. 4. Principle of mutation analysis by SSCP and sequencing. (Peltonen et al. 2006) Copied with permission from Sage Publications.

**Single-strand conformation polymorphism (SSCP)**

Mutations that are detectable by the SSCP method include base substitutions, small insertions, deletions, and rearrangements. In SSCP, the PCR-amplified products can be separated into single strands by denaturation and electrophoresed on non-denaturating polyacrylamide gels (Orita et al. 1989, Hayashi 1992). The separation is based on different electrophoretic mobility of a sequence-specific conformation of the single-stranded DNA fragment (Orita et al. 1989). During electrophoresis single-strand DNA fragments fold into a three-dimensional shape depending on their primary sequence (Orita et al. 1989, for reviews see Peltonen et al. 2006, Kakavas et al. 2008). Changes in the sequence (e.g., point mutations) can cause a shift in the electrophoretic mobility of the analyzed conformations.
compared with wild-type DNA (Peltonen et al. 2006, Kavakas et al. 2008). Because of radioactivity and the requirement for large gels, the original method (Orita et al., 1989) has been largely replaced by non-radioactive PCR-SSCP methods, such as the silver staining SSCP method (Welsh et al. 1997).

Several studies have shown that the optimization of SSCP conditions is essential for analytical sensitivity and efficiency (references are found in the review Peltonen et al. 2006). Single-stranded DNA fragments have more than one stable conformation under particular electrophoretic conditions. Under non-denaturating conditions, single-stranded DNA adopts a secondary structure that is dependent upon its sequence. Because some mutations are detectable only within a very narrow window of temperatures, more than one conformation may occur close to the critical transformation temperature, and therefore, several additional single-stranded bands may be seen (Orita et al. 1989, Sheffield et al. 1993). Thus, the gel temperature is the most critical parameter affecting the conformation and electrophoretic mobility of single strands. Because of this, it is essential to use two running temperatures to obtain good efficiency (Welsh et al. 1997). Several parameters have been empirically found to affect the sensitivity of SSCP analysis (Hayashi et al. 1993). Among them are (i) type of mutation; (ii) size of DNA fragment; (iii) G and C content in fragment; (iv) content of the polyacrylamide or other gel matrix composition; (v) gel size and potential; (vi) gel temperature during electrophoresis; (vii) DNA concentration; (viii) run time of the electrophoresis; (ix) buffer composition, including ionic strength and pH; and (x) buffer additives, such as glycerol or sucrose (Peltonen et al. 2006, Kakavas et al. 2008).

**Sequencing**

DNA sequencing has long been the golden standard for identification of TP53 mutations. It allows the identification of the exact location and type of sequence change. Current manual and automated applications for sequencing the TP53 gene are based on the dideoxy-chain-termination principle. In sequencing, with the use of DNA polymerase, the molecule of double-strand DNA that is to be analyzed acts as a template for the synthesis of DNA copies that all have the same beginning but terminate in different points of the strand (Sanger et al. 1977). The advances in technology have made it possible to study whole cancer genomes using large scale and high-throughput DNA sequencing (for a review see Drmanac et al. 2002). In comparison with microarray-based methods, NGS-based
(next-generation sequencing) methods offer digital reading, larger dynamic signal range and higher reproducibility (for a review see Mardis & Wilson 2009).

2.5.2 Analysis of the effect of TP53 mutations

Several experimental techniques are used to study the effect of mutation TP53 (Bullock & Fersht 2001). Hotspot mutations are at residues involved either in making contacts with DNA or in supporting the structure of the DNA-binding surface. Different hot-spot mutants show different structural changes. DNA-contact mutants retain the overall architecture of the DBD with loss of a critical DNA contact. They may actively prevent DNA binding if a large hydrophobic side chain is introduced (e.g. Ser241Phe, Arg248Trp, and Cys277Phe). Zinc-binding mutants affect the zinc coordination sphere (e.g. Cys176Phe, His179Arg, and Cys242Phe). This category includes Arg175His, the most frequent hotspot mutant, because introduction of histidine residue causes distortions that directly interfere with zinc binding. Substitutions introducing smaller residues at this position have been shown to be less destabilizing with partial retention of function (Bullock et al. 2000). Structural mutants cause distortions that create internal cavities or surface crevices in the protein scaffold, inducing conformational changes in the DNA binding surface. Overall, analysis of the mutations detected in human cancers not only allows definition of the functionally important regions of the protein, but their heterogeneity also allows more precise dissection of the various functions of p53 (Kato et al. 2003). However, mostly mutation frequencies in tumors have been reported, while less attention has been paid to the connection of functional state of the mutated p53 with clinical and environmental aspects of cancer.
2.5.3 p53 immunohistochemistry

As the p53 protein is the end-product of gene expression, it appears logical to try to directly visualize protein expression by immunohistochemical analysis linked with morphological analysis allowing qualitative evaluation of the cells presenting these defects (Dowell et al. 1994). Mutations of the TP53 gene result in the production of a protein with an abnormal structure and prolonged half-life. This protein accumulates in the nucleus, resulting in immunohistochemically (IHC) detectable overexpression. The half-life of wild-type p53 protein is short and it cannot be visualized by immunohistochemistry. Earlier observations have shown that the majority of TP53 mutations are missense mutations that accumulate in cancer cells and can thus be detectable by IHC. However, it is now admitted that p53 IHC is not suitable as a screening method for mutations, since not all types of TP53 mutations are detected (for reviews see Koch & Sidransky 2004, Olivier & Taniere 2011). Nonsense or frameshift mutations do not lead to
accumulation of p53 protein. This is due to instability of truncated proteins, which are generally not detectable despite the use of monoclonal antibodies that recognize an epitope situated in the amino-terminal domain of p53 (Soussi 2009). Sequencing of the complete coding sequence of TP53 shows that 10 to 25% of the mutations are truncating mutations (nonsense, frameshift or splice site mutations) that are not detected by IHC since they do not lead to a stable protein. (Olivier et al. 2007).
3 Aims of the present study

Head and neck squamous cell carcinoma is associated with considerable mortality and morbidity. Over 50% of the patients die of their disease within 5 years after diagnosis, with tumor recurrence, metastasis, and development of second primary neoplasms as major causes of treatment failure. Finding reliable molecular markers for early diagnosis, prognosis and prediction of response to treatment is a major challenge for cancer management. Although aberrations of p53 are the most frequent molecular events in human cancers, it has not been conclusively shown whether p53 aberrations are associated with environmental exposures in head and neck squamous cell cancers. The cancer-associated $TP53$ mutations are primarily missense mutations that may affect the transcriptional activity of the p53 protein. Not all mutations have equally deleterious effects on p53 function. This study pursued the role of $TP53$ and p53 aberrations as biomarkers of environmental exposure in head and neck cancer. The potential use $TP53$ and p53 aberrations as well as some other proteins known as cell cycle regulators in estimating survival and response to treatment were also assessed. More specifically, the objectives were:

- To validate the formerly established PCR-SSCP analysis method by screening for unknown mutations in head and neck squamous cell carcinoma samples
- To determine the frequency and functional consequences of $TP53$ mutations in exons 5-8 in head and neck squamous cell cancer with exposure data
- To establish the putative association between specific $TP53$ mutation types in head and neck squamous cell cancer and clinical outcome of the patients.
- To ascertain the role of other cell cycle regulators in predicting tumor behavior in head and neck squamous cell carcinoma.
4 Materials and methods

4.1 Strategy of the project

The study pursued the role of \textit{TP53} and p53 aberrations as biomarkers of environmental exposure in head and neck cancer, and their potential use in estimating survival and response to treatment. In formalin-fixed, paraffin-embedded head and neck squamous cell carcinoma samples the existence of a \textit{TP53} mutation was first confirmed with SSCP and the type of mutation was then separately analyzed in each studied exon by sequencing. The connection of the functional state of the mutated p53 with clinical as well as environmental aspects of cancer was analyzed. The study also aimed to find potential prognostic factors among the cell cycle regulating proteins that could predict biological aggressiveness of the tumor. The strategy used in the project found correlations between etiological and molecular factors and biological behavior of the tumor on head and neck squamous cell cancer (Figure 6).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{strategy.png}
\caption{Strategy of the project.}
\end{figure}

4.2 Summary of used methods

The combination of different methods utilized in this doctoral thesis and their use in the four original publications is shown in Table 2.
Table 2. Methods used in the original publications I-IV.

