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MOLECULAR BIOMARKERS IN HEAD AND NECK CANCER

Evaluation of biomarkers in prognostication and radiotherapy response prediction

Johannes Routila

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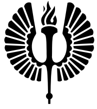

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To my family;

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JOHANNES ROUTILA: Molecular Biomarkers in Head and Neck Cancer.
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ABSTRACT

Radiotherapy plays an integral part in the treatment of head and neck squamous cell carcinoma (HNSCC). Despite rigorous investigation spanning several decades, no molecular biomarkers are currently available for the prediction of radiotherapy response of an individual HNSCC tumour. Several radioresistance mechanisms have been acknowledged, including p53 alterations, hypoxia, and cancer stem cells.

In this thesis, the overall purpose, role, and interpretation of molecular biomarkers in the context of HNSCC is discussed, and the clinical problem-field is emphasized. Putative radioresistance related molecular biomarkers were selected for investigation in HNSCC cell lines and patient materials. For clinical investigation, all HNSCC patients treated in the tertiary referral centre of Turku University Hospital during 2005-2010 were retrospectively collected. Clinical patient data was gathered, patient tumour samples were collected and processed into a tissue microarray. Immunohistochemistry and in situ hybridization were used for detection of biomarker expression and their relation to patient survival was analysed in multivariable survival models.

Copy number alterations of stemness associated cancerous inhibitor of protein phosphatase 2A (CIP2A) were demonstrated in HNSCC cell lines and the presence of copy number alterations was found to be associated with a poor prognosis in HNSCC patients. Putative radioresistance biomarkers were investigated in several HNSCC cell lines after construction of a cell microarray. Stem cell marker OCT4 was revealed to be significantly associated with intrinsic radioresistance. The representativeness of the clinical tissue microarray was carefully confirmed using a novel population validation method. Using immunohistochemical stains, putative prognostic biomarkers were shown to perform poorly in the population-validated tissue microarray (PV-TMA). Finally, using the PV-TMA, OCT4 was found to predict for poor radiotherapy response and improved chemoradiotherapy response.

In conclusion, using HNSCC cell line microarray and highly representative PV-TMA patient material, OCT4 was established as a stratification biomarker between radiotherapy and cisplatin-based chemoradiotherapy.

KEYWORDS: biomarker; stemness; radiotherapy; radiosensitivity; radioresponse; cisplatin; cell microarray; tissue microarray; population-validation; semiotics; head and neck cancer; squamous cell carcinoma

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Lääketieteellinen tiedekunta
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TIIVISTELMÄ

Sädehoidolla on keskeinen rooli pään ja kaulan alueen levyepiteelisyöpien hoidossa. Vuosikymmenten tutkimuksesta huolimatta yksittäisen pään ja kaulan alueen levyepiteelisyövän sädehoitovastetta ennustavaa molekulaarista biomarkkeria ei ole käytettävissä. Nykyisin tunnetaan lukuisia huonoa sädeherkkyyttä selittäviä mekanismeja kuten p53-geenin mutaatiot, kasvaimen hypoksiset olosuhteet ja syöpäkantasolut.

Tässä työssä tarkastellaan molekulaarisen biomarkkerin tehtävää, tarkoitusta ja tulkintaa pään ja kaulan alueen levyepiteelisyövän yhteydessä korostaen kliinistä ongelmakenttää. Sädeherkkyyteen liitettyjä biomarkkeriehdokkaita tutkittiin pään ja kaulan levyepiteelisyöpäsolulinjoja ja potilasaineistoja hyödyntäen. Kliinisenä tutkimuksena kerättiin kaikki vuosina 2005–2010 Turun yliopistollisessa keskussairaalassa hoidetut pään ja kaulan alueen levyepiteelisyöpäpotilaat käsittävä aineisto. Potilasnäytteet kerättiin ja niistä valmistettiin kudosmikrosiru. Biomarkkerien ilmentymistä tutkittiin immunohistokemiallisilla menetelmillä ja in situ hybridisaatiolla ja niiden yhteyttä potilaiden ennusteeseen selvitettiin monimuuttujaisilla ennustemalleilla.

CIP2A-proteiinin kopiolukumuutoksia todettiin pään ja kaulan alueen levyepiteelisyöpäsolulinjoissa, ja kopiolukumuutosten havaittiin olevan yhteydessä pään ja kaulan levyepiteelisyöpäpotilaiden heikentyneeseen ennusteeseen. Pään ja kaulan alueen levyepiteelisyöpäsolulinjoista kootussa solumikrosiruaineistossa havaittiin kantasolutekijä OCT4:n olevan merkitsevästi yhteydessä solujen sädeherkkyyteen. Kliinisen kudosmikrosiruaineiston todettiin olevan edustava. Biomarkkeriehdokkaat suoriutuivat huonosti potilaiden ennusteen määrittämisestä, mutta OCT4 ennusti levyepiteelikasvaimen huonoa sädehoitovastetta, mutta hyvää ennustetta sisplatiinipohjaisen kemosädehoidon jälkeen.

Tutkimuksessa todettiin pään ja kaulan levyepiteelisoluja ja edustavaksi havaittua väestövarmennettua kudosmikrosiruaineistoa hyödyntäen, että OCT4 soveltuu sädehoidon ja sisplatiinipohjaisen kemosädehoidon valintaa ohjaavaksi biomarkkeriksi.

AVAINSANAT: biomarkkeri; kantasolumaisuus; sädehoito; sädeherkkyys; sädehoitovaste; sisplatiini; solumikrosiru; kudosmikrosiru; väestövarmennus; semiotiikka; pään ja kaulan alueen syöpä; levyepiteelikarsinooma

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Abbreviations

AUC	Area under the curve
BAC	Bacterial artificial chromosome
CI	Confidence interval
CIP2A	Cancerous inhibitor of protein phosphatase 2A
CMA	Cell microarray
CRT	Chemoradiotherapy
C/RT	Radiotherapy with or without chemotherapy
DNA	Deoxyribonucleic acid
DMEM	Dulbecco's modified Eagle's medium
EGFR	Epidermal growth factor receptor
EBV	Epstein-Barr virus
FFPE	Formalin-fixed paraffin-embedded
FISH	Fluorescence in situ hybridization
HER2	Human epidermal growth factor receptor 2
HNSCC	Head and neck squamous cell carcinoma
HPV	Human papillomavirus
HR	Hazard ratio
LSS	Locus-specific signal
mRNA	Messenger ribonucleic acid
NDFIP1	Nedd4 family interacting protein
OCT4	Octamer-binding transcription factor 4 (pou5f1)
OR	Odds ratio
OS	Overall survival
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein-1
PD-L1	Programmed cell death ligand-1
PV-TMA	Population-validated tissue microarray
RT	Radiotherapy
shRNA	Short hairpin ribonucleic acid
SNB	Sentinel node biopsy

UT-SCC	University of Turku – Squamous Cell Carcinoma
TCGA	The Cancer Genome Atlas
TMA	Tissue microarray
TNM	Tumour – node – metastasis classification

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Routila J, Bilgen T, Saramäki O, Grénman R, Visakorpi T, Westermarck J, Ventelä S. Copy number increase of oncoprotein CIP2A is associated with poor patient survival in human head and neck squamous cell carcinoma. *Journal of Oral Pathology and Medicine*, 2016; 45(5): 329–37.

II Routila J, Suvila K, Grénman R, Leivo I, Westermarck J, Ventelä S. Cancer cell line microarray as a novel screening method for identification of radioresistance biomarkers in head and neck squamous cell carcinoma. *BMC Cancer*, 2021; 21(1): 868.

III Routila J, Leivo I, Minn H, Westermarck J, Ventelä S. Evaluation of prognostic biomarkers in a population-validated Finnish HNSCC patient cohort.
European Archives of Oto-Rhino-Laryngology, 2021; 278(11): 4575-85.

IV Routila J, Qiao X, Weltner J, Rantala JK, Carpén T, Hagström J, Mäkitie A, Leivo I, Ruuskanen M, Söderlund J, Rintala M, Hietanen S, Irjala H, Minn H, Westermarck J, Ventelä S. Cisplatin overcomes radiotherapy resistance in OCT4-expressing head and neck squamous cell carcinoma. *Oral Oncology*, 2022; 127: 105772.

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1 Introduction

Tumours classified as head and neck squamous cell carcinoma (HNSCC) arise from the epithelial lining of the mucosa of the upper aerodigestive tract. The main subsites of HNSCC are oral cavity, oropharynx, larynx, and hypopharynx. HNSCC represents over 90% of all head and neck cancers and is linked to the clinically important phenomenon of precancerous changes along the whole mucosal lining, the field cancerization phenomenon. One of the most disconcerting problems is the astounding diversity among HNSCC cases. (Gnepp 2009). Commonly encountered clinical surprises include the aggressive behaviour of a small, histologically indolent tumour, radiotherapy failure despite optimal planning, but also a radical radiotherapy response of an invasive tumour.

HNSCC has proven particularly resistant to endeavours of the scientific community to develop functioning biomarkers for therapy selection. Here, an emphasis on the word *functioning* is laid since many suggested biomarkers have proven to have little practical usefulness despite their perfectly feasible theoretical background. Such feasibility is apparent in analysis of the molecular diversity of HNSCC, which has not been able to produce practicable therapy-guiding molecular biomarkers. The inability to bring about functioning biomarkers is the more disconcerting as recent advances in other cancers and other fields of cancer therapy have provided survival advantage among cancer patients. The victory parade of human epidermal growth factor receptor 2 (HER2) and the targeted trastuzumab therapy for treatment of breast carcinomas and, more recently, lung carcinomas is probably the best-known example (Pegram et al. 1998; Singer et al. 2008; Wu et al. 2022).

Many studies and research groups approach HNSCC divided in parts based on *e.g.,* the site of the primary tumour. In this thesis, another view is adopted and HNSCC – rather than “HNSCCs” – is discussed based on several important considerations. First, HNSCC is treated by the same community of head and neck surgeons and head and neck oncologists responsible for treatment decisions, making discussions of HNSCC fruitful for the whole community rather than tailoring them to a subspecialty. Secondly, no compelling genetic or molecular difference between the subsites has been discovered (Camuzi et al. 2021; Zhu et al. 2022). Thirdly, boundaries between the subsites are somewhat artificial as demonstrated by the frequent involvement of

several distinct locations as well as multiple primary tumours (Coca-Pelaz et al. 2020). Fourthly, all HNSCC has a frequent tendency for metastatic involvement of neck lymph nodes, which dictates a ubiquitous emphasis of neck status and handling.

Whatever approach is taken, it is clear, that discovery of clinically useful therapy stratification biomarkers for HNSCC is currently an unmet need. While this claim is often repeated, very little effort has been provided to answer the exact nature of a *useful biomarker*.

1.1 Prolegomena

The first question to be answered in a thesis concerning biomarkers is the very nature of biomarkers themselves: What is a biomarker? Or to be more exact, what distinguishes a biomarker from other alterations or processes in the body. It is apparent from the vast literature on biomarkers, that the term biomarker is often used without much consideration to its definition. The concept of biomarker was adopted in the 1950s, supposedly by Porter, and it highlights the emergent biomechanical understanding, that alterations in normal processes can be measured to give indirect evidence of such alterations (Porter 1957). The purpose of a biomarker is to provide information about biological processes, that can be applied in evaluation of patients and their treatment. In the Biomarker Definitions Working Group paper, a definition of a biomarker was given as follows:

> A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention. (Atkinson et al. 2001)

In addition to the exact or numerical nature of biomarkers – a biomarker has an *objectively measured* value of some sort, albeit usually a dichotomous interpretation of *e.g.,* the presence or absence of a stain – it already by definition carries the role for the medical practice as an indicator of an underlying process or therapy response. For the purposes of this discussion, we will concentrate on molecular biomarkers, and leave clinically revealed signs and symptoms aside, even though it is apparent that they often serve a similar function in the role of a biological marker. For this understanding, the interpretation of molecular biomarkers happens in a specific patient-context. As is intuitively clear, a molecular biomarker provides information, that is applied as a change in the way the patient's problem is approached. However, this interpretation is not as simple a process than it seems.

For a molecular measurement to become a molecular biomarker, it needs to play a specific role for the medical practice: The biomarker needs to indicate a useful biological process. The objectivity of biomarkers would seem to suggest that a

biomarker is a passive indicator of the underlying process, while it is apparent, that there is an active process of interpretation before a biomarker starts to mean something. The process of interpretation can be called semiosis and the art and science of interpreting semiotics. The meaning-making and the interpretative task concerning biological phenomena play an increasing role in the current understanding of biomarkers.

The basis for biomarker function is often postulated to lie in the preclinical and mechanistic molecular study of tumour biology. It is universally acknowledged, that after a mechanistic understanding about a specific biological process is revealed, this process can be assessed using a suitable biomarker for evaluation of the individual patient. Useful and usable biological markers may provide information about the presence of disease, such as glycoprotein CA12-5 for ovarian carcinoma or prostate specific antigen (PSA) for prostate adenocarcinoma but may also indicate a treatment response such as HER2 analysis in breast carcinomas.

A typical classification divides biological markers into diagnostic, staging, prognostic, and predictive markers (Kim et al. 2014; Ludwig and Weinstein 2005). This understanding is very useful in the context of straightforward biomarker discovery. However, despite the apparent appeal of such classification, there are several astute criticisms of such division concerning HNSCC.

α. A diagnostic biomarker would aim at indicating the presence or absence of a disease. While this may be true for some biomarkers, the interpretative task with cancer biomarkers consists of several steps prior to the diagnosis of cancer could be made. Thus, the idea of a diagnostic biomarker is a theoretical one. A further complication in HNSCC is the relative ease in evaluating the mucosal lining of the upper aerodigestive tract, setting requirements on diagnostic biomarkers much higher than in more occult cancers.

β. A staging biomarker should aim at providing information about the tumour extent or spreading. However, the typical example of a staging HNSCC biomarker is the use of p16 immunohistochemistry to stage oropharyngeal HNSCC. Here, the staging rules are determined based on the etiological landscape of the tumour, not that p16 would in itself provide information about the tumour extent.

γ. The differentiation between prognostic and predictive biomarkers has produced a great number of literature (Ballman 2015; Sechidis et al. 2018). While the distinction should be discursively understood, it plays little role in practice, since prognostic biomarkers tend to change the way patients are treated.

δ. Thus, the true *well-defined* functional biomarker seems to be the predictive biomarker, which indicates a specific course of action for the actual patient.

An example of such biomarker use could be the HER2 detection, which allows for a dichotomous evaluation of HER2 therapy indication.