<table>
<thead>
<tr>
<th>Method</th>
<th>Target</th>
<th>Publication</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>sequencing</td>
<td>TP53 mutations</td>
<td>I,II,III</td>
<td>Vähäkangas et al. 1992</td>
</tr>
<tr>
<td>immunohistochemistry</td>
<td>p53, p16, p21, cyclin D1</td>
<td>II,III, IV</td>
<td>Rahko et al. 2006</td>
</tr>
<tr>
<td>structural changes</td>
<td></td>
<td></td>
<td>et al. 2003</td>
</tr>
</tbody>
</table>

4.3 Patients and samples (II-IV)

The study population (II-IV)

The study population consisted of North Finnish patients diagnosed with a primary head and neck squamous cell carcinoma in the University Hospital of Oulu, Finland between the years 1994 and 1996. The patients were recruited to the study when entering the hospital. The histological material of head and neck squamous cell carcinoma lesions was obtained during routine essential diagnostic procedures. Formalin-fixed, paraffin-embedded tumor samples were used for the study. The stage of the disease, tumor size and lymph node involvement was determined according to the International Union Against Cancer TNM classification (1997). The histological grade of the tumors was reviewed and classified according to the World Health Organization Classification of head and neck tumors (Barnes et al. 2005).

Exposure background in head and neck squamous cell cancer (II)

In each case, a questionnaire was filled about smoking, alcohol consumption and workplace with a possibility of exposure to chemicals at work, as well as family history (Study II). Questions were asked during the first contact with the cancer clinic by an experienced doctor or nurse. An exposure index was calculated using the data from the structured questionnaire on lifestyle and work history, as well as on the exposure to chemicals. In the case of tobacco, information on smoking history was provided by the patients via self-reporting. Tobacco exposure was defined as pack years. Exposure to alcohol was defined as the amount and frequencies of drinking. For each case, exposure to chemical/dust was assessed based on the entire work history.
Analyzed samples in different studies (II-IV)

Histological sections were obtained from 46 patients in study II and III and from 55 patients with HNSCC in study IV. Head and neck squamous cell carcinoma samples (n=46) were analyzed for TP53 mutations and p53 IHC. In study IV, in 56 cases p16 protein, in 50 cases p21 protein, and in 47 cases cyclin D1 protein was analyzed. All three proteins were analyzed in 44 cases.

Table 3. Summary of study population.

<table>
<thead>
<tr>
<th>Patients</th>
<th>n</th>
<th>Gender</th>
<th>Stage of the disease</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>male</td>
<td>female</td>
<td>Limited stage</td>
</tr>
<tr>
<td>Studies II, III</td>
<td>46</td>
<td>31</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Studies IV</td>
<td>55</td>
<td>37</td>
<td>18</td>
<td>15</td>
</tr>
</tbody>
</table>

*RT = radiotherapy

The treatment of patients

In this study, the treatment strategies for patients were carried out according to the local protocol for treatment, and the treatment line depended on the stage of the tumor. Different treatment modalities used in this study are summarized in Table 3. Postoperative radiotherapy of 50–60 Gy was delivered using conventional fractionation. Patients receiving definitive RT (50-64 Gy) had mostly laryngeal carcinoma (Study III). No patients were treated with adjuvant chemotherapy. Patients with advanced carcinoma received only palliative treatment (three patients in study (IV).

4.4 Analysis of TP53 gene mutations (I-III)

PCR-SSCP (I-III)

Both the background and procedure of the PCR-SSCP method have been described in detail in Study I. Exons 5-8 of the TP53 gene were separately amplified by PCR using two sets of intron primers, the second set internal to the first, i.e. nested primers (Lehman et al. 1991) (Study I, Table 1). The most important point in PCR is sterility. To check for possible contamination, the first
and the last reactions in each PCR series were controls with no template in the reaction. If a band appeared indicating contamination, the whole series of PCR reactions was discarded. The amplified products have to be carefully purified by agarose gel electrophoresis.

For SSCP analysis, purified heat-denatured single-stranded DNA fragments were analyzed using non-denaturating 20% polyacrylamide gels. The Pharmacia PhastSystem® semi-dry equipment was used for SSCP analysis. Two running temperatures were used for each exon. The efficiency increases greatly compared to the use of either temperature alone. Both negative and positive controls were included in each analysis to ensure the quality of the analysis. As a negative control, gel-purified, amplified normal TP53 DNA was used. Positive controls were amplified using mutated primers in the place of the left primers (Study I, Table 1) and only runs where the positive controls were clearly visible were accepted. The gels were stained with a silver staining kit (Pharmacia Biotech, Finland) according to the instructions from the manufacturer.

**PCR-Sequencing of the TP53 gene (II,III)**

Once a mutation was detected by the presence of similar band shifts in SSCP from two independent PCRs, the PCR amplified samples were sequenced with ABI PRISM 3100 sequencer and BigDye Terminator Sequencing Kit (Applied Biosystems, Foster City, CA).

**4.5 Immunohistochemistry (II-IV)**

The histological material was fixed in 10% formalin and embedded in paraffin after that. Paraffin sections of 4 μm were incubated at 37°C for at least 4 hours, usually overnight (except Cyclin D1 IHC sections were incubated in room temperature for 30 minutes), dewaxed in Histo-Clear, and hydrated in a descending alcohol series. Endogenous peroxidase activity was blocked by peroxide incubation, after which non-specific binding was blocked. For staining for p53, mouse monoclonal antibody was used as the primary antibody. The antibody recognizes both wild-type and mutant forms of human p53 and the epitope is located between the amino acid residue 19 and 26. For staining the Histostain-bulk kit® was used. Biotinylated antimouse IgG was used as the secondary antibody and peroxidase was introduced as a streptavidin conjugate. The antibody reaction was visualized by using a fresh substrate solution.
containing aminoethyl carbazol. The sections were counterstained with hematoxylin, dehydrated and mounted in glycerol-vinyl-alcohol (Study II, III). The immunoreactivity in the malignant cells in each section was graded according to the number of positively staining nuclei. For p16, p21 the IHC staining was continued using Post Primary Block (Novolink Polymer Detection System) according to the manufacturer’s protocol. The sections were counterstained with hematoxylin, dehydrated and mounted in HistoMount. For negative controls the primary antibody for p16 was replaced with mouse non-immuno IgG and each set of staining always included a separate known positive control sample (Study IV). The evaluation of p16, p21 and cyclin D1 stainings was performed estimating the percentage of positively staining nuclei and according to the intensity of staining (Study IV). The slides were analyzed separately by two independent observers blinded from the clinical data (Study II-IV). The cut-off value was determined using ROC analysis. The primary antibodies used in the study are listed in Table 4.

### Table 4. Immunohistochemical antibodies and antibody dilutions used in the studies.

<table>
<thead>
<tr>
<th>Study</th>
<th>Detected molecule</th>
<th>Antibody, manufacturer</th>
<th>Specificity</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>II, III</td>
<td>p53</td>
<td>DO-7, Novocastra Laboratories Ltd.</td>
<td>monoclonal, mouse</td>
<td>1:300</td>
</tr>
<tr>
<td>IV</td>
<td>p16</td>
<td>Boster Biological Technology</td>
<td>monoclonal, human</td>
<td>1:20</td>
</tr>
<tr>
<td>IV</td>
<td>p21</td>
<td>Neomarkers</td>
<td>polyclonal, mouse</td>
<td>1:200</td>
</tr>
<tr>
<td>IV</td>
<td>cyclin D1</td>
<td>DAKO</td>
<td>monoclonal, rabbit, anti-human</td>
<td>1:25</td>
</tr>
</tbody>
</table>

### 4.6 Statistical analysis (II-IV)

The correlations of tumor stage, TNM classification, histological grade, gender, age, and primary anatomical site were analyzed separately with Fisher’s exact test according to the cyclin D1, p16 p21, p53 immunoreactivity and TP53 mutations. Disease-free survival time, disease-specific survival and overall survival were analyzed for the HNSCC patients with respect to p53, p16, p21 and cyclin D1 protein staining and TP53 mutations using the Kaplan-Meier method, and the statistical differences in survival among subgroups were compared by a Log Rank test (Mantel Cox) and Breslow estimator. Survival was defined as the time from the date of diagnosis to death due to the cancer or the date of the last follow-up visit. Disease-free time was defined as the time from diagnosis of cancer to
recurrence or death to cancer. Overall survival and disease-specific survival time was calculated from the date of diagnosis until death, the date of the last follow-up examination or the date of the collection of clinical data, November 24, 2009. The cut-off values for evaluation of IHC results are based on statistically calculated ROC-analysis. Probability values <0.05 were considered to be statistically significant. All statistical analyses were performed using the SPSS software system (SPSS for Windows, version 16.0, Chicago, IL).

4.7 Ethical aspects (I-IV)

Ethics of the research process

Ethics involves the principles of conduct governing an individual or a group, and conforming to accepted professional standard of conduct is usually regarded as morally sufficient (Merlo et al. 2008). The special interest on the gene level in molecular cancer epidemiology is based on the realization that the process of carcinogenesis involves the accumulation of genetic damage, such as a mutation in a certain tumor suppressor gene e.g. TP53, and such a mutation when heritable may carry susceptibility to cancer (Vähäkangas 2003). Important practical considerations include the relevance of the marker used and the specificity and sensitivity of the assay, which affect the proportion of true positives (Rawbone 1999). Due to the small sample size, further studies are needed to clarify the role of potential prognostic markers.

Approvals from the official ethics committee

According to Finnish law (Medical Research Act 1999/488, 2004/295, 2010/794) based on principles outlined in the Declaration of Helsinki, studies of this kind need to be approved by an official research ethics committee. The study protocol was approved by the Ethics Committee of Oulu University in March, 1994, and the approval was renewed by the Ethics Committee of Oulu University Hospital in July, 2002 (EETTMK: 17/2002) and in September 2006 (EETTMK amendment 64), when including markers for Study IV.
Patients and samples

According to the principle of autonomy (Beauchamp & Childress 2001) people have the right to decide how their body, tissue and information about their health are used. Before interviewing and sample collection, all patients in this study signed a written informed consent that allowed the use of tissue material as well as the data from medical records for this study. Patients were interviewed by hospital personnel (a doctor or a nurse) and the coded data were stored in a safe locked place by designated researchers. The study did not interfere with the clinical treatment of the patients.
5 Results

5.1 TP53 mutation detection by PCR-SSCP (I-III)

5.1.1 Method development for TP53 mutation detection (I)

In the search for an efficient TP53 mutation method, optimization of the SSCP conditions was necessary. The critical factors include running conditions, specificity of the primers for amplification, coverage by the used primers of full exons with splice sites, and positive and negative controls for all strands amplified (Welsh et al. 1997). The most important factor context running conditions is the temperature. Precise temperature was gained in the PhastSystem® (Pharmacia) equipment.

5.1.2 TP53 mutation analysis by PCR-SSCP (II,III)

When collecting tumor samples, it is important to visualize the tumor area carefully before scraping paraffin-embedded tumor samples. This includes the use of hematoxylin-eosin stained tumor samples. Arbitrary scraping declines sensitivity of TP53 mutation analysis detection.

In mutation analysis for HNSCC samples two independent amplified DNA products judged to be positive in SSCP confirmed the TP53 mutations. Tumors with similar consistent, positive band patterns in SSCP analysis but negative by sequencing were found. Judging by SSCP the TP53 gene was mutated in a total of 26 primary tumors out of 46 HNSCC patients (57%) with altogether 39 TP53 mutations. Direct sequencing was successful in identifying the type and location of the TP53 mutation in 23 tumor samples. Thus 88% of mutated samples were further analyzed and correlated with chemical exposure data and clinical survival data.

5.2 p53 alterations in HNSCC (II-III)

5.2.1 TP53 mutations in HNSCC (II, III)

The overall frequency of TP53 gene mutation positive cases (57%, 26/46) was found in a series of histologically confirmed cases of head and neck squamous
cell cancer. The majority of the mutations were missense mutations (30/36, 83%) (Figure 7). Only one of the mutations was a nonsense mutation and two were silent. All silent mutations detected were in tumors with multiple mutations. Eleven tumor samples (11/26, 42%) were found to harbor multiple TP53 mutations. There was only a small difference in the prevalence of mutations between different tumor sites. Transversions (17/31, 55%) were more frequent than transitions (14/31, 45%). Codon 259 was mutated in 4 samples (GAC>GAA).

**Fig. 7. Types of the TP53 mutations.**

The mutations were classified as taking into account the functional and structural domains of p53 as described in the IARC TP53 mutation database (http://www-p53.iarc.fr/). In these Studies II and III, 22% (8/36) of the mutations affected the L2 domain (between codons 164 and 194), which is needed for the correct folding and stabilization of the central part of the protein, 11% (4/36) affected the LSH (loop-sheet-helix) motif (codons 119-135 and 272-287), while 8% (3/36) affected the L3 domain (between codons 237-250), directly involved in the interaction between the protein and DNA. According to the IARC database and based on experimental data, four of the missense mutations lead to non-functional proteins (Cys238Ser, Gly245Asp, Glu258Lys, Arg283Pro). Based on the predicted structure (by amino-acid conservation rules or structural analysis), several of the
found mutations probably have functional impact, leading to a non-functional protein (Leu130Ile, Leu130Phe, Thr155Ser, Val157Phe, Val172Ala, Arg175His, Met243Leu, Asp259Glu, Cys275Tyr). There were more numerous TP53 mutations leading non-functional p53 protein in the L3/LSH motif compared to the L2 motif, but no statistically significance difference was observed.

The majority of patients with a negative family history of cancer had a TP53 mutation, while patients with a positive family history of cancer had a TP53 mutation in only about half of the cases. When taking into account age, TP53 mutations occurred more commonly among younger head and neck squamous cell cancer patients, especially among patients with a tumor in the oral cavity. However, this difference was not statistically significant (p = 0.330). Otherwise TP53 mutation status was not statistically significantly associated with patient characteristics.

5.2.2 p53 immunohistochemistry in HNSCC (II,III)

Overall, about half of the 46 primary HNSCC cases, i.e. 24 (52%), showed positive staining for the p53 protein. No statistically significant associations between the p53 accumulation and TP53 mutations were found; however, in cases with intense tumor staining, 8 out of 10 cases (80%) also contained a TP53 mutation, while 17 out of 32 cases (53%) presenting with negative or weak p53 staining contained a TP53 mutation in the tumor (p = 0.16, Fisher’s exact test). There was no correlation between the type of the mutation and the positivity of p53 immunostaining.

5.3 HNSCC and environmental exposure (II)

5.3.1 Clinicopathological variables and environmental exposure

There was a higher proportion of women among never-smokers compared to men: 88% of non-smokers were women, while 14% of heavy smokers were women (p < 0.001 Pearson Chi-Square). The female patients with head and neck cancer also had a significantly lower mean exposure to alcohol and tobacco than the male patients (p < 0.05, Fisher’s Exact Test). However, no statistically significant difference between the sexes was found when the overall exposure (including work history) was considered (p = 0.09, Fisher’s Exact Test). When taking into
account tumor site, tobacco and alcohol exposures were significantly higher in laryngeal tumors than in tumors of the oral cavity (p < 0.005, Fisher’s Exact Test). 79% of the laryngeal carcinoma patients were men.

5.3.2 Environmental exposure and p53 alterations

In p53 IHC, there was a statistically significant difference in p53 positive staining result in heavy smokers compared to non-smokers (p < 0.05, Fisher’s Exact Test). However, there was no statistically significant correlation between smoking and TP53 mutation status. When taking into account total exposure p53 aberration appeared to be more prevalent in patients with higher exposure index (exposure index 6-8). There was no statistically significant correlation between p53 alterations and alcohol consumption. There was a trend that males had more often a tumor showing p53 overexpression in IHC. p53 overexpression was more prevalent in laryngeal tumor compared to other anatomical sites; however, the difference was not statistically significant.

Four individuals had similar TP53 mutation, Asp259Glu, most probably leading to a non-functional p53 protein. All patients with this missense mutation had chemical exposure to tobacco and alcohol, and 75% of the patients had documented work exposure to chemicals including pesticides, oil and asbestos.