1.2 The semiotic concept of a biomarker

In recent years, there has been a revived interest in the theory of interpretation put forward by Ch. S. Peirce in the late 19th century (Bell 2013; Peirce 1903). In biology and related sciences, a field called biosemiotics has arisen to offer a philosophically motivated backbone for understanding interpretation in biosciences (Aragno 2019). In accordance with Peirce's semiotic theory, such theories are focused on the concept of a sign – a carrier of meaning (Figure 1). Peirce's original concept of a triadic model of meaning consists of three parts: The object itself, the representamen, and the interpretant. Semiosis – the interpretation of the sign and the understanding of its meaning – consists of encountering the representamen such as the immunohistochemical stain of a protein and understanding the interpretant. A final interpretant would be the end results arising from examination of the biomarker status. The object of interpretation is not the encountered representamen, but the biological process that lies *behind* the representamen, which is in turn associated with a specific course of action, a habit. In Peirce's formulation, the meaning of a sign is the habit it produces. (Favareau 2015; Peirce 1903, 2011.) Accordingly, a meaning is derived about the patients' condition from the simple brown colour of an immunohistochemical stain. This triadic understanding of sign is unique to Peirce's semiotics and provides an interesting starting-point for the analysis of molecular biomarkers, which intuitively carry with themselves a deeper interpretative meaning about the individual patient.

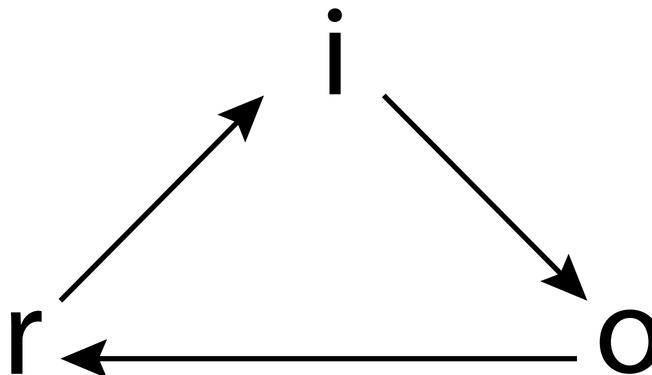


Figure 1. The triadic model of semiosis conceptualizes the three components of interpretation: the object (o), the representamen (r)l, and the interpretant (i).

In actual life, the process of semiosis is much more complicated both because of the multitudes of different kinds of signs and because of the multi-layered reality making up a complicated chain of signs, which may in most cases be an infinite series. Furthermore, the interpretation of a sign is not only dependent on the representamen, understood as the underlying biological process. Interpretation is crucially influenced by the specific *universe of discourse*, in which the interpretation takes place. Thus, the individual interpretative task related to the specific biomarker happens as a part of a complicated understanding of the totality of a diagnostic process, consisting of several interpretations of different clinical and molecular signs. The meaning of the biomarker depends on the overall understanding of the patient's situation. This highlights the importance of the surrounding clinical information.

While the meaning of a sign carries with itself a specific habit of action, the objective-looking biomarker does not dictate the action that is undertaken, but instead the actions reflect the way interpretation was made. Such a reversal of interpretation – a retroactive semiosis – gives rise to the appearance of objectivity in changing from manifestly subjective interpretation of patient condition to a fetishistically objective interpretation (Marx 1890; Wahrig 2018; Žižek 1989). The fetishistic reversal of object-relations demonstrates itself in the reliance on the objective-looking biomarkers and the inability to carry out biomarker interpretation independent of the patient's clinical state. This type of critique of pseudo-objectivism in medical and biological processes of interpretation has been common in continental philosophical scientific literature (Gadamer 1960; Heidegger 1927; Lukács 1968; Wahrig 2018; Žižek 1989), whereas even in hermeneutically oriented medical literature the objectivism is rarely questioned (Leder 1990; Svenaeus 2000). Accordingly, the typical context of medical philosophy approaches treatment problems as problems of evidence and its quality (Cochrane 1972; Guyatt et al. 1992; Stanley and Campos 2016).

What was said above holds true regarding all kinds of biomarkers, both clinical measurements, imaging biomarkers and molecular biomarkers. Molecular biomarkers are often thought to hold a special place in the complex system of relations of interpretation. Their nature is defined in the measurability and apparent objectivity (*cf.* Bell, 2013). However, this objectivity is superficial as molecular biomarkers carry with themselves similar interpretative problems as other types of biomarkers.

α. Firstly, there are issues in the reliability of the measurement, such as confounding factors. For the reliability of the biomarker, it is mandatory that the thing that is measured is what is thought to be measured, which is often not the case, when different isoforms or similar protein structures are present.

β. Secondly, there is the question of appropriateness, especially regarding timing of measurement. Measurements should be carried out at an exact time-point, *e.g.,* prior to radiotherapy or after irradiation, in the context of primary therapy or recurrence.

γ. Thirdly, there is the problem of interpretation of the biomarker result. The results are a continuous, but their interpretation is based on a cut-off value. This cut-off value may be seemingly objective such as the 70% limit used in p16 evaluation or openly arbitrary such as a semiquantitative +++ often used in preliminary studies. In both cases the continuous and complex real data expressed in *e.g.*, the immunohistochemical stain is reduced to a dichotomy of positive vs. negative.

In addition, the patients, cancers, and individual tumours behind the biomarkers are very different from each other, complicating the interpretative task. Finally, the medical practitioners carry their own prejudices and presuppositions, which affect the way biomarkers are interpreted and applied, into the process of interpretation (Figure 2). This radical subjectivity is well discussed as the concept of a hermeneutic circle, where the final interpretation depends on previous steps during the process (Gadamer 1960).

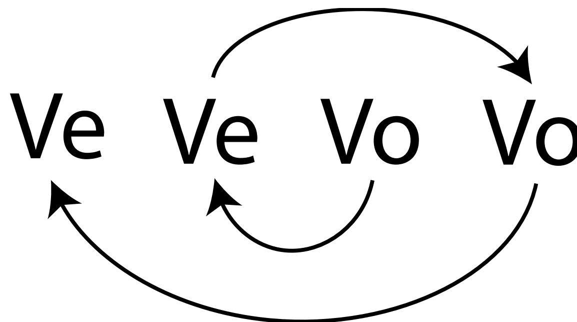


Figure 2. The hermeneutic circle illustrates the relationship between *Vorgriff (Vo)* and *Verständnis (Ve)*, fore-conception and understanding.

Superficially, the clinical interpretation of a molecular biomarker may seem very easy. A positive result would suggest a certain course of action and a negative result another. A perfectly feasible application of such a specificity of habit is demonstrated in the HER2 detection, which is used in breast carcinomas to decide, whether a patient could be treated with HER2 inhibitor trastuzumab. Interestingly enough, HER2 inhibitors are sometimes used in salivary duct carcinoma, in which the HER2 determination seems to function similarly (Uijen et al. 2022). The case of HER2 emphasizes, that while the biomarker is dichotomously reduced to a simple positive

vs. negative, the clinical treatment problem is not answered by this dichotomy. The interpretation of HER2 is straightforward, because it dictates a dichotomous treatment practice: Either the use of HER2 inhibitor or the use of standard therapy. Crucially, this information is obtained only when the possibility for consideration of HER2 is on the table. However, this does not unfortunately hold true for most biomarkers since the purpose of such biomarkers is not very well-defined. A dichotomously functional biomarker could be dubbed a *well-defined biomarker*.

According to the semiotic understanding of a molecular biomarker, the important question is the treatment decision, which the biomarker opens. The final interpretant of the semiosis of a molecular biomarker is the action it brings about. This meaning of the biomarker – the habit it produces – is in the transformation if causes to the playroom of medical experience. Thus, investigation into a putative or possible biomarker should be carried out with utmost regard to the surrounding clinical information. This understanding plays a significant role on the whole process of biomarker discovery: The specific clinical problem to be answered by the molecular biomarker takes supremacy in comparison to the biological processes reflected in the biomarker expression and regulation (Goossens et al. 2015).

2 Review of the Literature

2.1 Head and neck squamous cell carcinoma

Head and neck squamous cell carcinoma is an umbrella term for squamous cell carcinoma of the mucosal lining of the upper aerodigestive tract (Gnepp 2009). HNSCC affects all subsites of head and neck regions, the mucosal epithelial lining of the upper gastrointestinal and respiratory tracts. The most common sites are oral cavity, oropharynx, larynx, and hypopharynx, while HNSCC tumours originating in the sinonasal cavity or nasopharynx are relatively rare. Since the advent of modern imaging technology, carcinomas of unknown primary represent a small percentage of tumours.

The terminology of HNSCC is complicated by the fact, that this cancer provides an exceptional diversity of tumours, with exquisite and elaborate differences in behaviour as well as molecular profile. These squamous cell carcinomas encompass several histological subtypes, and the copious subsites have many remarkable features. Clinical classification of HNSCC is based on the tumour – node – metastasis (TNM) classification, the concept of which was originally introduced by Pierre Denoix (Denoix 1950). The first TNM classification for laryngeal cancer appeared in 1958, while the latest update of the TNM classification of HNSCC was published in 2017 (UICC 1958, 2017). In the 8th edition, the most significant changes were the inclusion of p16 immunohistochemistry in classification of oropharyngeal squamous cell carcinoma and a novel classification of metastatic carcinoma of unknown primary.

The histopathological appearance of HNSCC is one of squamous differentiation with possible keratinization, nuclear pleomorphism, and frequent mitosis (Figure 3). A lymphocytic infiltrate is common (Mandal et al. 2016). The clinical significance of histological grade is in dispute (Anderson et al. 2021; Flörke et al. 2021; Fortin et al. 2001) and was recently abandoned in high-risk human papillomavirus (HPV) positive oropharyngeal squamous cell carcinoma (UICC 2017). Important histological subtypes of HNSCC include verrucous HNSCC, which frequently exhibits local recurrence but does not metastasize in the absence of an invasive component (Ackerman 1948; Alonso et al. 2018; N. Wang et al. 2020). Papillary, basaloid, and spindle cell HNSCC are relatively rare subtypes (El-Naggar et al. 2017). Such HNSCC tumours are treated according to usual guidelines, since differences in aggressiveness

have not been unequivocally proven (Russell et al. 2011; Shah et al. 2014). Lymphoepithelial carcinoma presents peculiar diagnostic problems since it has a strong lymphatic component and may be confused with lymphomas (Gnepp 2009). Epstein-Barr virus (EBV) detection is used especially in the evaluation of nasopharyngeal carcinoma, lymphoepithelioma, and carcinoma of unknown primary, whereas EBV positivity has no direct therapeutic implications (Goon et al. 2022).

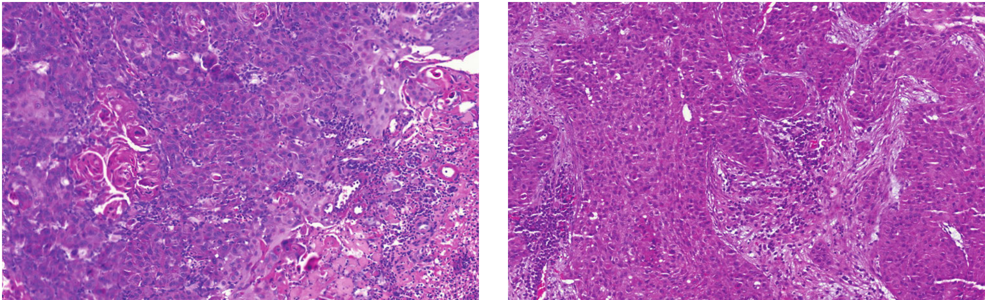
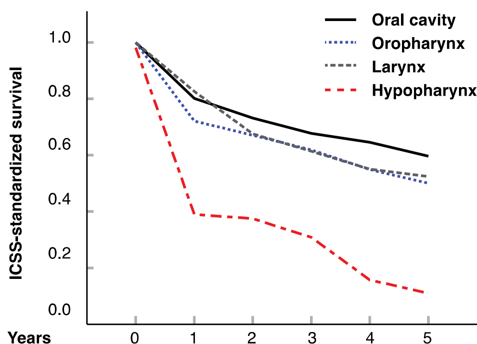


Figure 3. *left*: The histological appearance of grade I HNSCC of tongue margin with its typical keratinization, nuclear pleomorphism, and lymphocytic infiltrate. *right*: A grade III, HPV-negative oropharyngeal HNSCC.

The survival rates of HNSCC have improved negligibly during several decades (Bray et al. 2018; Gatta et al. 2015). Importantly, carcinomas of unknown primary may carry a favourable prognosis (Axelsson et al. 2017), while hypopharyngeal tumours are associated with a particularly dismal survival rate (Figure 4). The changing epidemiology of oropharyngeal HNSCC with an increase in HPV-related disease is probably associated with an improvement in survival.

Turku University Hospital in 2005-2015



EUROCARE-5 in 2000-2007

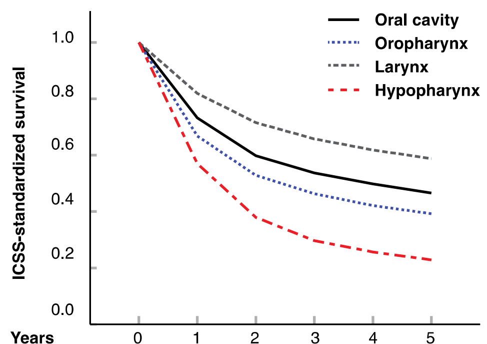


Figure 4. Age-standardized survival rates in main sites of HNSCC (oral cavity, oropharynx, larynx, and hypopharynx) in *left:* Turku University Hospital and *right:* EUROCARE-5 data.

While there are some differences of therapeutic strategy between the subsites of HNSCC, several considerations support discussing HNSCC in its entirety. Overall, the boundaries between HNSCC subsites are somewhat artificial as demonstrated by the frequent involvement of several distinct locations as well as multiple primary tumours within the same subsite (Coca-Pelaz et al. 2020). Despite important genetic and molecular heterogeneities, no compelling literature is available concerning a genetic or molecular difference between the subsites of HNSCC (Camuzi et al. 2021; Saba et al. 2020). The traditional risk factors, mainly alcohol and tobacco exposure, affect the complete mucosal lining of the upper aerodigestive, giving rise to the important phenomenon of field cancerization (Slaughter et al. 1953). While viral causative agents are similarly distributed along the aerodigestive tract, evidence suggests that there is a lesser risk for synchronous second primary tumours in HNSCC related to HPV infection (Rietbergen et al. 2014; Xu et al. 2013). However, the viral risk factors often present in conjunction with a canonical risk profile (Bouland et al. 2021; Jouhi et al. 2018).

Emphasizing the clinical consideration of HNSCC, the same multi-specialty tumour boards are responsible for treatment decisions. Thus, the decisions between surgical and oncological treatment approaches are decided similarly between the different subsites. Finally, HNSCC tumours have a frequent tendency for metastatic involvement of neck lymph nodes, which dictates a ubiquitous emphasis of neck status and handling. In conclusion, the umbrella concept of HNSCC should not be abandoned, while both the clinical and the research community should remain appreciative of the distinctive features making up clinical and pathological subcategories.

Despite a growing body of literature concerning the genetic and molecular events in HNSCC, and the advancing knowledge about the different risk factors for HNSCC, discovery of clinically useful molecular biomarkers has proven an exceptionally challenging task. Thus, treatment decisions of HNSCC are based on clinical and histological evaluation alone.