There was no significant difference between a high exposure index and TP53 mutation status according to the p53 protein function (functional vs. non-functional). When taking into account structural and functional domains of p53, there was a statistically significant difference between patients’ exposure to tobacco and alcohol and TP53 status. Patients (83% of patients) with tumor containing wild-type p53 mostly had only light total exposure to tobacco and alcohol and occupational agents, while patients with a tumor containing TP53 mutation in DNA-binding regions (L2, L3/LSH motif) mostly had higher total exposure to chemicals (p < 0.032). A mutation possibly linked to tobacco exposure, Val157Phe missense mutation, was found in a patient who was a heavy smoker.
5.4 Potential prognostic factors (III,IV)

5.4.1 Prognostic significance of TP53 mutations

In this material, TP53 mutations in the DNA-binding domains L2, L3 or LSH were found to be indicators of poor prognosis in Kaplan-Meier analysis when the disease-specific survival was analyzed (p < 0.05, Log Rank). Patients who had TP53 mutations in L2, L3 and LSH motifs died of cancer more often (78% 5-year disease-specific survival) than patients with a wild-type p53 in their tumor (44%). There was a statistically significant 5-year overall survival rate of 31% in patients with TP53 mutation in DNA-binding motif compared to 70% in other patients (p < 0.05). This was also seen as regards the disease free-time (p < 0.05, Log Rank).

An interesting finding was that the patients with TP53 mutations in DNA binding surface region had a higher number of late recurrences than the patients with wild-type p53 or patients with TP53 mutation outside the functional domains of p53. As figure 8 illustrates, there was a trend that patients with mutations in the L3/LSH motif had more nodal metastasis than patients with a wild-type p53 in their tumor (p = 0.2, Pearson Chi-Square). TP53 mutations status (with or without TP53 mutations) was not associated with survival or disease recurrence. Strong p53 staining was associated with a better disease-free time, but no correlation was found between the overall survival and p53 staining or disease-specific survival.

Fig. 8. Association between TP53 mutation status and nodal metastasis.
Patients with TP53 mutations in important DNA-binding motifs L2, L3, or LSH received less combined treatment (surgery and radiotherapy) compared to patients with a TP53 mutation outside the L2/L3/LSH motif or a wild-type p53 (14% vs. 61%, p = 0.009, Fisher’s Exact Test). On the other hand, patients treated with combined treatment had longer disease-free-time (5 years in 71% compared to 38%, p = 0.045, Log Rank), cancer-specific-survival (5-yr survival 72% compared to 34%, p = 0.013, Log Rank), and overall survival (5-yr survival 58% compared to 31%, p < 0.05, Log Rank). When taking into account the age and health of patients, there was no difference in the treatment modalities between the different age groups or basic diseases.

Patients with TP53 mutations in the L3/LSH motif had significantly poorer response to radiotherapy than patients with wild-type p53 or TP53 mutation outside the DNA-binding regions (11% vs. 49% vs. 40%, p < 0.05, Fisher’s Exact Test) (Figure 9). When comparing different treatment modalities and survival, the 5-year disease-specific survival was 75% in the group treated with preoperative radiotherapy, 70% in the postoperative radiotherapy group and only 40% in the group with surgery alone. This difference between the treatment groups reached the border of statistical significance (p = 0.05, Fisher’s Exact Test).

Fig. 9. Association between TP53 mutation status and response to radiotherapy.
5.4.2 Other cell cycle regulators in HNSCC

The survival analysis showed a statistically significant correlation between the tumor immunohistochemical staining of p16 protein and the overall survival of the HNSCC patients. The Kaplan-Meier analysis showed that the cumulative survival rate of patients with p16 negative tumor was 41% after 5 years’ follow-up, whereas the cumulative survival for p16 positive cases was 74% (p < 0.05, Log rank) (Figure 10). However, there was no statistically significant correlation between the p16 status and 5-year disease-specific survival, which was 59% in those with a p16 negative tumor and 72% in p16 positive cases (p = 0.373, Log Rank). A similar finding was obtained for the progression-free survival time in these groups (p = 0.398).

Cyclin D1 overexpression was significantly associated with poorer overall survival and the association was also observed with cyclin D1 and disease-specific survival, although this correlation did not reach statistical significance. The five-year overall survival in patients with a tumor showing low expression of the cyclin D1 was 72%, being only 45% in the patients with a tumor showing high expression (p < 0.050, Log Rank) (Figure 11). The disease-specific 5-year survival rate was 83% compared to 59% in the case of tumors with low expression of cyclin D1 compared to tumors with high expression, respectively, (p = 0.234, Log Rank; Breslow p = 0.158). However, there was no difference in the progression-free time between patients with tumors showing different expression of cyclin D1.

The Kaplan-Meier analysis identified patients with dismal overall survival (Figure 12). In patients with a high expression of cyclin D1 but a p16 negative tumor the five-years overall survival rate was only 25% while in all other cases it was 64% (p = 0.028, Log Rank) (Table 5). However, there was no statistically significant correlation in the disease-specific survival; 5-year survival rates were 59% compared to 75% in tumors showing cyclin D1 high expression but being p16 negative and in other cases, respectively (p = 0.220 Log rank; Breslow p = 0.111) (Table 6), and there was no difference in progression-free time.
Fig. 10. Kaplan-Meier analysis showing overall survival in patients with head and neck squamous cell carcinoma according to immunohistochemical staining of p16 protein.

Fig. 11. Kaplan-Meier analysis showing overall survival in patients with head and neck squamous cell carcinoma according to immunohistochemical staining of cyclin D1 protein.
When analyzing the disease-specific five-year survival, there was a trend towards poorer survival in patients with a tumor showing low expression of p21 in immunohistochemical staining compared to patients with a high expression of p21 in tumors, although the difference was not statistically significant (53% vs. 72%, respectively, p = 0.150, Log Rank) (Table 6). Also in the overall 5-year survival rate there was no statistically significant difference (44% compared to 59%; p = 0.460, Log Rank) (Table 5). However, when the analyzed tumors were negative for both p16 and p21 proteins, there was a more distinct trend towards a worse overall survival compared to other patients; the 5-year overall survival was only 20% in cases of p16 and low expression of p21 in tumors compared to 61% in other tumors (p = 0.158, Log Rank) and the 5-year disease-specific survival was 30%, compared to 71% in other tumors, (p = 0.229, Log Rank). In the progression-free survival analysis, however, such a difference was not seen. In the subgroup of patients with a tumor showing low expression of p21 protein and high expression of cyclin D1 protein compared to other tumors there was a trend towards worse disease-specific survival rate (38% versus 68%, respectively, p = 0.137 Log Rank). However, there was no difference in 5-year overall survival
time between patients with tumors showing low expression of p21 protein and high expression of cyclin D1 compared to other tumors (50% versus 60%, \( p = 0.145 \), Log Rank).

In this study, Kaplan-Meier analysis showed that taking together the patients with tumors showing p16+/p21+/cyclin D1 in immunohistochemical staining there was a trend towards a better 5-year disease-specific survival rate of 88%, while survival rate in other cases was 55%. The difference was, however, not statistically significant (\( p = 0.158 \), Log Rank). A similar trend was also seen in overall survival rates, 88% in patients with a tumor showing p16+/p21+/cyclin D1- compared to 53% in other cases’ survival rates (\( p = 0.158 \), Log Rank). There was no difference in recurrences between these two groups. Tables 5 and 6 summarize the association between immunohistochemical markers’ combination and overall survival (Table 5) and disease-specific survival (Table 6).

**Table 5. Comparison between immunohistochemical staining and patients’ 5-year overall survival.**

<table>
<thead>
<tr>
<th>Immunohistochemical staining result</th>
<th>5-year overall survival (%)</th>
<th>p-value (Log Rank)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cyclin D1</td>
<td>p16</td>
<td>p21</td>
</tr>
<tr>
<td>+ - ND</td>
<td>25%</td>
<td>64%</td>
</tr>
<tr>
<td>ND - -</td>
<td>20%</td>
<td>61%</td>
</tr>
<tr>
<td>+ ND -</td>
<td>50%</td>
<td>60%</td>
</tr>
<tr>
<td>- + +</td>
<td>88%</td>
<td>53%</td>
</tr>
</tbody>
</table>

**Table 6. Comparison between immunohistochemical staining and patients’ 5-year disease-specific survival.**

<table>
<thead>
<tr>
<th>Immunohistochemical staining result</th>
<th>5-year disease-specific survival (%)</th>
<th>p-value (Log Rank)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cyclin D1</td>
<td>p16</td>
<td>p21</td>
</tr>
<tr>
<td>+ - ND</td>
<td>59%</td>
<td>75%</td>
</tr>
<tr>
<td>ND - -</td>
<td>30%</td>
<td>71%</td>
</tr>
<tr>
<td>+ ND -</td>
<td>38%</td>
<td>68%</td>
</tr>
<tr>
<td>- + +</td>
<td>88%</td>
<td>55%</td>
</tr>
</tbody>
</table>