2.1.1 Molecular diversity of HNSCC

HNSCC classification attempts include *e.g.,* the anatomical site-subsite divisions describing the specific localities and regions affected by the disease and the histopathological features of the tumours, which define both the originating tissue type and other features visible under the microscope. Novel molecular studies have provided interesting insights into the molecular and genetic diversity of HNSCC. An undisputed hallmark of HNSCC is genomic instability, leading to a high rate of copy number alterations and DNA mutations (Gollin 2014).

Copy number increases of genomic regions at the long arm of chromosome 3 (3q) have consistently been reported in over 50% of HNSCC originating from the main subsites (Huang et al. 2002; Patmore et al. 2007; Pickering et al. 2013; Speicher et al. 1995). High-level amplifications of bands 3q26 and 3q13 are especially frequent (Huang et al. 2002; Oga et al. 2001; Singh et al. 2001; Wreesmann et al. 2004) and have, furthermore, been linked to patient prognosis (Bockmühl et al. 2000; Singh et al. 2002). Copy number increase of several individual genes such as type 1 activin receptor and epidermal growth factor receptor (EGFR) have been reported to associate with survival and chemoresistance of HNSCC (Ambrosio et al. 2011; Nakata et al. 2011). Interestingly, while low-rate copy number alterations are often suggested to associate more with generalised genomic instability than actual oncogenic locus-specific driver amplification event (Korkola and Gray 2010), modest EGFR copy number increase was published to be a significant prognostic indicator in oral cavity squamous cell carcinoma (Nakata et al. 2011). Despite the widespread understanding, that genomic instability of HNSCC leads to copy number increase, no genomic test for identification of oncogenic activation has been adopted to clinical practice. The detection of DNA ploidy and S phase fraction by flow cytometry has largely been abandoned in spite of some positive results (Pekkola-Heino et al. 1994; Zätterström et al. 1991). However, detection of viral integration of EBV and high-risk HPV using in situ hybridisation methods is used in clinical pathology departments (El-Naggar et al. 2017; Ruuskanen et al. 2019).

HNSCC tumours and HNSCC-derived cell lines exhibit highly variable genomic changes (Lepikhova et al. 2018; Ludwig et al. 2018; Mann et al. 2019; Ruutu et al. 2005). The most mutated gene in HNSCC is the tumour suppressor and cell cycle regulator p53 (Lawrence et al. 2015). Both activating and inactivating mutations may be present, and the specific type of mutation may have significant implication to HNSCC behaviour (Klinakis and Rampias 2020; Servomaa et al. 1996). The prognostic significance of p53 mutations has been established, while no definitive clinical implications have been fixed (Pekkola-Heino et al. 1998; Servomaa et al. 1996; Zhou et al. 2016). Inactivation of p53 may also happen through HPV-related p16, and HPV-positive HNSCC tumours often carry wild-type, unmutated p53 gene (Oh et al. 2013). Accordingly, a more commonly mutated gene in HPV-positive HNSCC is PIK3CA, encoding a catalytic subunit of PI3K/AKT/mTOR pathway (Janecka-Widła et al. 2021). Another important site for mutations is the EGFR gene (Vatte et al. 2017).

Much of the molecular diversity in HNSCC is not directly explained by the genomic events (Farah 2021). Copious changes in protein expression, methylation rates, and protein phosphorylation also take place. An important tool for understanding of the impact of such molecular changes is pathway analysis, which puts the individual alterations in a clinically meaningful context. However, in HNSCC, alterations are present in virtually every pathway, making definite

interpretation and signification of such observations difficult (Dietz and Wichmann 2011; Kim et al. 2020; Zhao et al. 2018). In addition, such results have proven poorly reproducible (Marret et al. 2021).

The mechanisms of malignant transformation at cellular level, the causes of therapy failure, and the manifold genetic and molecular alterations responsible for therapy resistance have been the focus of HNSCC study for decades (Farah 2021; Marret et al. 2021). Despite an increased understanding in all these areas, HNSCC resists the attempts at tackling the important issues of therapy selection using molecular biomarkers. However, genetically and behaviourally heterogeneous HNSCC tumours cannot satisfactorily be treated without regard for the molecular variation (Zhang et al. 2021). This is reflected in the surprising failures in treatment of inconspicuous and small tumours, the early metastasis of some tumours, and most importantly, in our inability to evaluate the risk for appearance of tumour recurrence after initially successful therapy. Such questions offer a backbone for discovery of molecular biomarkers for HNSCC therapy stratification.

2.1.2 Clinical diversity of HNSCC

In addition to tumour-related molecular and genetic diversity, there are also differentiating clinical patient characteristics, such as tobacco and alcohol consumption, occupational mutagenic exposures, and sexual practices associated with high-risk HPV infection. The traditional or canonical risk profile associated with mutagenic exposures explains a significant amount of variability in prognosis and treatment success (Denissoff et al. 2022; Ingarfield et al. 2019; Leoncini et al. 2015). On the other hand, the causative role of HPV infection seems to be related to prognostic benefit (Bryant et al. 2018; Chung et al. 2014; Jouhi et al. 2017). While the presence of high-risk HPV infection does not mandate sexual risk behaviour, epidemiological studies have established a significant association between oral sexual behaviour and oropharyngeal HPV infection (Rettig et al. 2015). Despite the landmark observation, that HPV is associated with favourable prognostic impact in oropharyngeal HNSCC, several trials have demonstrated a loss of such prognostic benefit, when de-escalation strategies are adopted (Chera and Amdur 2018; Gillison et al. 2019; Mehanna et al. 2019).

Roughly 50% of HNSCC patients presents with nodal metastasis, while distant metastasis is rare (Gatta et al. 2015). Frequent comorbidities are associated with patient age, and include cardiovascular disease, alcohol-related hepatic and neurovascular compromise, and tobacco-related pulmonary disease (Ruud Kjær et al. 2021). In addition to history of previous malignancies, second primary lung and oesophageal carcinomas are also frequently encountered (Sawaf et al. 2021). Based on epidemiological changes in HNSCC profile in recent years, however, routine

upper panendoscopy for evaluation of upper aerodigestive tract has gradually been abandoned in patients with a favourable risk profile (Metzger et al. 2019; Rodriguez-Bruno et al. 2011; Valentin et al. 2021).

Despite differentiating features, the diverse HNSCC tumours share common characteristics. A careful clinical evaluation of the tumour is mandatory to discover its extension and the risks posed by therapy for surrounding tissue, which may ultimately lead to a significant loss of both functional and cosmetic requirements. Currently, treatment decisions are made in multidisciplinary head and neck tumour boards, where treatment protocols are optimized, treatment is meticulously planned, and therapy response is monitored (Grégoire et al. 2010; Machiels, René Leemans, et al. 2020). Most centres include dentists, nutritionists, speech therapists, pathologists, head and neck surgeons, and radiation oncologists in such boards (Machiels, René Leemans, et al. 2020).

The history of treatment of HNSCC is characterized by attempts to overcome the limitations of surgery. Sacrifice of normal or even functionally compromised tissue must be negotiated against the risks caused by loss of essential functionalities (Al-Qurayshi et al. 2022; Lane et al. 2022; Nichols et al. 2019). The introduction of radiotherapy in 1920s presented a revolutionary possibility in the treatment of previously inoperable tumours, and as was later to be seen, offered some advantages even in the treatment of resectable tumours (Coutard 1932; Shirinian et al. 1994). Despite the early successes and significant refinements in methodologies, it was soon to become apparent, that neither surgery, radiotherapy nor combination of both could be used to cure all patients. The appearance of a tumour recurrence after an initial success is the most common type of such failure and carries a poor prognosis. Treatment of tumour recurrence is often dictated by the delimitation of therapeutic options by previous radiotherapy (Mehanna et al. 2016). While an established option in select cases, reirradiation of an HNSCC recurrence may result in fatal complications (Kreinbrink et al. 2022).

The clinical stratification of HNSCC patients to different curative therapeutic strategies is based on evaluation of tumour spread in the form of TNM classification, histological evaluation, and patient overall condition. The primary tumour is treated with a local operation alone, radiotherapy alone, or local operation followed by radiotherapy. Less frequently, neoadjuvant radiotherapy followed by surgery is used. In all indications, based on tumour characteristics, radiotherapy may be combined with concurrent radiosensitizing chemotherapy. In addition to handling the primary tumour, management of the neck lymph nodes is a key issue and may be performed using neck dissection surgery or radiotherapy (Chow 2020; Machiels, René Leemans, et al. 2020). Treatment options of distant metastasis, previously considered an incurable disease, include, in select cases, both surgery and radiotherapy (Debbi et al. 2022; Vartanian et al. 2022).

Table 1. Summary of the Finnish national guidelines for treatment of HNSCC. *

ORAL CAVITY		T1-2N0	T1-2N1	T3-4N0	T3-4N1	T1-4N2-3
	primary	SX	SX	SX	SX	SX
	neck	none/SNB/L I-III	L I-III(IV)	L I-III	L I-IV	L I-IV(V)
	adjuvant	none	if indicated	if indicated	C/RT	C/RT
OROPHARYNX, P16+		**T1-2N0**	**T1-2N1**	**T3N0-1**	**T4N0-1**	**T1-4N2-3**
	primary	SX or C/RT	SX or CRT	SX or CRT	CRT	CRT
	neck	L II-IV or C/RT	L II-IV or CRT	L II-IV or CRT	CRT	CRT
	adjuvant	if indicated	CRT	CRT if SX	N/A	N/A
OROPHARYNX, P16-		**T1–2N0**	**T1-2N1**	**T1–2N2–3**	**T3N0–3**	**T4N0–3**
	primary	SX or C/RT	SX or C/RT	CRT	CRT	SX or CRT
	neck	LII-IV or C/RT	LII-IV or C/RT	CRT	CRT	CRT or LI-IV(V)
	adjuvant	if indicated	if indicated	N/A	N/A	CRT if SX
GLOTTIS		**T1N0**	**T2N0**	**T1-2N+**	**T3**	**T4**
	primary	SX or RT	SX or C/RT	CRT or SX	CRT	SX (or CRT)
	neck	none	(C/RT)	CRT or L II-IV	CRT	L II-VI
	adjuvant	none	none	if indicated	N/A	CRT
SUPRAGLOTTIS		**T1N0**	**T2N0**	**T1-2N+**	**T3**	**T4**
	primary	SX or RT	SX of CRT	SX or C/RT	CRT or SX	SX or CRT
	neck	L II-IV or RT	L II-IV or CRT	LII-IV or C/RT	CRT or L II-IV	L II-IV
	adjuvant	none	if indicated	CRT if SX	CRT if SX	CRT if SX
SUBGLOTTIS		**T1N0**	**T2N0**	**T1-2N+**	**T3**	**T4**
	primary	C/RT	CRT	CRT	surgery	surgery
	neck	C/RT	CRT	CRT	L II-VI	L II-VI
	adjuvant	N/A	N/A	N/A	CRT	CRT
HYPOPHARYNX		**T1-2N0**	**T1-2N1**	**T1–2N2–3**	**T3N0–3**	**T4N0–3**
	primary	SX or C/RT	CRT	CRT	CRT	SX or CRT
	neck	LII-IV or C/RT	CRT	CRT	CRT	CRT or LI-IV(V)
	adjuvant	if indicated	N/A	N/A	N/A	CRT if SX

*T: tumour classification, N: nodal status, SX: surgery, SNB: sentinel node biopsy, L: neck dissection levels, RT: radiotherapy, CRT: chemoradiotherapy, C/RT: radiotherapy with or without chemotherapy, N/A: not available. If indicated: treatment option based on the histological evaluation of the surgical samples.

Separate guidelines for the treatment of oral cavity, oropharyngeal, laryngeal, and hypopharyngeal tumours are available. The laryngeal tumours are further divided anatomically in glottic, supraglottic, and subglottic squamous cell carcinomas, whereas oropharyngeal squamous cell carcinomas are offered distinct guidelines based on p16 status. The typical therapeutic strategies of the major HNSCC subsites in Finland are summarized in Table 1 based on the guidelines set by the Finnish Society for Head and Neck Oncology (FSHNO 2020; Grénman and Joensuu 2011). Recent advances in the surgical treatment of head and neck tumours include the introduction of transoral robotic surgery for oropharyngeal squamous cell carcinoma (Mydlarz et al. 2015; Nichols et al. 2019) and refinement of reconstruction techniques (Bozec et al. 2019; Yao et al. 2019). Novel radiotherapy methods such as intensity-modulated or volumetric modulated radiotherapy and volume reduction in small tumours have been successful in limiting toxicity (Grégoire et al. 2015). However, the introduction of novel chemotherapy agents and immunotherapies have yet to decisively change the basic therapeutic schemes (Solis et al. 2022; Wong et al. 2022).

Local HNSCC tumours are preferably treated with single-modality therapy using either surgery or radiotherapy, while metastatic and locally advanced tumours are usually treated with combination of surgery and radiotherapy. In fact, there is little unequivocal evidence for the superiority of different therapeutic strategies. (Chow 2020; Machiels, René Leemans, et al. 2020.) In the treatment of verrucous carcinoma, radiotherapy is discouraged based on incidental reports of histological deterioration after radiotherapy and a recent retrospective prognostic evaluation (Mohan et al. 2017). Despite harmonization and guidelines, treatment stratification decisions are still commonplace. Importantly, considerable variation exists in the addition of concomitant chemotherapy to radiotherapy (Goel et al. 2022; Krishnamurthy et al. 2022). Recently, the acceptance of transoral robotic surgery in the treatment of oropharyngeal and supraglottic tumours has made surgery a feasible option to standard radiotherapy in therapy stratification of these HNSCC tumours (Nichols et al. 2022). Similarly, the treatment selection in early glottic HNSCC is based more on institutional practice than actual superiority of either therapy (Al-Qurayshi et al. 2022; Ferreira et al. 2020; Pakkanen et al. 2022). Radioresistance of HNSCC

Since the first discovery of X-rays (Röntgen 1895) and radioactivity in radium (Becquerel 1896; Curie and Sklodowska-Curie 1898), both radium therapy and X-ray therapy have been widely applied for the purposes of cancer treatment. Early observations of the applicability of radiotherapy in the context of non-operable tumours has had a profound effect on all cancer therapy. Henri Coutard's work on external beam radiotherapy using X-irradiation was so impactful, that the protracted

fractionation approach presented in 1922 was established as the foundation of all future experimentation (Holsti 1995).

Henri Coutard, a pioneer in the field of external beam radiotherapy, began experiments treating head and neck carcinomas using Roentgen rays in the 1920s (Coutard 1932). Previously inoperable and thus incurable HNSCC tumours of larynx, hypopharynx and oropharynx were treated using fractionation schemes, providing basis for future development of radiotherapy. After these revolutionary experimentations, radiotherapy has gained a strong role in all treatment of head and neck cancer and is currently used in the treatment of approximately one half of HNSCC patients (Chow 2020; Machiels, René Leemans, et al. 2020).