6 Discussion

6.1 Detection of p53 alterations in cancer

6.1.1 TP53 mutation detection in cancer research

Methods for the analysis of mutations in TP53 gene are widely used in cancer research. PCR-SSCP (single-strand conformation polymorphism) and PCR-sequencing are the most frequently used methods for TP53 mutation analysis. Any mutation analysis method will have a large variation in the quality of the results, depending on how it is used (Welsh et al. 1997, Wikman et al. 2000). The need of standardized methods for the assessment of TP53 mutation status in human tumors has been recognized as a starting point for developing p53 as a biomarker (Soussi & Beroud 2001). The use of the optimized and validated SSCP method may increase the sensitivity and specificity. This is in line with previous studies (reviewed by Kavakas et al. 2008). Important practical considerations are specificity and sensitivity of the assay, which affect the proportion of true positives. The use of automated temperature control in electrophoresis guarantees exact running temperatures and enables reproducibility of the analysis. High efficiency is possible to obtain using two temperatures in combination with other optimized conditions (Castren et al. 1997, Welsh et al. 1997, Peltonen et al. 2006). Even 15–30% of the mutations are missed if only one temperature is used (Castren et al. 1998). Another critical point is the use of primers. The developed SSCP method requires exactly the same primers for PCR that were used for the development. To include all putative mutations it is important to use intron primers that include all splice sites of the exons. In this study artefacts due to formalin-fixation or the infidelity of polymerase-enzyme in PCR were ruled out by repeated analysis using newly isolated samples. Also sterile working and careful purification of DNA is necessary to obtain a good background and readable result in SSCP. In head and neck cancers primary tumors in different sites are quite small, especially in laryngeal tumors. This brings additional challenges to the screening methods concerning formalin-fixed paraffin-embedded tumor samples in humans (Vähäkangas et al. 1992, for a review see Ren et al. 2000, Gnanapragasam 2010).

The sensitivity of manual dideoxy sequencing to detect mutated fragments against wild-type background does not reach fully developed and validated SSCP
sensitivity (Castren et al. 1997, Welsh et al. 1997, Meinhold-Heerlein et al. 2001, reviewed by Peltonen et al. 2006). In the present study some TP53 mutations in lesions based on reproducible SSCP positivity were found to be negative in sequencing. This is actually the worst disadvantage of the highly sensitive SSCP: all samples cannot be confirmed by sequencing, which is why location and type of such mutations may remain unresolved. It is generally accepted that direct sequencing requires at least 20% of mutant alleles in the sample, but this can vary considerably according to the quality of the sample (Soussi 2007). In the present study, further specified analysis after SSCP of TP53 mutations was needed to study the correlation with the biological aggressiveness of a tumor. Chip techniques, which have capacity for automation, are promising in terms of clinical applications. However, chips do not detect insertions or large deletions, but single-base deletions can also be missed (Lu et al. 2002, Bosch et al. 2004)

Certainly, when mutation detection is used for clinical purposes, e.g., as a prognostic marker, there is no place for mistakes (Jiang et al. 2004) and sequencing is the most accurate method (www-p53.iarc.fr). In the future, a cost-effective next-generation sequencing-method that offers higher reproducibility could be a useful method for clinical purposes (for a review see Mardis & Wilson 2009).

6.1.2 TP53 mutations influence to p53 protein function

In this study, according to the predicted structure (by amino-acid conservation rules or structural analysis), several of the found mutations were likely to have functional impact leading to a non-functional protein (Leu130Ile, Leu130Phe, Thr155Ser, Val157Phe, Val172Ala, Arg175His, Met243Leu, Asp259Glu, Cys275Tyr). These findings are in line with TP53 database functional data (http://www-p53.iarc.fr). Structural and functional effects in TP53 gene are important for p53 transcriptional activity. Experimental assays have been performed in yeast and human cells to measure functional properties (Petitjean et al. 2007 a, b). In this study, according to the database validation, one mutation, Arg175His, has a gain of oncogenic function properties.

Based on recent data that indicate that structural mutations alter the stability of the protein (Olive et al. 2004, Yip et al 2006), we conclude that in seven tumors there was a mutation in the current study that was more likely to disrupt all functions of the protein. In four other tumors there were mutations within a
contact residue that was probably more selective in affecting the transcriptional activity of p53.

### 6.1.3 Detection of p53 alterations by IHC in HNSCC

In this study, there was no statistically significant correlation between p53 overexpression in immunohistochemical analysis and TP53 mutations analyzed by PCR-SSCP and sequencing. The overexpression and accumulation of p53 protein has been widely used as a surrogate marker for detection of p53 abnormalities in tumors. However, positive IHC will not recognize tumors with TP53 gene null mutations, which lead to non-existent p53 protein, in fact accounting for a significant number of somatic TP53 mutations (Olivier & Taniere 2011). The correlation between missense mutations in the gene and nuclear accumulation on the protein appears to be 80%, with variation from one type of cancer to another (Casey et al. 1996). A major difficulty with IHC is that tumor biopsies are not always representative for the whole tumor, and the cores obtained from a biopsy might even enlarge this selection bias. Especially in small biopsies, such as those obtained from small primary tumors, e.g. laryngeal tumors in this study, the cores relatively often do not contain enough tumor tissue for reliable scoring. Another problem is that standardization of IHC scoring is difficult (Takes et al. 1998).

### 6.2 Association of chemical exposure with the frequency and functional consequences of TP53 mutations in HNSCC

#### 6.2.1 Molecular linkage of TP53 mutations with exposure

TP53 mutation analysis is a useful methodology in the field of carcinogenesis research (for a recent review see Olivier et al. 2010). The focus has been in reporting mutation frequencies in tumors while less attention has been paid to the connection of functional state of the mutated p53 with clinical and environmental aspects of cancer. The TP53 mutational pattern has been suggested to be a clinically relevant “molecular sensor” of genotoxic exposure to environmental carcinogens and endogenous mutagens (Hollstein et al. 1991, Hussain & Harris 1999). Although smoking and alcohol have been reported to be linked with TP53 mutations in head and neck cancer (Brennan et al. 1995, Liloglou et al. 1997,
Koch et al. 1999, Ko et al. 2001, Ronchetti et al. 2004), environmental exposure, including occupational exposure, has not been reported to have been studied previously. An interesting finding of the present study was that four patients with a tumor containing a similar missense mutation, Asp259Glu, had high exposure index. All four patients were smokers and used alcohol. Moreover, most of these patients (75%) had environmental exposure to e.g. pesticides. According to this study and the IARC TP53 database, this is a very rare mutation with a total of nine Asp259Glu mutations having been described worldwide. Almost half of those nine described mutations were found in larynx tumors, suggesting that the larynx is the preferential site for this mutation. The other reported tumor sites were skin, lung, and lymph node. This implies that the Asp259Glu missense mutation is more common in squamous cell carcinomas than in other histologies.

In this study the Val157Phe missense mutation, which is typically induced by the cigarette smoke carcinogen benzo(a)pyrene (BP) in experimental systems (Hainaut & Vähäkangas 1997), was found in a patient who was a heavy smoker. In this study transversions were more frequent than transitions. However, the difference was quite small (transversions 55%, transitions 45%). It is possible that the differences could be due to an etiological factor, e.g. exposure to smoking. However, due to small numbers of this study, it was not possible to determine differences in mutation patterns between non-smoking and smoking patients.

### 6.2.2 p53 alterations and chemical exposure

The present study shows, in line with the literature (Bosch et al. 2004, Cabanillas et al. 2007), that p53 overexpression is a common event in HNSCC. In this study p53 overexpression was more common among heavy smokers than among non-smokers, in accordance with previous studies (Cruz et al. 2002, Field et al. 1994). p53 may be induced in cells by exposure to DNA-damaging chemicals (Hainaut & Vähäkangas 1997) through post-translational modifications (Hollstein & Hainaut 2010). There was no correlation in this study, however, between the TP53 mutations and smoking. Likely explanations are the small number of patients and insufficient exposure data. Data in the literature are discrepant, with some previous studies showing a positive association between TP53 mutation and tobacco smoke in patients with HNSCC (Brennan et al. 1995, Liloglou et al. 1997, Koch et al. 1999, Ko et al. 2001, Ronchetti et al. 2004), but the results are in line with those of (Obata et al. 2000, Chaves et al. 2004, Poeta et al. 2007). No statistically significant association between p53 alterations and exposure to
alcohol consumption was found. The specific role of alcohol in the occurrence of \textit{TP53} mutations is hard to prove, since alcohol abuse is frequently associated with smoking habit (reviewed by Blons & Laurent-Puig 2003). The specific positive impact of alcohol on the incidence of \textit{TP53} mutations was significant after adjustment for cigarette smoking in only one series, a Taiwanese series of patients (Hsieh \textit{et al.} 2001).