Already the first attempts with radiotherapy revealed a differential sensitivity among different types of tumours (Regaud et al. 1922; Schwarz 1914). The term radiosensitivity (ger. Strahlenempfindlichkeit, fr. radiosensibilité) was first formally used by Regaud, replacing the earlier suggestion of idiosyncrasy (Regaud and Ferroux 1927). Cancer radiosensitivity can be defined as a tumour characteristic that makes the tumour responsive to ionizing radiation. The terminological counterpart is radioresistance, which means, that the tumour does not respond to radiotherapy. In clinical practice, the meaningful interpretation is the fine line between a radiosensitive tumour that can be successfully treated with radiotherapy and a radioresistant, which either does not directly respond to radiotherapy or recurs after an initially successful therapy. Thus, the radioresistance of a tumour is determined by the radiotolerance of the surrounding normal tissue. (Cohen-Jonathan-Moyal et al. 2020; Nix et al. 2004.)

Several advances have been made in the following decades in the identification of radioresistance, implications of radioresistance, and the adoption of radiosensitization strategies. Due to the field cancerization phenomenon (Slaughter et al. 1953), radiotherapy remains a mainstay treatment option in HNSCC in spite of problems in predicting radioresistance of individual tumours.

2.1.3 Current understanding of radioresistance

The first suggestion of a radioresistance mechanism was most likely related to tumour proliferation (Albers-Schönberg 1896; Bergonie and Tribondeau 1906), and is customarily referred to as Bergonie and Tribondeau's law. After this crude original observation, Regaud made very important progress in defining radiosensitivity between different tumour and tissue types. Importantly, tonsillar carcinoma was soon recognized as being particularly sensitive to irradiation (Ewing 1929).

Paving way for a molecular explanation of radioresistance, the phenomenon of acquired or induced radioresistance was recognized early in the 20th century (Lasseur 1904), while debate over the localisation of such characteristics continued over the

next several decades (Windholz 1947). Early discussion of this phenomenon offered several hypotheses, including the selection of radioresistant cell population, induction of radioresistance after irradiation challenge and damage to nontumorous tissue.

The cellular causes of radioresistance define the intrinsic radioresistance of the tumous cells. The intrinsic radioresistance has been tracked to copious phenomena in human tumours, ranging from p53 mutations to cancer stem cells (Perri et al. 2015). An intrinsic or in vitro radioresistance was originally evaluated using the clonogenic growth assay (Puck and Marcus 1956), and its relation to in vivo characteristics was noted by Fertil and Malaise (Fertil and Malaise 1981).

Several important mechanisms have currently been accepted to play a role in tumour radioresistance. However, it is important to note, that many of these mechanisms are interlinked, and thus the isolation of the functional role of and individual molecular alterations responsible for radioresistance has been challenging. In fact, most factors, that contribute to hallmarks of cancer, also increase radioresistance by one or another mechanism.

The first identification of the effects of hypoxic environment on carcinoma cells by Otto Warburg was based on observations of metabolic changes (Warburg 1924). Hypoxia is a common method for evasion of oxygen-exacerbated DNA damage in different cancers and is understandably caused by reduced blood flow in fast-growing tumour as well as general poor oxygenation associated with tobacco smoking (Gatenby and Gillies 2004). Furthermore, hypoxia induces changes in most other mechanisms or radioresistance. Alterations in tumour suppressor p53 are common and play a definite role in radioresistance along with malignant transformation (Pekkola-Heino et al. 1998; Servomaa et al. 1996). However, results on radioresistance of p16-positive, p53 wild-type carcinomas is conflicting (Nagel et al. 2013; Ziemann et al. 2015).

EGFR overexpression is nearly ubiquitous in HNSCC, and numerous molecular changes along EGFR signalling pathways have been shown to play a role in radioresistance development (Ang et al. 2002; Rieckmann and Kriegs 2019). Epithelial-mesenchymal transition, characterized by the loss of cell surface adhesion molecule E-cadherin expression, is crucial for immune evasion, metastatic and invasive behaviour, and, further, radioresistance. Protein trafficking associated Nedd4 family interacting protein (NDFIP1) regulates one of the crucial controllers of cell proliferation, epithelial-mesenchymal transition associated tumour suppressor PTEN. Importantly, NDFIP1 was recently recognized as a prognostic factor in HNSCC (Ahmad et al. 2017; Uhlen et al. 2017). Angiogenesis is a recognized hallmark of cancer and develops in response to tumour growth. Angiogenic response, typically increased by hypoxic environment, is a potential target for radiosensitization (Denaro et al. 2012).

Cancer stem cells were first identified in hematopoietic malignancies (Park et al. 1971). In 1976, Nowell proposed, that cancer is developed in a multistep progression through different phases of genetic variation allowing different features to manifest (Nowell 1976). Fidler's and others' studies demonstrated, that only a limited number of tumour cells are capable of initiation of metastasis or regeneration of tumour tissue (Fidler 1970, 1973; Hamburger and Salmon 1977). Current understanding of cancer stem cells in radioresistance revolves around a multi-faceted nexus of stemness factors (Nathansen et al. 2021; Reya et al. 2001; Wiechec et al. 2022). The role of cancer stem cells in HNSCC radioresistance is well established, although radiosensitization strategies based on stemness are lacking (Peitzsch et al. 2019; Singh et al. 2021).

The importance of intrinsic radioresistance is not to be underestimated, while it is also apparent, that the clinical phenomenon of radioresistance cannot be totally explained by the intrinsic qualities of the tumour (Perri et al. 2015). Patient nutritional status, competence of the immune system, and other clinical factors also play an important role in defining response to radiotherapy. In recent years, the role of tumour infiltrating T cells and macrophages as well as the immunological activation in general has been an important focus of research (Almangush et al. 2022, 2020; De Meulenaere et al. 2017).

The most common type of tumour infiltrating lymphocytes, CD8-positive cells, may be localized in tumour stroma or directly in interaction with the HNSCC cells, which may affect the specific immunological response they elicit (Borsetto et al. 2021). Several subtypes of tumour associated macrophages have been recognized, and detection of CD68 and CD206 have been investigated in HNSCC (Liu et al. 2021). Importantly, both the presence of tumour-infiltrating lymphocytes and tumour associated macrophages has been associated with radiosensitivity in HNSCC tumours (Balermpas et al. 2014; Fu et al. 2020; Larionova et al. 2019).

Enrichment of the pathway associated with the programmed cell death protein-1 (PD-1) and programmed cell death ligand-1 (PD-L1) is thought to be associated with immunogenicity of carcinomas, and accordingly, has been associated with good radiosensitivity of HNSCC. (Lyu et al. 2019). However, demonstration of PD-1/PD-L1 pathway activity remains problematic due to issues with the reproducibility in the staining results (Qiao et al. 2020). Importantly, this pathway is a target for novel immunotherapeutic drugs (Burtness et al. 2019; Ferris et al. 2016; Tao et al. 2020).

Finally, many of the important refinements in radiotherapy technique aim at delivering higher radiotherapy dose to the tumour and its surroundings, since many recurrences and residual tumours can be explained by failures in the radiotherapy targeting. Importantly, however, in studies taking this type of target failure into account, recurrences in the full dose field have also been reported, even in HPV-

positive HNSCC. (Chen, Chin, et al. 2017; Geretschläger et al. 2012; Johansen et al. 2017; Nissi et al. 2021.)

Despite the plethora of molecular mechanisms of radioresistance which are frequently at play in HNSCC, these tumours are regarded to be relatively radiosensitive. Accordingly, radiotherapy is used in definitive treatment of HNSCC either alone, or as an adjuvant or neoadjuvant treatment in combination with surgery. The elective treatment of neck lymph nodes without evidence of metastatic spread is an important part of radiotherapy use. (Chow 2020; Machiels, René Leemans, et al. 2020.)

2.1.4 Radiosensitization strategies

Even though HNSCC is relatively radiosensitive, and radiotherapy plays an integral part in HNSCC management, the clinical phenomenon of radiotherapy failure is the more devastating since radiotherapy is applied in situations where surgical treatment is not feasible or leads to functional compromise. Such is the case in laryngeal HNSCC, where radiotherapy is used as an organ-preservation strategy (Eita et al. 2022; Foote et al. 2006). Thus, the identification of clinical radioresistance is of utmost importance, as it would allow for proper adoption of radiosensitization.

Nutritional issues are mostly mentioned in the context of radiotherapy in the important role that a balanced diet plays in limiting toxicity from radiotherapy and radiosensitizing chemotherapeutic agents. Diet also modulates immune response and thus make the treated tumour more susceptible to radiation (Soldati et al. 2018). Several studies have investigated the role of caloric restriction or ketogenic diet in the alteration of the hypoxic environment (Klement and Champ 2014; Klement and Sweeney 2022; Ma et al. 2021; Schroeder et al. 2013).

Since tumour hypoxia is related to both tumour aggressiveness and radioresistance, several strategies have been developed to tackle hypoxia during radiotherapy. Hyperbaric oxygen therapy can be used to increase the oxygen partial pressure and saturation in tumours. Despite several positive studies, the current consensus based on systematic review of literature is, that hyperbaric oxygen therapy is not a standard approach to the radiosensitization of HNSCC (Overgaard 2011). Other hypoxic modifications of the usual radiotherapy include the use hypoxic radiosensitizer nimorazole, the use of which has provided promising patient benefit in recent trials (Hassan Metwally et al. 2015; Saksø et al. 2020).

Surgically removing tumour prior to radiotherapy, called debulking, may expose tumours to immunological attacks and decrease hypoxic volume. Such an approach is currently mostly limited to recurrent or locally very advanced HNSCC. However, small studies have demonstrated the benefit of a debulking strategy (Knegt et al. 2002; Ohguri et al. 2008). In addition to surgical debulking, preoperative induction

chemotherapy has been evaluated in numerous combinations but has not been adopted widespread except for nasopharyngeal carcinoma (Forastiere 2004; Ghi and Paccagnella 2019).

Several fractionation schemes have been evaluated in the attempt to optimize radiotherapy response. Standard definitive radiotherapy for HNSCC is administered five times a week for six to seven weeks until the effective tumour dose of 66 to 70 Gray has been achieved. This schedule can be hastened as in accelerated radiotherapy, divided into smaller doses as in hyperfractionation, or divided into higher doses as in hypofractionation. No definite benefit has been demonstrated from any of these approaches and the standard radiotherapy scheme remains the most widely accepted. (Grégoire et al. 2022.)

2.1.4.1 Chemotherapy-based radiosensitization

Cisplatin (cis-diamminedichloroplatinum, CDDP) was first synthesized in the 1840s by Michele Peyrone, and it was originally known by the eponymic name Peyrone's chloride (Peyrone 1844). The Nobel prize winner Alfred Werner discovered the exact chemical coordination of cisplatin and the related stereoisomer transplatin (Werner 1893). Interest in the medical use of cisplatin was awakened in 1960s, when Barnett Rosenberg accidentally discovered, that electrical current created platinum salts, which inhibited the bacteria Escherichia coli division (Rosenberg et al. 1965). Early experimentation revealed that testicular germ line cancer is particularly susceptible to cisplatin even in disseminated stage, making cisplatin an integral part of testicular cancer treatment. Later, it has been established that testicular cancer is intrinsically hypersensitive to cisplatin due to a defective DNA repair mechanism (Fichtner et al. 2022).

The mechanism of action of cisplatin is based on its ability to bind DNA, thus causing DNA damage and cell cycle arrest. Cisplatin resistance may develop through several mechanisms, including defects in cell transport, apoptotic cell death, tolerance of cisplatin-induced oxidative stress, and modulation of calcium-signalling.

Earliest experiments with cisplatin in advanced HNSCC therapy date to the 1970s and were done with cisplatin alone or in combination with other chemotherapies (Prestayko 1979). The use of cisplatin in radiosensitization was forwarded based on the observation of hypoxia reversal (Yan and Durand 1991). In the first trials using concurrent cisplatin with radiotherapy, complete response was observed in 70% of advanced HNSCC patients (Al-Sarraf et al. 1987; Marcu et al. 2003). The true landmark observation of cisplatin is its use in organ-preservation strategies (Koch et al. 1995; Parmar et al. 2021). However, in spite of these important observations, the patient benefit from cisplatin cannot currently be predicted accurately, making the

indiscriminate use of cisplatin in radiosensitization of frail and elderly patients problematic (Blanchard et al. 2011; Forastiere 2004; Pignon et al. 2009).

Toxicity is an important issue which limits the use of high-dose cisplatin. Since kidneys accumulate cisplatin, nephrotoxicity is a major concern and the most common cause for cisplatin refusal. Ototoxicity is common and affects at least 30% of patients treated with standard chemotherapeutic regimes. The typical clinical picture consists of tinnitus and high-frequency hearing loss (Frisina et al. 2016). Vertigo and dizziness are less common symptoms (Prayuenyong, Baguley, et al. 2021), and are probably multisensory in origin with a reported association of polyneuropathy (Prayuenyong, Kasbekar, et al. 2021). Myelosuppression and severe gastrointestinal problems are relatively uncommon (Guan et al. 2016).

Eligibility issues are related to treatment toxicity. The standard high-dose cisplatin regime world-wide is based on three biweekly cycles of 100 mg/m^2, which is completed by only two thirds of the patients. Weekly cycles of 40 mg/m^2 may be beneficial in terms of cisplatin toxicity and especially in reducing long-term radiation-induced damage. Since the weekly low-dose cisplatin has been proven non-inferior in recent trials, it remains a popular regimen especially in Nordic countries due to lower incidence of side effects (Jacinto et al. 2017; Mashhour and Hashem 2020; Noronha et al. 2018; Szturz et al. 2019).

The next-generation platinum compound carboplatin, that is chemically and functionally closely related to cisplatin, was developed in the 1980s in response to concerns over cisplatin toxicity (Calvert et al. 1982). Currently, it has effectively been side-railed in the therapeutic schemes of HNSCC. In a recent meta-analysis, cisplatin was found to offer superior survival benefit with less haematological adverse reactions, albeit a higher frequency of gastrointestinal toxicity and nephrotoxicity was noted (Guan et al. 2016).

In addition to the established platinum-based radiosensitization in HNSCC therapy, several other chemotherapeutic agents have been investigated. Other classical chemotherapeutic agents used in the radiosensitization of HNSCC include taxane group medications paclitaxel and docetaxel, which are mainly used in case of platinum contraindications (Mody et al. 2016; Vokes et al. 1995). Approved therapies hydroxyurea, fluorouracil, and methotrexate are used primarily in combination therapies (Argiris et al. 2003).