There was significantly higher tobacco and alcohol exposure in patients with laryngeal tumors than in those with oral cavity tumors in the present study. \textit{p53} overexpression was indeed more prevalent in laryngeal tumors than in other anatomical sites. \textit{p53} overexpression has previously been reported in laryngeal squamous cell carcinomas at a frequency ranging from 38\% to 79\% of the tumor samples analyzed (Kazkayasi \textit{et al.} 2001). Similarly De Paula \textit{et al.} (2009); a large material of over 700 patients has reported a correlation between \textit{p53} immunohistochemically positive tumors and anatomical site (De Paula \textit{et al.} 2009). The sensitivity to carcinogen exposure, e.g. to tobacco smoke, may vary in different anatomical sites (De Paula \textit{et al.} 2009). There was only a small difference in the prevalence of mutations between different tumor sites in this study.

In the present study, \textit{TP53} mutations were more common in young patients. Most of these young patients had been exposed to tobacco and alcohol. Based on the literature, oropharyngeal cancer in non-smoking males is strongly associated with HPV infection (Toner & O’Regan 2009). Furthermore, previous studies have shown that HPV correlates with wild-type \textit{p53} (Dai \textit{et al.} 2004, Gillison \textit{et al.} 2000). This might exclude HPV infection as an etiological factor in the group of young patients in this study. Previously, a case-control study has indicated that risk factors in patients younger than 45 were similar to older patients (Llewellyn \textit{et al.} 2004).

Interestingly, in the present study, young patients had a negative family history for HNSCC. In the literature family history of cancer has been associated with an earlier onset of HNSCC (reviewed by Toner & Regan 2009). In the whole study population, tumors in patients with sporadic cancer had more often \textit{TP53} mutations than tumors in patients with a positive family history. Most likely there are interindividual differences in susceptibility to carcinogens (Vähäkangas \textit{et al.} 2003) that are based in part on genetic differences in the metabolism of carcinogens (Wogan \textit{et al.} 2004). Carcinogen activation leads to formation of DNA adducts and potentially mutations. Considering this with the age correlation
and family history data, the results may be interpreted as supporting environmental etiology of the TP53 mutations in HNSCC.

Interestingly, in this study females with head and neck cancer had less exposure to chemicals than males, supporting the reported higher susceptibility of women to carcinogens such as cigarette smoke (Toyooka et al. 2003). A small proportion of individuals exposed to carcinogens may develop the disease, and intrinsic susceptibility to environmental exposure no doubt plays a role in head and neck cancer as well (see e.g. Vähäkangas et al. 2003, Llewellyn et al. 2003).

6.2.3 TP53 mutation frequency and functional consequences in HNSCC

In this study TP53 mutation frequency did not differ from that reported by the IARC TP53 database in head and neck squamous cell carcinomas (http://www-p53.iarc.fr). Point mutations altering p53 function are distributed over a large region of the gene, especially in the hydrophobic midportion where many substitutions alter p53 conformation and sequence-specific transactivation activity (Levine et al. 1991, Hollstein et al. 1991, Greenblatt et al. 1994). In the present study the hot spot middle region of the TP53 gene was underrepresented.

In the present study more than half of the TP53 mutations possibly changed the gene to encode a non-functional protein. There are several possible mechanisms leading to non-functional mutant p53 protein; e.g. zinc is essential for the function of p53, because p53 does not adopt the correct conformation in the absence of zinc (Bullock et al. 2000, Hainaut & Mann 2001).

Structural mutants altering the stability of the protein were detected in the present study (Martin et al. 2002, Cuff & Martin 2004, Joerger et al. 2006, Yip et al. 2006, Lubin et al. 2010). Some were capable of disrupting all functions of the p53 protein. The DNA contact mutations often selectively affect the transcriptional activity of p53 due to reduced binding affinity without affecting the stability (Lubin et al. 2010). Both classes of mutant p53 proteins commonly accumulate to high levels in tumor cells and are defective in wild-type p53 functions (Kato et al. 2003, Olive et al. 2004).

In this study patients with wild-type p53 protein had significantly lower exposure to tobacco, excessive levels of alcohol and occupational chemicals, while patients with tumor containing TP53 mutation in DNA-binding regions (L2, L3/LSH motif) mostly had higher exposure to all of those risk factors. In the present work no correlation was observed between the functionality of TP53
mutations and exposure to chemical carcinogens. Unfortunately, in 37.5% of cases evaluated for functional consequences of the TP53 mutations it was not possible to calculate the exposure index due to partly lacking exposure data. It remains to be clarified whether there is any correlation between the functional effects of the mutations to exposure types or total exposure. These data suggest that further studies on whether a mutation in structural and functional domains of the TP53 gene serves as a potential molecular marker for chemical exposure are warranted. Thus TP53 mutations certainly need to be interpreted very carefully in terms of biological and functional consequences.

6.3 Prognostic value of specific TP53 mutations in HNSCC

6.3.1 TP53 mutations and clinicopathological variables

Advances in the understanding of the molecular and biological basis of HNSCC may increase the opportunities for identification of new markers indicating biological aggressiveness of these tumors. TP53 mutations are being studied as a clinical marker (for a recent review see Robles & Harris 2010). TP53 mutations are commonly found in head and neck cancers, and most of the published mutations affect the p53-DNA interactions, resulting in partial or complete loss of the transactivation functions (Soussi & Beroud 2001). In this study there were 83% missense mutations, which is in line with the literature. The resulting amino acid substitution is usually within the central DNA-binding domain (Vousden 2009).

No statistically significant correlation was observed between p53 aberrations and clinicopathological variables such as histological grade, TNM classification or the stage of the disease. However, in the present study, there was a trend of TP53 mutation to be more common in young patients. While most head and neck squamous cell carcinomas (HNSCC) occur in older people, an increasing number of young patients are being affected worldwide, with up to 5.5% among population under 40 years (for a review see Toner & O’Regan 2009). Previously TP53 mutations have been shown to be linked to the young age of patient (Koch et al. 1995). In the present study 2.2% of patients suffering from head and neck cancer were younger than 40 years old and, noteworthy, all these patients had cancer in the oral cavity with TP53 mutation. These data are in line with a previous observation made in head and neck cancer patients by De Paula et al.
(2009), who found significantly higher p53 expression (p < 0.05) and a higher incidence of oral cavity tumors among younger patients (De Paula et al. 2009).

On the other hand, Regezi et al. (1999) evaluated and compared the expression of the cell cycle proteins p53, p21, Rb and MDM2 in tongue cancer patients aged 35 and younger and those aged 75 or older. They reported no difference among young and older patients in terms of p53 protein expression (Regezi et al. 1999). The possible association with age in different head and neck cancers still requires confirmation in larger patient materials. Interestingly, males had a p53 positive tumor at IHC more often than females, possibly due to high exposure to tobacco and alcohol. However, there was no difference between gender and TP53 mutations.

Furthermore, even when comparing the specific TP53 mutations, e.g. TP53 mutations in DNA-binding domains (L2, L3/LSH motif) and TP53 mutations outside those regions, no differences in stage, TNM-classification or tumor histological grade were found. However, in the present study there was a difference in the frequency of node metastasis between the patients with TP53 mutations in the L3/LSH motif (83%) and patients with a wild-type p53 tumor (50%), but the difference was not statistically significant. Erber et al. (1998) have also reported that patients with contact TP53 mutations present lymph node metastasis significantly more often than patients with a structural mutation or wild-type p53 (Erber et al. 1998).

### 6.3.2 Clinical relevance of the TP53 mutations in DNA binding domain

In this study it was shown that tumors containing specific TP53 mutations in DNA-binding surface regions (L2, L3 + LSH) were more aggressive than tumors with mutations outside of those regions. Patients who had TP53 mutation in the L2, L3 or LSH motif died of cancer more often than patients with a wild-type p53 in their tumor. More specifically, the Kaplan-Meier analysis showed that only 22% of patients with a tumor containing TP53 mutations in DNA-binding domains were alive without cancer, while over half (56%) of the patients with wild-type p53 in their tumor were alive. A clear difference was also found in the 5-year overall survival between patients with a TP53 mutation affecting the DNA-binding domain compared to patients with other p53 status (31% and 70%, respectively). Furthermore, TP53 mutations in domains L2, L3 + LSH were
associated with more relapses and statistically significantly shorter disease-free time, indicating a more aggressive phenotype.