2.1.4.2 Novel targeted therapies

EGFR inhibitor cetuximab was introduced in early 2000s and was adopted in radiosensitization of HNSCC due to a favourable toxicity profile in the early trials (Bonner et al. 2006; Herbst and Langer 2002; Robert et al. 2001). After the preliminary results, however, the role of cetuximab has become limited due to less

successful treatment outcomes (Bauml et al. 2019; Gillison et al. 2019; Riaz et al. 2016; Rischin et al. 2021). Cetuximab is currently used more often in the context of recurrent and metastatic disease than primary curative intent therapy. In patients with major contraindications such as renal failure, cetuximab offers an important adjunct in the treatment arsenal (Krishnamurthy et al. 2022).

Antiangiogenic therapies provide a feasible molecular rationale for HNSCC radiosensitization (Salama et al. 2011). Bevacizumab is an inhibitor of vascular endothelial growth factor A. It has shown promise in the radiosensitization of HNSCC in combination with other chemotherapeutic agents (Argiris et al. 2019; Fury et al. 2012, 2016; Yao et al. 2015).

Emerging immunotherapies targeting PD-1/PD-L1 checkpoint inhibition aim et increasing the radiosensitivity of HNSCC tumours by immunogenicity. They are currently mainly used in palliative therapy of inoperable HNSCC after platinum-based chemoradiotherapy. Interestingly, PD-L1 expression may increase in response to cisplatin chemotherapy, suggesting an added benefit of combination chemotherapy (Paolino et al. 2021). Combination of several immunotherapies is another avenue under investigation (Economopoulou 2016). PD-1 inhibitor nivolumab has yet not been evaluated in clinical trials for radiosensitization (Vos et al. 2021), but retrospective analyses have been promising (Altay-Langguth et al. 2021; Leidner et al. 2021; Sari et al. 2022). A phase I trial with ipilimumab, an inhibitor of cytotoxic T-lymphocyte-associated protein-4, was recently completed (Ferris, Moskovitz, et al. 2022).

The PD-L1 inhibitor avelumab has not been approved in the treatment of HNSCC, but has shown preliminary promise in combination chemoradiotherapy (Tao et al. 2020). The approved PD-L1 inhibitor pembrolizumab has provided significant survival benefit in several trials (Burtness et al. 2019). Providing the most convincing evidence supporting the standard use of immunoradiotherapy thus far, efficacy of radiosensitization using pembrolizumab was recently investigated in the primary therapy of cisplatin ineligible patients (Weiss et al. 2020). Another further trial of primary curative therapy was recently launched (Machiels, Tao, et al. 2020). Despite positive findings, immunological therapies are associated with significant and sometimes fatal adverse effects, limiting their clinical application (Clarke et al. 2021). Importantly, the patient benefit cannot reliably be predicted using immunohistochemical evaluation of PD-L1 expression (Burtness et al. 2019; Haddad et al. 2022).

2.2 The purpose of molecular biomarker in HNSCC

Despite a profound understanding of molecular and genetic mechanisms of cancer behaviour, biomarker studies of HNSCC have produced little concrete results.

Numerous putative biomarkers have been suggested, and it is noteworthy, that there exist over 5 000 publications indexed in PubMed/MEDLINE database concerning biomarkers of HNSCC (*cf.* Falco et al. 2022; Kim, McShane, and Conley 2014; Polanska et al. 2014; Schaaij-Visser et al. 2010). While the number of suggested biomarkers is staggering, their impact on treatment decisions has consistently remained poor, replicability non-existent, and performance poor.

Thus, unsurprisingly, no biomarkers are currently available for identification of radiotherapy benefit. In line with the semiotic understanding on biomarker function, a molecular biomarker plays its role in a specific clinical environment. Thus, assessment of clinical factors simultaneously with biomarker discovery should be performed within well-defined clinical problem-fields.

2.2.1 Radiotherapy response prediction

The radioresistance phenomenon has important repercussions on two main questions: First, when is radiotherapy advisable as opposed to surgical treatment or in conjunction with surgery. And secondly, when should radiotherapy be augmented with radiosensitization using cisplatin or other chemotherapy or immunotherapy agents. A considerable overlap exists between these two questions, since the advisability of radiotherapy is significantly influenced by the expected impact it shall have on the HNSCC tumour, which is, in turn, dependent on the radiosensitivity. Therefore, these fundamental questions can duly be understood as an umbrella for all the questions that regard the patients' overall condition, radiotolerance of normal tissue, surgical methodologies, nutrition, ability to tolerate the positions of radiotherapy, and endless other problems. Thus, radioresponse prediction is the single most important task for HNSCC molecular biomarkers.

A clinically and surgically oriented approach to the question of radiosensitivity reveals another set of questions, which should be answered by an appropriate biomarker-augmented clinical framework. The most fundamental question to be answered is whether curative treatment is at all possible. This question can relatively reliably be answered by observing the clinical picture, imaging studies and tumour extension in detail. Analysis of the second set of questions is much harder, and can be formulated in two questions:

α. Should the primary tumour be resected before radiotherapy?

β. Should a neck dissection be carried out before radiotherapy?

In these questions, there is an underlying assumption, that if surgical therapy be successful alone, postoperative radiotherapy is not needed: This is the case in small tumours, where radical surgical therapy alone is sufficient. The question of primary tumour treatment is primarily affected by the expected response by radiotherapy and

the ease of surgical treatment. The evolutions of both radiotherapy methodology and surgical techniques constantly changes the scales of this emphasis. Thus, a molecular biomarker may offer critical information about the probability of success with a radiotherapy-based curative treatment, but the feasibility of surgical therapy still limits the eagerness to begin treatment with surgery.

The second question is related to the risk of metastatic spread, which has proven enigmatic. While it is apparent in metastatic cancer, that the metastasis needs to be treated, the problem lies in cases where no demonstration of metastasis is available, since occult metastasis are relatively common. The staging of the neck using sentinel node biopsy is an inefficient yet functioning way to evaluate the need for neck dissection (Panula et al. 2021). Thus, neck metastasis is a problem-field where biomarkers could very well play a role.

It is important to note, that if curative treatment for the primary tumour or the neck is the goal, the exact procedural details are well standardized based on tumour extension and reconstructive needs. The construction and delimitation of radiotherapy fields and definition of target doses are similarly well-characterized. However, the question of radiosensitization with cisplatin or cetuximab remains more open to individual considerations and cannot currently be reliably answered based on clinical information. The conceptual questions in the clinical problem-field are summarized in Figure 5 based on current HNSCC therapeutic guidelines.

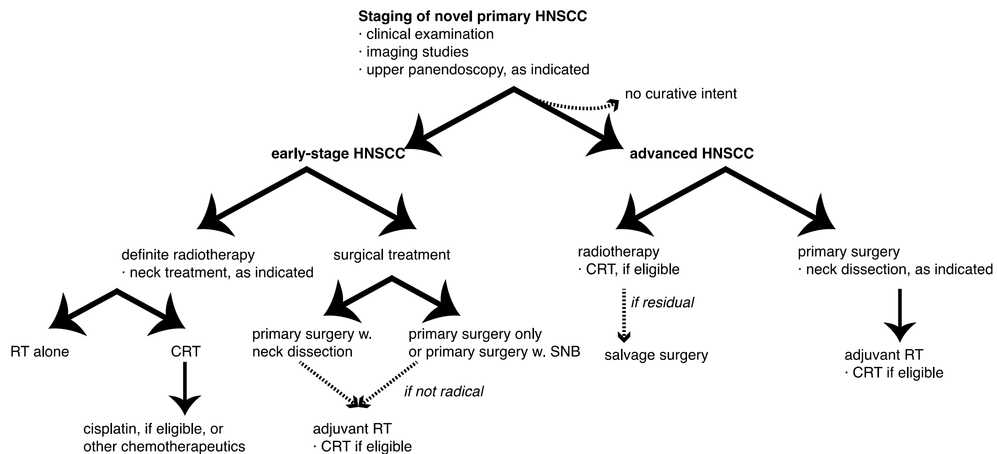


Figure 5. Conceptual decision-tree of current head and neck squamous cell carcinoma (HNSCC) stratification. After tumour staging, early-stage tumours are treated with radiotherapy (RT), chemoradiotherapy (CRT), or surgery. Surgery may be followed by adjuvant RT or CRT, and it may include sentinel node biopsy (SNB) for neck staging. Advanced HNSCC is treated with RT, CRT, or surgery. A small proportion of patients are either ineligible for curative treatment or refuse therapy, in which case only palliative treatment is available. Crossroads indicate potential for molecular biomarker enhanced decision-making.

An optimal molecular biomarker for HNSCC would provide a rationale for a specific treatment protocol (Kim et al. 2014). Since the most unanswered question is the radiosensitivity of the individual tumour, radically changing the odds in favour or against radiotherapy-based curative therapy, biomarkers for radiotherapy and chemoradiotherapy are urgently needed. Reliable molecular biomarkers for the purposes of this therapy stratification are currently lacking. It is to be emphasized, that the actual overall aim is to identify radiotherapy biomarkers – not radioresistance biomarkers. Thus, radioresistance is just one aspect of the complete biomarker framework. The complete assessment of radiotherapy response as a clinical question mandates an investigation of factors influencing radiotherapy response in the clinic.

2.2.2 De-escalation studies

In want of a biomarker to clearly indicate the therapeutic strategy needed, p16 immunohistochemistry and HPV in situ hybridization for identification of HPV-related HNSCC have gained popularity in the context of treatment de-escalation or deintensification of oropharyngeal squamous cell carcinoma therapy. The prognostic value of HPV is well established (Bryant et al. 2018; Chung et al. 2014; Jouhi et al. 2017), providing the rationale for de-escalation studies investigating different chemotherapeutic agents, smaller radiotherapy doses, and transoral robotic surgery instead of radiotherapy. Despite the clear prognostic value of HPV detection, results concerning the intrinsic radiosensitivity of HPV-related HNSCC is conflicting (Nagel et al. 2013; Ziemann et al. 2015). However, in vitro studies are complicated by the loss of HPV in cell culture (Doorbar 2016). Another key issue is the selection of patients and the reliability of the selected method for high-risk HPV detection, since there are considerable discrepancies between HPV-DNA detection, HPV-RNA detection, and p16 immunohistochemistry (Evans et al. 2011; Hashmi et al. 2020; Larsen et al. 2014; Prigge et al. 2017).

Numerous clinical trials have been conducted for evaluation of de-escalation in HPV-positive HNSCC. The use of radiotherapy without standard radiosensitizing chemotherapy has been successful in reducing toxicity in preliminary studies (Sher et al. 2021; Takemoto et al. 2021). The preference of transoral robotic surgery over radiotherapy can well be motivated by the remaining open possibility for adjuvant radiotherapy, since no conclusive benefit from either approach has been shown (Nichols et al. 2019). Similar justification can be offered for the delimitation of the irradiation field to elective neck (Swisher-McClure et al. 2020), while the combination of robotic surgery with low-dose radiotherapy closes the playroom of possibilities (Ferris, Flamand, et al. 2022), The use of cetuximab instead of platinum-based radiosensitization has proven detrimental to patient outcome (Mehanna et al.

2019; Swiecicki et al. 2020). Finally, the combination of low-doses of both cisplatin and radiotherapy has proven beneficial in preliminary studies (Chera et al. 2018, 2019). Some preliminary studies have also demonstrated positive results with induction chemotherapy using different chemotherapeutic combinations and reduced radiotherapy intensity (Chen, Felix, et al. 2017; Misiukiewicz et al. 2019).

2.2.3 The process of biomarker discovery

The process of biomarker discovery can be conceptualized in phases of development, which was originally suggested for diagnostic biomarkers. The suggested phase succession is based on drug screening studies and thus progresses through preclinical discovery towards clinical testing, and finally, after laborious process, to clinical validation. (Pepe et al. 2001.) Suitable phases may vary based on the investigated phenomenon, especially due to rarity of certain diseases and specific requirements for the individual molecular biomarker (Figure 6).

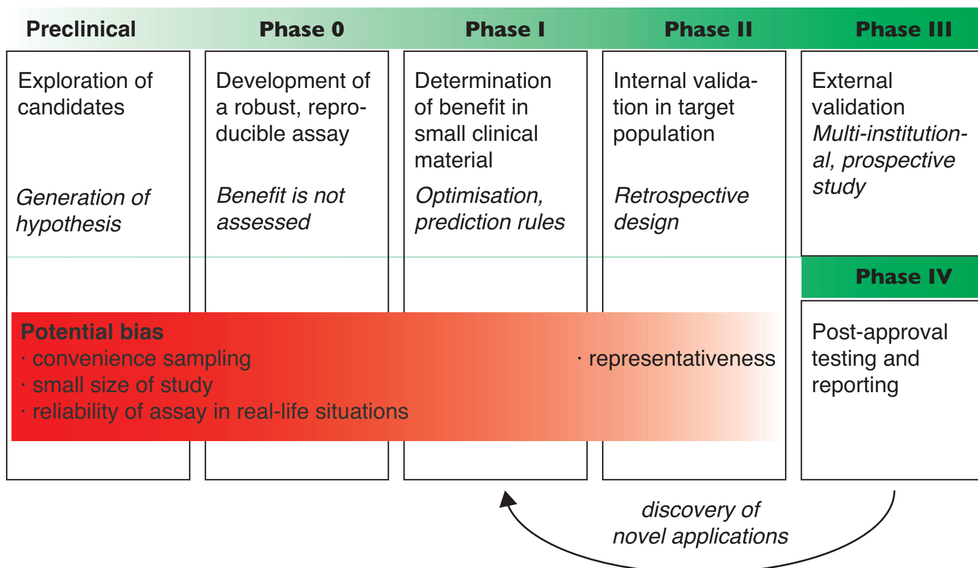


Figure 6. Conceptual phases of biomarker discovery for HNSCC. Modified for HNSCC based on Pepe MS, Etzioni R, Feng Z, Potter JD, Thompson M Lou, Thornquist M, et al. Phases of Biomarker Development for Early Detection of Cancer. J Natl Cancer Inst. 2001;93(14):1054–61.

The early attempts at formulating uniform biomarker discovery and validation phases emphasized the need for preclinical biomarker discoveries. Such discovery-based biomarker studies however led to an inflation after “omics”-based approaches at biomarker discovery became more widespread. It is noteworthy, however, that

despite the simplicity in simultaneously examining a larger number of potential candidates using these novel techniques and the important preclinical information derived from such studies, very little actual biomarkers have emerged in the process (Rossi et al. 2022).

Most preclinical studies begin with the examination of tumour type specific cell lines. While the issue of representativeness and reliability is a major concern, carefully designed cell line studies provide important information on cell signalling and relationship between molecular events and cellular characteristics. (Gillet et al. 2013; Mirabelli et al. 2019)

Cell microarray (CMA) analysis offers a novel method for the evaluation of multiple cell lines cost-efficiently, allowing many cell lines to be investigated simultaneously to correlate findings against previous information, such as intrinsic radioresistance or chemotherapy sensitivity (Ferrer et al. 2005; Gately et al. 2011; Jonczyk et al. 2016; Wu et al. 2014). The CMA methodology offers also a simple but powerful way to implement antibody validation, since staining protocol optimization, evaluation of differences in expression and localization are easy to evaluate. While the CMA technique requires a large quantity of cells for each of the included cell lines, even slowly growing cell lines can be amalgamated by mixing the cells with a morphologically dissimilar cell line (Waterworth et al. 2005).

Since immunohistochemistry is the most often used method in tumour tissue biomarker exploration, its problems with antibody reliability are of special importance. In recent years, interest in the proper validation process has increased in recognition of problems encountered in antibody specificity and reliability. *E.g.* in HNSCC, the reliability of p16 immunohistochemistry is highly dependent on selected antibody (Sawicka et al. 2013). Antibody validation is possibly the most important step in the preliminary analysis of a putative biomarker for immunohistochemistry, and great care should be taken in the selection of a suitable antibody. A minimum requirement for antibody validation is the demonstration of specificity in *e.g.,* Western blot analysis, demonstration of proper sensitivity for positive and negative control staining, and demonstration of proper localization of the immunoreactivity. In addition, the suitability of the antibody and the immunohistochemical protocol for the expression levels within the tumour of interest is of paramount importance. (Bussolati and Leonardo 2008; Weller 2018.)

In recognition of the inflation of preclinical study and the numerous suggested biomarkers, a further development called for systematic sampling of patients to address recruitment bias issues, as opposed to traditional convenience sampling (Pepe et al. 2008). Retrospective registry-based studies, by definition, introduce bias in patient inclusion due to poor data reporting and registry exclusion, which are unavoidable to some extent. Accordingly, an emphasis was made on prospective recruitment, conceptualized in the PRoBe-guidelines (Pepe et al. 2008). Prospective

recruitment is no less free from sample bias, on the contrary. While there are little systematic evaluations on the subject, it is clear, that many prospective recruitment environments carry a significantly higher risk for exclusion of patients from lower socioeconomic classes or patients with high alcohol-tobacco intake (Berger 2005; Ellenberg 1994; Rothwell 2005).

2.3 Biomarker expression and detection in HNSCC

Any protein or gene, that is expressed, overexpressed, or underexpressed in HNSCC may be used as a biomarker combined with a specific interpretation of its meaning. The expression of such potential biomarkers is based on specific biological processes, which it reflects. In some cases, indeed, the connection between the molecular biomarker and the biological process it reflects is apparent. This is the case in targeted therapies, where the presence of target indicates a potential role for the targeted therapy. However, despite the obvious appeal of this type of matter-of-fact thought, there are many situations in which the usefulness of targeted therapy cannot reliably be inferred from the related putative biomarker. An important example is the limited benefit from using PD-L1 immunohistochemistry in stratifying patients to treatment with pembrolizumab, as high PD-L1 expression does not directly indicate and low PD-L1 expression does not preclude benefit from targeted therapy (Burtness et al. 2019; Haddad et al. 2022). In contrast, other therapies such as HER2 targeting biological therapy can well be guided with HER2 genomic detection (Pegram et al. 1998; Singer et al. 2008).

Based on the above consideration, the performance of the biomarker in the specific clinical context in which it is to be applied, is more important than the biological process which it reflects and which it is related to. Thus, while biomarker discovery often begins with hypothesis-forming preclinical steps, studies into the clinical applicability, robustness, and reproducibility are particularly necessary steps for biomarker discovery (Kim et al. 2014; Pepe et al. 2001, 2008). Despite such limitation in translational biomarker discovery, the laws governing expression of proteins and genes that can be used as biomarkers play a fundamental role for understanding tumour biology.

When an individual biological event is chosen as a target for biomarker discovery, the selection of proper methodology for its evaluation is encountered. The first dichotomy is the selection between relatively constant genomic events such as copy number alterations and mutations and less consistent changes, which include expression of either gene product mRNA or translated proteins and post-translational modifications such as phosphorylation, glycosylation, or methylation. While each of these steps may be used as biomarkers, the appropriate method of choice will often be discovered only after extensive profiling of both the biological event and cancer

under investigation. Despite the multitude of methods, most clinical applications of biomarkers work with either immunohistochemistry with specific antibodies, or in situ hybridization to evaluate genomic status.

The constant genomic copy number alterations may be detected using several methods. Particularly interesting are methods that allow for investigation of routine formalin-fixed paraffin-embedded (FFPE) samples, such as fluorescence in situ hybridization (FISH) and array-based comparative genomic hybridization (Al-Mulla 2011; Saramäki et al. 2001). In clinical laboratories, chromogenic and silver-enhanced in situ hybridization methods, which can be evaluated under conventional microscope, have mostly superseded the traditional FISH methodology. Novel methods such as next generation sequencing and single nucleotide polymorphism allow for simultaneous evaluation of copy number alterations, mutations, and methylation (Solis et al. 2022). The gold standard method for identification of mutations in tumours consists of PCR-based amplification and sequencing of the amplified gene product. This method has a limited performance in FFPE samples, which has led to discovery of more straightforward methods such as the droplet digital PCR (Borkowska et al. 2021) in addition to investigation of other sample matrices such as serum and plasma (Ginkel et al. 2017).

The detection of mRNA is usually accomplished through PCR, the usefulness of which is, however especially limited in the typical FFPE samples (Cazzato et al. 2021). While not suitable for clinical practice, RNA sequencing is increasingly being used for investigation of mRNA expression. For protein level detection, Western blot can be used for fresh tumour tissue lysates. However, protein detection using immunohistochemistry of immunofluorescence is a powerful tool, and accordingly, has reached standard practice in copious clinical indications. The reliability of immunohistochemistry is strongly limited by the reliability of the antibody used for protein identification and by a proper validation process (Fitzgibbons et al. 2014; O'Hurley et al. 2014; Simpson and Browning 2017). Despite the need to control such factors, immunohistochemistry is easy, repeatable, automated, and simple. Accordingly, p16 immunohistochemistry has reached clinical practice as the primary method for high-risk HPV detection (Prigge et al. 2017). The reproducibility of immunohistochemical analysis can further be increased by advanced scoring systems such as the tumour and combined proportion scores used in PD-L1 analysis, which tend to turn the process much more laborious (Crosta et al. 2021).

Detection of post-translational modifications in proteins is relatively problematic, and the usual phosphoproteomic and methylomic analyses are highly experimental and distant from clinical practice (Carlson and Gozani 2014; Kauko et al. 2020). In addition, phosphorylation, methylation, and glycosylation status of proteins may fluctuate, making the acquired sample a single time-point reflection, especially when chemotherapeutic agents are used (Kałafut et al. 2021; Lima de

Oliveira et al. 2022). Some phosphorylation and methylation modifications can readily be identified using specific antibodies, such as EGFR phosphorylation (Kriegs et al. 2019). However, development of such antibodies necessitates intimate knowledge of the metabolism of the protein of interest and is prone to specificity issues (Carlson and Gozani 2014).

Novel glycoproteomic methods have been developed for mapping glycosylations of interest. The detection of individual glycosylated proteins is currently based on sandwich-type assays, and thus, cannot currently be performed using FFPE samples (Islam et al. 2021). However, based on the recognition of the ubiquity and implications of glycosylation modifications, such as relationship between PD-L1 glycosylation and PD-L1 immunotherapy (Chen et al. 2022; Y. N. Wang et al. 2020), novel detection methods are to be expected.

2.3.1 Regulation of gene and protein expression in HNSCC

The development of cancer happens canonically through multiple precancerous enabling or disabling mutations in cellular genetic environment (Senga and Grose 2021). Typical observations include the loss of tumour suppressor expression and the acquisition of tumour promoter expression. However, the correlation between mutations, gene expression, and protein expression has proven unpredictable (Chen et al. 2002; Kosti et al. 2016; Lundberg et al. 2010). Thus, a shift in focus can be observed from genetic alterations towards expression alterations (Jarnuczak et al. 2021; Sager 1997). Discordance between gene and protein events is especially important for cancers with numerous genetic alterations, such as HNSCC, which is characterized by its genomic instability (Califano et al. 1996).

An important addition to analysis of gene or protein expression is the pathway analysis, which concentrates on metabolic or signalling pathway alterations surpassing the mere individual genetic event. Using high-throughput technologies, key pathway alterations can be identified, reducing the risk of finding incidental passenger alterations or expression changes (Fang et al. 2021; Wei et al. 2016; Zhao et al. 2012). However, interpretation of such results must be carried out with caution, since culture conditions and tumour environment may alter the observations (Heid et al. 2022; Kähkönen et al. 2021). Importantly, in vivo, there may be regions of the same tumour with vastly different metabolic and oxidization conditions, altering the expression events. Hypoxia conditions are affected by the distance to feeding vasculature (Idel et al. 2021), whereas tumour infiltrating lymphocytes are distributed to immunologically cold and hot fields (Pretscher et al. 2009; Zaidi et al. 2019).

The molecular alterations in cancer are not restricted to the mere expression of genes and proteins. Several post-translational modifications are, at least to some

extent, specific to cancer. Such is the fucosylation observed across most cancers (Keeley et al. 2019) as well as many phosphorylation events revealed by novel phosphoproteomic analyses (Kauko et al. 2020). The post-translational modifications have important repercussions on both intrinsic and acquired cancer therapy resistance and radioresistance, providing interesting future investigation avenues.

2.3.2 Cancer stem cell hypothesis in HNSCC

Several mechanisms of HNSCC radioresistance have been enumerated in the attempt to discover potential radioresistance biomarkers and novel targets for radiosensitization. Cancer stem cells have an established role in the current understanding of radioresistance, while the exact method for the clinical identification of stemness in HNSCC is not unequivocally accepted (Picon and Guddati 2021; Yu and Cirillo 2020). Previous studies have demonstrated the usefulness of *e.g.* CD44 and MET in prognostic evaluation of HNSCC (Linge et al. 2016; Slavik et al. 2019), while the applicability in therapy response prediction seems questionable (M. Khan et al. 2020; Nisa et al. 2020).

A particularly interesting field for cancer stemness investigation is offered by the genes and proteins required for stem cell maturation. An important example of such factors is the stem cell marker octamer-binding transcription factor 4 (OCT4, *Pou5f1*), which is highly expressed in stem cells while playing a well-recognized role in cancer stem cells (Mohan et al. 2021).

2.3.2.1 Cancerous inhibitor of PP2A

Cancerous inhibitor of PP2A (CIP2A) is a 90 kDa endogenous inhibitor of PP2A, which interacts with the alpha subunit of PP2A (Junttila et al. 2007; Khanna, Pimanda, et al. 2013). CIP2A is involved in numerous PP2A regulated signalling pathways associated with various cancers, such as p53, mTOR, and c-Myc signalling (M. M. Khan et al. 2020; Khanna, Pimanda, et al. 2013; Puustinen et al. 2014). The gene encoding CIP2A is located in the band 3q13.13 in the long arm of chromosome three, which is commonly altered in HNSCC.

CIP2A was first discovered investigating autoantibodies in hepatocellular carcinoma (Soo Hoo et al. 2002), indicating a high immunogenicity of the protein. The physiological role of CIP2A is linked to stem cell regulation, more precisely to the regulation of spermatogonial proliferation of progenitor stem cells (Laine et al. 2013; Ventelä et al. 2012; Zeng et al. 2021). A splicing variant of CIP2A, associated with poor survival and therapy resistance in leukaemia, was recently reported (Mäkelä et al. 2021)

Since CIP2A is physiologically associated with spermatogenesis, its expression is associated with the expression of stem cell markers. CIP2A was found to be regulated by OCT4, which also plays a significant role in the stemness regulation of embryonic stem cells and testicular stem cells (Ventelä et al. 2012). Importantly, the CIP2A/OCT4 double positive spermatogonial stem cell population was resistant to irradiation (Ventelä et al. 2015). Further, CIP2A/OCT4 double positive cells were identified in HNSCC cell lines and CIP2A/OCT4 double positivity in HNSCC patient samples was associated with impaired prognosis after radiotherapy (Ventelä et al. 2015).

Regarding chemotherapy response, CIP2A is required for intestinal regeneration in response to both radiotherapy and cisplatin (Myant et al. 2015). CIP2A expression has further been shown to determine cellular response to checkpoint kinase inhibitors (Khanna, Kauko, et al. 2013) and doxorubicin (Choi et al. 2011). In serous ovarian carcinomas, high CIP2A expression has been linked to impaired patient survival after platinum-based chemotherapy (Böckelman et al. 2011) and, in cell line investigation, increased cisplatin resistance (Li et al. 2019; Zhang et al. 2015). Similar associations between cisplatin resistance and CIP2A expression have been reported across various cancer types such as renal cell carcinoma, gastric carcinoma, and lung carcinoma (Ji et al. 2018; Wei et al. 2014; Zhang et al. 2018).

In several studies, increased CIP2A expression has been associated with HNSCC aggressiveness or poor HNSCC patient prognosis (Alzahrani et al. 2020; Böckelman et al. 2011; Junttila et al. 2007; Katz et al. 2010; Ventelä et al. 2015) CIP2A was also shown to be highly expressed in salivary gland adenoid cystic carcinoma samples, while no prognostic role was detected (Routila et al. 2016). Importantly, CIP2A is involved with radioresistance mechanisms of HNSCC and rectal carcinoma (Birkman et al. 2018; Kim et al. 2019; Ventelä et al. 2015). Despite several prognostic observations, clinical studies into the use of CIP2A in therapy response prediction of HNSCC have not been published.

2.3.2.2 Stem cell marker OCT4

Stemness is associated with an uncontested increase in radioresistance, while the exact mechanisms remain elusive (Olivares-Urbano et al. 2020). There exist several strategies for the identification of stemness, including expression of the cell surface transmembrane glycoproteins CD44 and CD133, and expression of pluripotency markers such as OCT4, SOX-2, and NANOG (*cf.* Mohan et al. 2021).

OCT4 is a transcription factor and a stem cell marker, which plays an important role in stem cell pluripotency preventing stem cell differentiation. Association between OCT4 and radioresistance has been demonstrated in several previous studies and are explained by cancer stem cell phenomenon and epithelial-

mesenchymal transition (Villodre et al. 2016). One of OCT4's downstream targets is CIP2A (Ventelä et al. 2015). Detection of nuclear OCT4 expression using immunohistochemistry is well established in the identification of germ cell origin in metastasis of testicular cancer (Cheng et al. 2007; Jones et al. 2004).

The mechanistic function of OCT4 is under some debate. In a recent study, the mechanisms of OCT4-related radioresistance were investigated (Nathansen et al. 2021). It was shown, that OCT4 knockdown led to downregulation of the cell cycle checkpoint kinases CHK1 and WEE1 as well as downregulation of homologous recombination repair associated genes, leading to increased radiosensitivity. A similar increase in radiosensitivity was, however, observed also in response to OCT4 overexpression, supposedly due to deficiency in DNA repair mechanisms. These OCT4-linked DNA repair mechanisms are a crucial response to DNA damage induced by radiotherapy (Schulz et al. 2019).