These results suggest that TP53 mutations in the DNA-binding domain may be a strong indicator for prognosis in patients with HNSCC. These results support some previous studies showing an association between TP53 mutations affecting the DNA-binding region and unfavorable survival in head and neck cancer patients (Erber et al. 1998, Yamazaki et al. 2003, Poeta et al. 2007). Similar findings have been also reported in gastric cancer (Migliavacca et al. 2004), colorectal carcinoma (Bazan et al. 2005), esophageal cancer (Kihara et al. 2000) and in several studies in breast cancer (Børresen et al. 1995, Berns et al. 1998, Geisler et al. 2001, Nagai et al. 2003, Alsner et al. 2008). Interestingly, this rather small group of patients with mutations outside the DNA-binding domains of p53 had a better survival than the patients with a wild-type p53. No correlation was, however, observed between patients with and without TP53 mutations and disease-free, disease-specific or overall survival.

Contradicting results have been published about the presence or absence of TP53 gene mutations in exons 5-9 and the outcome in HNSCC (Obata et al. 2000, Sisk et al. 2002, Russo et al. 2006, Poeta et al. 2007). Some researchers have not found any correlation between TP53 mutations and survival (Erber et al. 1998, Bosch et al. 2004, Ronchetti et al. 2004, Eriksen et al. 2005, Gunduz et al. 2008), while others have found a positive association between the TP53 mutations and poor prognosis (Mineta et al. 1998, Obata et al. 2000, Sisk et al. 2002, Russo et al. 2006, Poeta et al. 2007). Furthermore, the loco-regional control rate and disease-free survival rate have been shown to be inferior in patients with a TP53 mutation compared to the patients with a wild-type TP53 (Alsner et al. 2001b). Taken together, the findings of this study and the published data suggest that the nature and location of the mutation are connected to tumor aggressiveness and prognosis, and it is not sufficient to analyze merely the presence or absence of the TP53 mutations. Thus, the evaluation of the usefulness of TP53 mutations in human tumors for clinical purposes requires larger studies and much more detailed analysis.

6.3.3 TP53 mutations in DNA-binding domain and correlation to the treatment of the patient

A statistically significant difference was found in the present study between the patients receiving combined treatment with curative intent (preoperative or treatment of the patient
postoperative radiotherapy) and TP53 mutation compared to wild-type p53 protein and TP53 mutation outside the DNA-binding region. Logically, patients treated with combined treatment survived significantly better according to disease-free time and cancer-specific survival. It may be possible that specific TP53 mutations present in the L2, L3 and LSH motifs could lead to inherently more aggressive tumors than mutations outside of these regions. The mechanism of action of a mutant p53 appears to be complex (Petitjean et al. 2007, Olivier et al. 2009). Many studies have shown that although mutant p53 proteins often lose the ability to activate the expression of genes that are responsive to wild-type p53, they may not be inert when it comes to the regulation of transcription (Vousden 2009). Indeed, a mutant p53 can acquire the ability to regulate gene expression both positively and negatively, which clearly contributes to some of the pro-tumorigenic functions of a mutant p53, such as enhanced survival and resistance to cancer therapy (Vousden 2009, Weisz et al. 2007). In this study the treatment of the patients was carried out according to the stage of the disease. The age of the patients or other diseases did not affect the treatment modalities.

Studies about the predictive role of response to radiotherapy in patients with TP53 mutations in head and neck cancer are rare. Loss of p53 function from mutation or deletion has been shown to influence radiation response and progressive growth of tumor cells in most human cancers (Soussi et al. 2005). In this study, tumors containing TP53 mutations in DNA-binding surface had significantly poorer response to radiation than other tumors. There were only a few complete responses to radiation in the patients with TP53 mutation in the DNA-binding region, while almost half of the patients (49%) with wild-type p53 responded to treatment. These data are in line with previous observations in head and neck cancer. Alsner et al. (2001) reported a strong relationship between TP53 mutation and poor prognosis in head and neck cancer patients given primary radiotherapy (Alsner et al. 2001b). Furthermore, Eriksen et al. (2005) had investigated the importance of TP53 mutations for the overall treatment time of radiotherapy in HNSCC patients (Eriksen et al. 2005). They noticed that patients with carcinomas containing a wild-type p53 did not benefit as much as the patients with a mutated TP53 from an increase in the number of weekly fractions (i.e. a reduction in the overall treatment time), as judged by local control at T-site, disease-specific or crude survival (Eriksen et al. 2005). This may be due to the decreased ability of some mutant p53 proteins to initiate apoptosis (Fei & El-Deiry 2003). Consequently, there may be less delay in G1 resulting in increased progress through the cell cycle and increased uncontrolled proliferation (Eriksen
Moreover, Alsner et al. (2001a) had noticed that the majority of head and neck tumors with a mutated TP53 had a tumor potential doubling time below that in wild-type tumors (Alsner et al. 2001a). Head and neck squamous cell carcinomas with a mutated TP53 had higher cell proliferation potential compared to tumors with wild-type p53 protein. These data suggest that TP53 mutation status may thus identify patients where the dominating factor associated with outcome is intrinsic radioresistance. However, it is possible that new treatment modalities may overcome radioresistance and other detrimental biological tumor characteristic and thus influence the prognosis.

6.4 Clinical relevance of the cell cycle regulators cyclin D1, p16 and p21 in HNSCC

6.4.1 Cell cycle regulators in HNSCC

The dysregulation of the molecular events governing cell cycle control is emerging as a central role of head and neck pathogenesis. Genetic abnormalities in HNSCC have been extensively studied to elucidate prognostic indicators of patient survival. In addition, they could be used as a marker for identifying high-risk patients who might benefit from different treatment modalities. The obvious advantage of immunohistochemical staining of cell cycle regulator proteins is that the technique can be applied on tissue used for routine histopathological assessment.

Head and neck squamous cell carcinoma frequently carries numerous genetic alterations, such as mutations, chromosomal losses, gains and amplifications (Perez-Ordonez et al. 2006). Common genetic alterations in HNSCC involve TP53, CDKN2A/p16 and chromosomal band 11q13. Amplification of 11q13 and overexpression of cyclin D1 is seen in 30–60% of HNSCC (Perez-Ordonez et al. 2006). Cyclin D1 is thought to play a role in head and neck cancer tumorigenesis (Meredith et al. 1995, Michalides et al. 1997, Maruya et al. 2004).

6.4.2 Cyclin D1 in HNSCC related to prognosis

In this material tumor tissue cyclin D1 expression was found to be significantly linked to unfavorable prognosis. Only 45% of the patients with a high expression of cyclin D1 in the tumor survived for 5 years after the diagnosis of HNSCC,
whereas in patients with a lower expression of cyclin D1 in tumor a 72-percent overall 5-year survival rate was observed. Furthermore, in the present study, the disease-specific survival was 59% in patients with tumors showing high expression of cyclin D1, whereas in patients with tumors showing low expression of cyclin D1 an 83-percent disease-specific 5-year survival rate was observed. These results are in line with studies reporting the role of cyclin D1 immunoreactive protein as a prognostic indicator in HNSCC (Akervall et al. 1997, Michalides et al. 1997, Namazie et al 2002, Maruya et al. 2004, Higuchi et al. 2007, Hafkamp et al. 2009). However, no correlation was observed here between the cyclin D1 expression levels and progression-free survival time, but Akervall et al. (1997) and Fracchiolla et al. (1997) have reported that cyclin D1 overexpression in HNSCC would be associated with shortened disease-free time (Akervall et al. 1997, Fracchiolla et al. 1997).

No significant associations were found between cyclin D1 staining and the following clinicopathologic parameters: age, sex, histology, T classification, N classification, or clinical stage in the present study. These results are also in line with one previous study (Higuchi et al. 2007). On the contrary, Namazie et al. (2002) have reported that poorly differentiated tumors show higher cyclin D1 amplification.