OCT4 and CIP2A are expressed in a radioresistant testicular cell population, which expresses stem cell biomarkers, and co-expressed in testicular seminoma and embryonal carcinoma samples (Ventelä et al. 2012, 2015). OCT4 further regulates CIP2A expression in testicular cancer cell lines and embryonic stem cell model. In addition, OCT4 depletion leads to inhibition of MYC serine 62 phosphorylation, the first recognized protein phosphatase 2A function of CIP2A (Ventelä et al. 2015). In HNSCC patient-derived UT-SCC cell lines, a positive correlation between CIP2A and OCT4 expression has been observed (Ventelä et al. 2015).

In testicular germ cell tumours, which are efficiently treated with cisplatin even in metastatic advanced setting, loss of OCT4 expression is associated with increased cisplatin resistance (Koster et al. 2013; de Vries et al. 2020). Some testicular cancers are highly sensitive to cisplatin despite not expressing OCT4, while cisplatin-resistant cell lines may express OCT4. OCT4-related sensitivity to DNA damaging chemotherapy may be related to the DNA repair mechanisms, that have been implicated in OCT4 related radioresistance (Nathansen et al. 2021; Schulz et al. 2019).

Contrary to the reports in testicular cancer, in lung carcinoma cell lines, loss of OCT4 was associated with cisplatin sensitivity (Liu et al. 2017) and radiosensitivity (Xing et al. 2015). Similarly, in nasopharyngeal carcinoma models, cisplatin resistance was associated with increased Oct4 (Gao et al. 2017). In cervical squamous cell carcinoma, OCT4 expression in patient samples has been associated with radiotherapy resistance (Shen et al. 2014), and increased OCT4 expression in cell lines led to increased cisplatin resistance (Yang et al. 2021).

A prognostic role for OCT4 has been suggested in a general HNSCC cohort (Koo et al. 2015) and hypopharyngeal carcinoma cohort (Ge et al. 2010). Several studies have investigated the association between OCT4 and HNSCC cisplatin resistance. In cell lines, induction of OCT4 expression has been associated with chemotherapy,

including cisplatin, resistance (Harada et al. 2016). In clinical patient materials, an OCT4-NANOG expressing phenotype has been associated with cisplatin treatment failure in oral HNSCC in two studies (Mishra et al. 2020; Tsai et al. 2011). OCT4 expression has been associated with impaired survival in two mixed cohort of radio- and chemoradiotherapy treated HNSCC patients (Sawant et al. 2016; Ventelä et al. 2015) and in another cohort of HNSCC patients treated with postoperative cisplatin- or oxaliplatin-based hypofractionated accelerated radiotherapy (Koukourakis et al. 2012).

3 Aims

The general aim of this study is the evaluation of radioresistance linked and stemness related biomarkers in HNSCC in order to increase our common understanding of biomarker function and purpose.

The specific aims of the four studies were:

α. The evaluation of copy number increases of radioresistance-linked CIP2A and its applicability in HNSCC prognostication (Study I).

β. The evaluation of putative radioresistance biomarkers using a cell microarray (CMA) of UT-SCC cell lines (Study II).

γ. The construction of a highly representative population-validated tissue microarray (TMA) for prognostic analysis of HNSCC (Study III).

δ. The assessment of the predictive value of OCT4 in radiotherapy and chemoradiotherapy stratification using the population-validated TMA (Study IV).

4 Materials and Methods

An overview of the materials used in the four individual studies is presented in Table 2. The appropriate ethical permits and considerations are collected in Section 4.8.

Table 2. An overview of the cell line and patient materials used in the four studies.

STUDY	CELL LINE MATERIAL	PATIENT MATERIAL
I	6 UT-SCC cell lines	52 patient TMA
II	26 UT-SCC cell lines + 3 CIP2A-shRNA-silenced UT-SCC cell lines	none
III	none	PV-TMA: 264 patients validated against cohort of 476 patients
IV	10 UT-SCC cell lines (antibody validation)	PV-TMA: 166 patients validated against cohort of 288 patients

*UT-SCC: University of Turku – Squamous Cell Carcinoma, TMA: tissue microarray, PV-TMA: population-validated TMA.

4.1 UT-SCC cell lines (I-II)

The characteristics of the University of Turku – Squamous Cell Carcinoma (UT-SCC) cell lines included in the study are summarized in Table 3. The included UT-SCC cell lines were established at the Department of Otorhinolaryngology – Head and Neck Surgery, University of Turku between 1990 and 2000. All UT-SCC cell lines were cultured at passage 15 to 20, except for UT-SCC-8 which was at passage 30. None of the cell lines is considered HPV-positive.

Cells were cultured in suitably sized cell culture flasks at 37 °C and humidified 5% CO_2. The cell culture medium was Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% foetal calf serum, and 1% antibiotics (penicillin and streptomycin).

Table 3. The UT-SCC cell lines included in the study. *

CELL LINE	SEX	AGE	PRIMARY	G	T	N	TYPE	AUC	STUDY
UT-SCC-2	m	60	floor of mouth	2	4	1	primary	1.8 ± 0.2	II
UT-SCC-5	m	58	tongue	2	1	1	primary	2.3 ± 0.3	II
UT-SCC-7	m	67	temporal skin	2	1	0	metastasis	2.0 ± 0.2	II
UT-SCC-8	m	42	supraglottic	1	2	0	primary	1.9 ± 0.1	II
UT-SCC-9	m	81	glottic	1	2	1	metastasis	1.4 ± 0.1	I-II
UT-SCC-14	m	25	tongue	2	3	1	primary	1.7 ± 0.3	I-II, IV
UT-SCC-16A	f	77	tongue	3	3	0	primary	1.8 ± 0.1	II, IV
UT-SCC-17	m	65	supraglottic	3	2	0	metastasis	1.8. ± 0.1	II
UT-SCC-19A	m	44	glottic	2	4	0	primary	1.7 ± 0.1	II, IV
UT-SCC-20A	f	58	floor of mouth	2	1	0	primary	2.1 ± 0.2	II
UT-SCC-22	m	79	glottic	2	1	0	recidive	1.8 ± 0.1	I
UT-SCC-24A	m	41	tongue	2	2	0	primary	2.6 ± 0.3	II
UT-SCC-25	m	50	tongue	1	2	0	recidive	N/A	II
UT-SCC-30	f	77	tongue	1	3	1	primary	2.0 ± 0.1	II, IV
UT-SCC-32	m	66	tongue	2	3	0	primary	1.7 ± 0.3	II
UT-SCC-34	m	63	supraglottic	1	4	0	primary	2.0 ± 0.1	II
UT-SCC-36	m	46	floor of mouth	3	4	1	primary	2.2 ± 0.2	II, IV
UT-SCC-40	m	65	tongue	1	3	0	primary	2.3 ± 0.2	I, IV
UT-SCC-45	m	76	floor of mouth	3	3	1	primary	2.0 ± 0.1	II, IV
UT-SCC-46A	m	62	gingiva	3	1	0	primary	1.6 ± 0.1	II, IV
UT-SCC-47	m	78	floor of mouth	3	2	0	primary	2.0 ± 0.2	II
UT-SCC-50	m	70	glottic	3	2	0	recidive	N/A	II
UT-SCC-60B	m	59	tonsil	1	4	1	metastasis	2.2 ± 0.3	II
UT-SCC-65	m	68	tonsil	2	4	2	primary	N/A	I
UT-SCC-72	m	50	gingiva	2	4	2	primary	2.8 ± 0.2	II, IV
UT-SCC-74A	m	31	tongue	2	3	1	primary	N/A	II
UT-SCC-76A	m	52	tongue	2	3	0	primary	2.5 ± 0.2	II
UT-SCC-79A	f	80	parotid	2	0	2	metastasis	2.4 ± 0.2	II
UT-SCC-79B	f	80	parotid	2	0	2	metastasis	2.5 ± 0.1	II

*m: male, f: female, G: primary tumour histologic grade, T: T class, N: N class, AUC: area under the curve of cell survival (intrinsic radioresistance), N/A: not available.

The intrinsic radioresistance data of UT-SCC cell lines determined previously was used in the study. Radioresistance was determined using the 96-well clonogenic assay (Grenman et al. 1988; Grénman et al. 1991; Pekkola-Heino et al. 1998; Puck and Marcus 1956; Servomaa et al. 1996). In short, the cells were cultured and plated on 96-well plates. Each cell line was irradiated with several photon irradiation doses ranging from 0.75 to 7.5 Gy. The number of dividing cells was counted, and area under the curve (AUC) of cell survival was calculated. For analysis, the AUC of the cell survival curve is interpreted as the mean inactivation dose.

4.1.1 shRNA cell lines (II)

UT-SCC-14 and UT-SCC-24A cell lines were selected for CIP2A sh-RNA silencing based on high CIP2A expression. shRNA-silenced cell lines were generated using puromycin resistant, GFP-tagged pGIPZ lentiviral vectors (Open Biosystems). For production of control cells, non-silencing pGIPZ.NS shRNA was used. CIP2A expression was silenced using pGIPZ.shRNA (#556) containing CIP2A antisense sequence TACATCAGCAGCAAGTTTG and pGIPZ.shRNA (#557) containing CIP2A antisense sequences TACTCAATGTCTTTATGTG. After lentiviral transfection, the cells were selected applying puromycin resistance and GFP tag. Finally, all cell lines were tested negative for replication competent viruses (RCV test) and Mycoplasma (The MycoAlert TMMycoplasma Detection kit, Lonza). CIP2A silencing was confirmed using Western blot with anti-CIP2A antibody (dilution 1:1000, 2G10-3B5, sc-80659, Santa Cruz Biotechnology, Inc.).

4.2 CMA construction (II)

An overview of the CMA method and an example of immunohistochemical staining of the CMA is presented in Figure 7. UT-SCC cell lines with previous data on radiosensitivity were preferred (*cf.* Table 3). Six cell lines represented metastasis and two cell lines represented recurrences. One cell line was derived from cutaneous squamous cell carcinoma (UT-SCC-7) and others from HNSCC tumours. The three CIP2A shRNA-silenced cell lines (*cf.* Section 4.1.1) were also included.

For each cell line, approximately 40 x 10^6 cell were needed for a FFPE cell pellet. After culture, cells were harvested by trypsinization and pelleted. After wash with phosphate-buffered saline (PBS), the cell pellet was resuspended in 120–160 µl of 10% neutral-buffered formalin. A conical fill made of 2% agarose in PBS was made on microfuge tubes. The formalin-suspended cells were the added into the tubes. The tubes were spun at 1000 rpm for 5 minutes, and the supernatant was discarded. Ten millilitres of buffered formalin was added for 48 hours, after which the pellets were stored in PBS at +4 °C until paraffin-embedding.

For paraffin-embedding, the microfuge tubes were cut open, and the pellets transferred into a tissue cassette. Services of Auria Biobank (Turku, Finland) were obtained for microarray assembly. Haematoxylin-eosin stained 6 μm sections of FFPE cell pellets were scanned using Pannoramic 250 Flash scanner (3DHISTECH Ltd., Budapest, Hungary) and 0.6 mm cores were annotated in Pannoramic Viewer software (3DHISTECH Ltd.). The cores were transferred into receiver blocks using TMA Grand Master (3DHISTECH Ltd.). Samples from normal human liver were included for orientation. The resulting CMA can be stained and analysed similar to patient FFPE samples and TMAs.

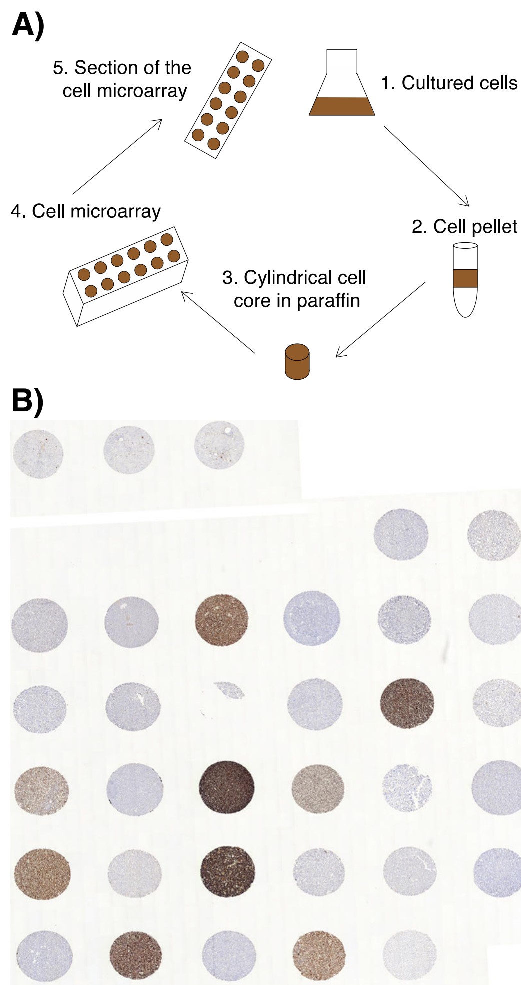


Figure 7. **A)** Overview of the CMA methodology and **B)** below, as an example of the practicality of the CMA, immunohistochemical staining for p16, demonstrating clear differences in staining intensity between the cell lines. (Reproduced from study II)

4.3 Patients (I, III-IV)

4.3.1 Patient cohort of study I (I)

For the first study, a previously collected retrospective patient material consisting of clinical data and tissue samples from 52 HNSCC patients was used (Ventelä et al. 2015). The basic characteristics of the patients are summarized in Table 4. The patients were operated with curative intent for new HNSCC tumour between 1995 and 2005. Adjuvant or neoadjuvant radiotherapy was used in 28 cases. Tumour staging was done according to TNM classification applicable at the time of diagnosis. Overall survival (OS) was defined from date of diagnosis to end of follow up or death. Based on patient chart review, tobacco exposure was dichotomously interpreted as current smoking at the time of diagnosis (yes/no) and alcohol consumption was dichotomized as hazardous alcohol use at the time of diagnosis (yes/no).

4.3.2 Population-based HNSCC cohort (III-IV)

Electronic patient database of the Hospital District of Southwest Finland was screened to include all HNSCC patients treated at Turku University Hospital region during years 2005–2010. Based on the electronic screen, altogether 952 patients' records were accessed and reviewed. After exclusion of patients with no primary HNSCC diagnosed during 2005-2010, the final cohort included 476 patients.

Patient data, tumour characteristics, comorbidities, and tobacco and alcohol exposure were meticulously charted. Tumour staging was done according to TNM classification applicable at the time of diagnosis. Patient treatments and TNM class were decided in multidisciplinary head and neck tumour board. Overall survival (OS) was defined from end-of-treatment to end-of-follow-up or death. Cause of death and survival status were determined by chart review.

End of follow-up was indicated by the last entry in the hospital patient records. End of treatment was defined as the last date of surgery of oncological treatment. When no treatment was offered, the day of diagnosis was used in survival time calculations.

Cumulative tobacco use was estimated as pack-years and the cut-off of 20 pack year smoking history was applied. In addition, details on current smoking were recorded. Current problem-level alcohol use was defined as patient-reported weekly alcohol consumption of at least 10 units of 12 grams. Current problem-level alcohol use or history of several alcohol related somatic complications were dichotomously used in analysis.