6.4.3 p16 in HNSCC

A wealth of information has been generated about p16 as a tumor suppressor that is down-regulated in cancer (for a recent review see Romagosa et al. 2011). However, p16 can also be overexpressed in tumors. In fact, p16 overexpression in some types of tumors, such as cervical and head and neck cancer (especially oropharyngeal squamous cell carcinoma) is used as a diagnostic tool and has been directly associated with infection by high-risk genotypes of HPV (Mulvany et al. 2008). The use of p16 IHC as a surrogate for HPV is, however, still somewhat controversial. This method has been shown to have 100% sensitivity in screening for transcriptionally active infection (Smeets et al. 2007, Singhi & Westra 2010). In turn, HPV independent pathways of oncogenesis can also lead to increased expression of p16 and thus the specificity of p16 IHC turns out to be only 79% (Smeets et al. 2007, Singhi & Westra 2010, Lundberg et al. 2011). HPV-associated HNSCCs have been shown to have a better prognosis in terms of both recurrence and survival (Romagosa et al. 2011). Actually, based on this study and literature, p16 seems to be quite a strong candidate as a prognostic marker. The
survival analysis showed here a statistically significant correlation between the tumor immunohistochemical staining of p16 protein and the overall survival of the HNSCC patients. The Kaplan-Meier analysis showed that the cumulative survival rate of patients with a p16-negative tumor was 41% after 5 years’ follow-up, whereas the cumulative survival for p16-positive cases was 74%. However, there was no statistically significant correlation between the p16 status and 5-year disease-specific survival, which was 59% in those with a p16-negative tumor and 72% in p16-positive cases. Furthermore, there was no difference in the present study between p16 immunohistochemical staining result and progression-free survival. There is a growing body of studies showing inverse correlation between p16 immunohistochemical staining and poor overall survival in HNSCC (Harris et al. 2010, Young et al. 2011, reviewed by Romagosa et al. 2011), which is in line with the present study.

No correlation was observed between p16 expression and clinicopathological variables such as histological grade, TNM classification, the stage of the disease or the age of the patients. This is in line with the study by Namazie et al. 2002. On the contrary, Yuen et al. (2002) have reported correlation between p16 expression and T stage. They found a significant inverse correlation between the p16 expression and advanced stage in HNSCC (Yuen et al. 2002). In the literature it is also suggested that p16 immunohistochemical expression can function as a surrogate marker to predict treatment outcome in HNSCC (Kumar et al. 2008, Nichols et al. 2009, for a review see Romagosa et al. 2011).

6.4.4 p21 in HNSCC

p21 plays an important role in modulating cell cycle control, inducing apoptosis, and inhibiting cell growth, subsequently affecting cancer risk. Since p21 expression is one of the most prominent markers for the functional activity of p53, many studies have analyzed p21 expression in different types of human cancer, but the results are conflicting (Lei et al. 2010). Given the functional importance of p21 in critical p53 pathway, genetic alteration of p21 could be associated with prognosis in SCCHN patients. There is a growing number of studies showing correlation between p21 immunohistochemical staining and prognosis in HNSCC (Hafkamp et al. 2009, Kapranos et al. 2001, Fischer et al. 2011). Kapranos et al. (2001) as well as Hafkamp et al. (2009) have reported that the lack of p21 was shown to correlate with tumor progression and negative prognosis in head and neck cancers (Kapranos et al. 2001, Hafkamp et al. 2009). In the present study,
there was a trend, but not a significant association, between p21 immunohistochemical staining and disease-specific survival. The cumulative 5-year disease-specific survival rate was 72% for the patients presenting with a tumor with high p21 expression, whereas it was only 53% for patients with a low expression of p21. No significant correlation was observed between p21 immunohistochemical staining and overall survival in this study. This is in line with a previous study by Korkmaz et al. (2005), as well as Chung et al. (2009), which found no correlation to overall survival (Korkmaz et al. 2005, Chung et al. 2009). On the contrary, however, Fischer et al. (2010) have reported that p21 expression does correlate significantly with poor overall survival (Fischer et al. 2010). The prognostic value of p21 overexpression remains thus conflicting.

Interestingly, in the present study it was not possible to detect any significant correlations between the immunoreactions for p21 and the traditional clinical or histopathological prognostic factors at the time of diagnosis, such as the stage or grade of the tumor. Neither was there any correlation between patient’s age or sex or anatomical site of the tumor and the expression of the p21 protein in the primary tumor. These data contradict some previous results but are in line with those of Fischer et al. (2010).

### 6.4.5 Cell cycle regulators as potential prognostic markers in HNSCC

Based on the literature and this study, a combination of markers seemed to show better prognostic value than any single marker alone (Chung et al. 2009). There is a growing amount of evidence indicating that cyclin D1 and p16, which are biologically closely related in the G1/S phase of the cell cycle, might have an important role in the clinical evaluation. The Kaplan-Meier analysis showed that only 25% of patients with a high expression of cyclin D1 but a p16-negative tumor survived 5 years after diagnosing the malignancy, while more than half (64%) of the group including all other cases with different staining results survived that time. Although the size of these subgroups was relatively small, we were able to observe that the patients with a tumor where cyclin D1 was positive and p16 negative had a significantly worse overall survival rate compared to the other subgroups of patients. In the present study, the combination of p16 negativity plus high expression of cyclin D1 was the strongest indicator for poor prognosis. These findings are in line with the literature (Namazie et al. 2002, Higuchi et al. 2007). The prognostic value of these markers has previously been reported in HNSCC patients treated with concurrent radiotherapy and weekly
docetaxel (Higuchi et al. 2007). Namazie et al., (2002) have also shown that the combination of cyclin D1 amplification and p16 deletion serves as a better biological marker than either of the aberrations alone in identifying aggressive tumor behavior (Namazie et al. 2002). However, there was no statistically significant correlation in the disease-specific survival and no significant difference in progression-free survival in the present study.

In this study, patients with p16- and p21-positive and cyclin D1-negative tumors at IHC showed a trend towards a better 5-year disease-specific survival rate of 88%, while the survival rate in other cases was 55%. A similar trend was also seen in overall survival rates, 88% of the patients with tumors showing expression of p16+/p21+/cyclin D1- survived for 5 years after the diagnosis of HNSCC, whereas in patients with tumors showing other staining results a 53-percent overall survival rate was observed. In addition, there was no difference in relapse rates between these two groups in the present study. The difference in survival suggests that grouping the markers according to their function (cell cycle regulators) could aid in finding potential prognostic scores predicting outcome in HNSCC. The major limitation in the present study was the small number of tumor samples; further studies are thus needed to clarify the connection.
7 Summary and conclusions

Survival of patients with locally advanced HNSCC remains poor despite improved treatment modalities. New markers indicating biological aggressiveness are thus warranted to help select candidates for novel intensive approaches such as neoadjuvant chemotherapy. In the present study, p53 alterations and some other cell cycle regulating proteins were explored in tumors of patients with head and neck squamous cell carcinoma. The association between environmental exposure and p53 alterations was evaluated. The prognostic role of p53 alterations (especially specific TP53 mutations) and cyclin D1, p16 and p21 proteins as immunohistochemical markers was also pursued. Important considerations for feasibility of a marker are its biological impact and the specificity and sensitivity of the assay to detect it in an appropriate clinical setting.

The specific conclusions are summarized as follows:

1. Validation of the analytic method used, e.g. PCR-SSCP, is essential for obtaining good sensitivity, specificity and efficiency, especially when using human tumor samples. The diversity of the type and functional consequences of TP53 mutations clearly need to be taken into account when analyzing the prognostic value of TP53 mutations. IHC detects protein with antibodies and is not a method for analyzing TP53 gene mutations.

2. Specific TP53 mutations were linked with chemical exposure and indicate their usefulness as molecular markers in HNSCC. Patients with tumors containing TP53 mutations in the DNA-binding domain had significantly higher exposure to chemicals compared to patients with a wild-type p53 in tumor. Especially the TP53 mutation Asp259Glu correlated to exposure and was more prevalent in laryngeal tumor, which may represent a preferential site of this mutation.

3. Patients with a tumor containing TP53 mutation in the DNA-binding domain had a significantly less favorable prognosis and response to radiotherapy than patients with wild-type p53 or a mutation of TP53 outside the DNA-binding region. TP53 is a potentially useful marker for prediction of the patient’s outcome and response to radiotherapy in head and neck cancer.

4. High expression of cyclin D1 and lack of p16 protein expression in IHC were the strongest prognostic indicators of a poor outcome. A combination of markers seemed to have better prognostic value than any single assay alone.
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