The patient cohort for Study IV included all 288 patients of this cohort treated with either adjuvant, neoadjuvant, or definitive radiotherapy, including 12 patients treated with non-curative intent.

Table 4. Characteristics of the 52 patient TMA. (Modified from study I)

PARAMETER		***N***	***%***
AGE (YEARS)			
	< 60	*22*	*42*
	≥ 60	*27*	*52*
	Not available	*3*	*6*
GENDER			
	Male	39	75
	Female	13	25
SMOKING AT DIAGNOSIS			
	Yes	21	*40*
	No	26	*50*
	Not available	5	*10*
HAZARDOUS ALCOHOL USE AT DIAGNOSIS			
	Yes	5	*10*
	No	42	*81*
	Not available	5	*10*
T CLASS			
	T1-2	24	46
	T3-4	24	46
	Not available	4	8
N CLASS			
	N0	31	60
	N+	21	40
SURVIVALSHIP AT THE END OF FOLLOW-UP			
	Alive	30	58
	Dead	22	42
PRIMARY SITE			
	Oral cavity	30	58
	Larynx	12	23
	Oropharynx	6	12
	Other	4	8

4.4 TMA construction (I, III-IV)

For all three studies, appropriate patient FFPE samples were acquired from Turku University Hospital pathology archives through Auria Biobank. After review of the archived slides by a pathologist, new haematoxylin-eosin stained 6 µm sections of each potential tumour block were scanned using Pannoramic 250 Flash scanner (3DHISTECH Ltd.), with a special focus in the inclusion of small tumour samples. Duplicate 0.6 mm cores were annotated in Pannoramic Viewer software (3DHISTECH Ltd.), representative of both tumour centre and tumour margin or invasive front. The annotated cores were transferred into receiver blocks using TMA Grand Master (3DHISTECH Ltd.). Samples from normal human liver were included for orientation.

4.5 Immunohistochemistry (I-IV)

For analysis of protein expression in patient samples, TMAs, and the CMA, immunohistochemical stainings were used. For each staining, a semiquantitative scoring protocol was developed.

4.5.1 Staining protocols (I-IV)

Six µm sections of FFPE blocks were used for all immunohistochemical stainings. The antibodies and concentrations are listed in Table 5. The routine diagnostic protocols for p16, p53, and EGFR immunohistochemistry used at Turku University Hospital clinical pathology laboratory were used in the study. The stainings were carried out in Ventana staining automate (Ventana, Tucson, AZ).

For DPPA4, PME-1, SET, NDFIP1, and OCT4 stainings, immunohistochemical stainings were done at the Turku Centre for Disease Modelling (TCDM, Institute of Biomedicine, University of Turku, Turku, Finland) according to previously established staining protocols (Routila et al. 2016; Ventelä et al. 2015). The CIP2A staining of Study I were also stained according to these protocols. The MET staining of Study IV was done in the laboratory of a collaborator according to previously reported protocol (M. Khan et al. 2020). Positive and negative tumour samples were selected for use as control for each staining.

Table 5. Summary of antibodies used for the study.

Target	Name of antibody	Manufacturer	Catalogue No.	Species	Dilution
PME-1	anti-PME-1 (B-12)	Santa Cruz Biotechnology	sc-25278	mouse monoclonal	1:1000
SET	anti-SET (H-120)	Santa Cruz Biotechnology	sc-25564	rabbit polyclonal	1:1000
NDFIP1	anti-NDFIP1	Sigma-Aldrich	HPA009682	rabbit polyclonal	1:500
OCT4	anti OCT3/4 (C-10)	Santa Cruz Biotechnology	sc5279	mouse monoclonal	1:200
CIP2A	anti-CIP2A	(Soo Hoo et al. 2002)	in-house	rabbit polyclonal	1:10000
CIP2A	anti-CIP2A (2G10-3B5)	Santa Cruz Biotechnology	sc-80659	mouse monoclonal	1:25
DPPA4	anti-DPPA4	Abcam	ab31648	rabbit polyclonal	1:100
MET	anti-MET (SP44)	Spring Bioscience	ab227637	rabbit monoclonal	1:100
P16	anti-p16ink4A (E6H4)	Ventana Medical Systems	705–4713	mouse monoclonal	prediluted
P53	anti-p53 (DO-7)	Ventana Medical Systems	790–2912	mouse monoclonal	prediluted
EGFR	anti-EGFR (5B7)	Ventana Medical Systems	790–4347	mouse monoclonal	prediluted

In short, after deparaffinization, the slides were rehydrated with an ascending ethanol series. Heat-induced epitope retrieval was performed in 10 mM Tris-EDTA-buffer (pH 9.0) using a microwave oven or a pressure cooker. After cooling down for 20 min at room temperature, the slides were rinsed with water. The slides were then treated for 10 min in 3% bovine serum albumin and PBS to block protein activity and rinsed in Tris-HCl (pH 7.4). The slides were incubated with the indicated primary antibody overnight, treated with the appropriate secondary antibody (Dako EnVision anti-rabbit or Dako EnVision anti-mouse K-4001) for 30 min, and incubated for 10 min in DAB+ liquid (Dako K3468). Between each reagent, slides were rinsed with water. Mayer's haematoxylin was used for counterstaining, and the slides were mounted after final rinse and dehydration.

For CIP2A immunohistochemical staining of Studies III-IV, a commercially available antibody was used (*cf.* Table 5). The staining protocol was optimized using both manual an automated stainings with different dilutions and detection kits. For

final analysis of the TMA, Ventana BenchMark XT staining automate (Ventana) was used with OptiView DAB kit and with 64-min CC1 preparation and 32-min antibody incubation.

4.5.2 Scoring of immunohistochemistry (I-IV)

All immunohistochemical stainings were analysed independently by JR and at least one further author (KS, IL, SV). After semiquantitative scoring, contradictory cases were discussed until consensus was reached.

p16 immunostaining was regarded positive, when at least 70% of carcinoma cells demonstrated strong nuclear and cytoplasmic staining intensity. Unclear cases were resolved by comparing the p16 immunostaining with an HPV chromogenic in situ hybridization result (data not shown).

A previously established three-tier system was used for p53 staining of CMA and TMA slides (Köbel et al. 2016). In this system, the immunohistochemical view correlates with genetic abnormalities, with absence of p53 staining being classified as deletion-like and moderate expression as wild-type pattern of staining. Aberrant overexpression is associated with p53 mutation or amplification.

EGFR staining intensity of CMA and TMA slides was scored in a four-tier system (0, +, ++, +++) for cytoplasmic/membranous staining pattern. Cytoplasmic/membranous MET, DPPA4, and CIP2A expression of the TMA stainings were scored in a three-tier system (0/+, ++, +++). In the CMA staining, CIP2A expression was not uniform, and thus the percentage of positive carcinoma cells was analysed together with the staining intensity.

A three-tier scoring system (0, +, ++) was used for nuclear PME-1 and SET staining of CMA and TMA slides, as previously established (Routila et al. 2016). Nuclear NDFIP1 staining was scored positive, when strong and uniform staining pattern was observed.

In CMA stainings for OCT4, the presence of strong nuclear immunoreactivity of individual cells was regarded positive, since OCT4 positivity was present only in a fraction of cells. In TMA stainings, however, a definite subpopulation of carcinoma cells with strong positive nuclei was required for a positive score.

4.6 Fluorescence in situ hybridization (I)

Bacterial artificial chromosome (BAC) clones RP11-184M19 for CIP2A and RP11-958C4 for DPPA4 were used to prepare the locus-specific FISH probes (Figure 8). The alpha-satellite probe pα3.5 for chromosome 3 centromere was used as centromere-specific reference. Nick translation was used to create the digoxigenin-labelled locus-specific probes and the fluorescein-isothiocyanate-labelled

centromere-specific probes. Six UT-SCC cell lines were analysed with both CIP2A and DPPA4 probes. The percentage of cells with increased copy number was calculated.

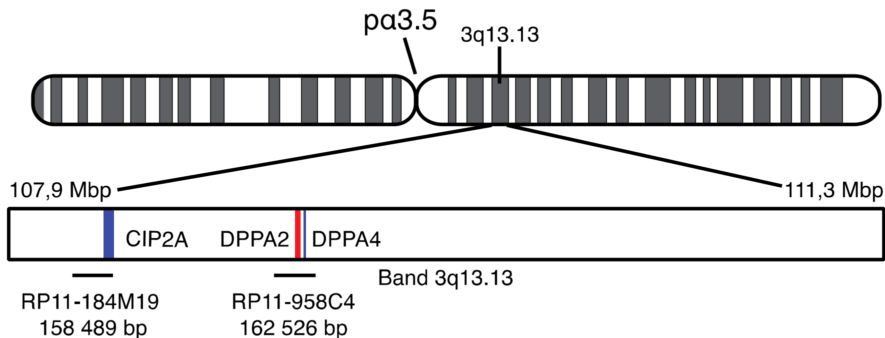


Figure 8. Chromosome 3 and the position of CIP2A and DPPA4 at chromosomal location 3q13.13. Location of BAC clones used for the creation of probes for CIP2A (RP11-184M19, position 108147111-108305600), DPPA4 (RP11-958C4, position 108930124-109092650) and the alpha-satellite probe for chromosome 3 centromere (pa3.5). (Reproduced from study I)

The 52 patient TMA was used for FISH analysis. Paraffin-embedded 6 µm sections were deparaffinized and FISH was performed (Saramäki et al. 2001). Briefly, the deparaffinized slides were treated in 1M sodium thiocyanate (NaSCN) at 80°C for 10 min, rinsed in H2O and incubated in 120 mg/40 ml pepsin (from porcine stomach, Sigma P-7012) for 15 min. Slides were washed and dehydrated in an ascending ethanol series, after which the slides were hybridized with the probes over two nights in a humidity chamber. The slides were then stringently washed, counterstained with an antifade solution (Vectashield; Vector Laboratories, Burlingame, CA, USA) containing 0.5 mmol/l 40,6-diamidino-2-phenylin-dole (DAPI) and the locus-specific probes were detected with anti-digoxigenin-rhodamine.

Copy numbers of CIP2A and DPPA4 were counted and classified as deletion, normal, gain or amplification. Gain was defined as having 3–4 copies and amplification as having 5 or more copies of the probe in question. Both gain and amplification were regarded as increased copy number. In addition, the cell lines were analysed for polysomy, and polysomy was defined as having increased number of both locus-specific signals and centromeric signal. Unsuccessful hybridisation results were excluded from further analysis.

4.7 Statistical methods (I-IV)

Statistical analyses were carried out in SPSS Statistics for Mac, version 25 (IBM Corp., Armonk, N.Y., USA). For each study, the data was entered in the software and formatted according to customary practices. The pertinent statistical methods were chosen based on the hypothesis under investigation. Throughout, p values less than 0.05 were deemed significant.

4.7.1 Correlation analysis

Correlation was determined by Spearman's method, using one-tailed test of significance. The strength of the association was interpreted in the usual Evans' scale of very weak, weak, moderate, strong, very strong (Evans 1996).

4.7.2 General Linear Model statistics (II)

Relationships between cell line radioresistance and immunohistochemical staining results were analysed using General Linear Model statistics, observing both main effects and interactions. Estimated marginal means were calculated, and bootstrapping was used to determine 95% confidence intervals (CI).

4.7.3 Logistic regression (III-IV)

Logistic regression analysis was used to analyse TMA inclusion bias. First, univariate associations were calculated, and based on preliminary variable selection, multivariable model was constructed using backward stepwise elimination method based on likelihood ratios. Odds ratios with 95% CI and p values are reported.

4.7.4 Survival analysis (I, III-IV)

Overall survival estimations were plotted using the Kaplan-Meier method (Kaplan and Meier 1958). Plots were drawn with sticks to indicated censoring. Five-year overall survival was used, except for nasopharyngeal carcinoma, where 10-year survival period is more appropriate. Overall survival was defined from end-of-treatment to end-of-follow-up or death.

The survival effects were analysed using Cox proportional hazards model (Cox 1972). Hazard ratios (HR) with 95% CI are reported, and p values were calculated. The proportionality of hazards was tested using log-minus-log plotting or plotting Schoenfeld residuals against survival time, when appropriate. For construction of multivariable survival models, a backward stepwise approach based on likelihood ratio method was applied with p value limits for inclusion and exclusion at 0.05 and

0.10, respectively. When the survival impact of molecular biomarkers was analysed in multivariable models, identified prognostic covariates were entered first, and the biomarkers entered in a further block.

4.8 Ethical considerations (I-IV)

For each study, appropriate ethical approval was obtained, and institutional guidelines and the declaration of Helsinki were followed. UT-SCC cell lines have previously been established from tumour samples of human HNSCC after individual patient informed consent according to ethical approval by the Ethics Committee of the Hospital District of Southwest Finland (Grenman et al. 1988).

For use of human HNSCC tumour samples in study I, approvals by the Finnish national authority for medicolegal affairs (Dnro 889/04/047/08) and regional Ethics Committee of University of Turku (Dnro 146/2007) were obtained. For use of human HNSCC tumour and liver samples in TMA construction of studies III and IV, approvals by the Finnish National Supervisory Authority for Welfare and Health (V/39706/2019), Ethics Committee of the Hospital District of Southwest Finland (51/1803/2017) and Auria biobank scientific board (AB19-6863) were granted.

4.9 Publication images

Microscopic photographs of the CMA and TMA stainings were obtained using the CaseViewer software (3DHISTECH Ltd.) after slides were scanned using Pannoramic 250 Flash whole slide scanner (3DHISTECH Ltd.). Photographs of either 10-fold or 20-fold magnification were exported, images were cropped in image editing software, and no colour adjustment or filtering was performed.

5 Results

5.1 CIP2A and DPPA4 fluorescence in situ hybridization (I)

5.1.1 UT-SCC cell lines

The novel FISH method for CIP2A and DPPA4 copy number alterations was tested in six UT-SCC cell lines. UT-SCC14 showed polysomy for both CIP2A and DPPA4, while CIP2A copy numbers were increased in 4/6 UT-SCC cell lines. DPPA4 copy number increase was observed in 5/6 cell lines, while in three cell lines copy number increase was present in less than 10% of cells. Furthermore, copy number increase of DPPA4 did not correlate with CIP2A copy number increase (Table 6).

Table 6. UT-SCC cell lines showed variable degrees of copy number increase of CIP2A and DPPA4. Results expressed as percentage of cells showing either increased LSS to centromere ratio or high-grade polysomy. (Adapted from Study I)

UT-SCC CELL LINE	-9		-14		-22		-40		-50		-65	
FISH STATUS	CIP2A	DPPA4	CIP2A	DPPA4	CIP2A	DPPA4	CIP2A	DPPA4	CIP2A	DPPA4	CIP2A	DPPA4
INCREASED LSS RATIO	26	4	0	4	20	14	42	25	35	0	0	3
HIGH POLYSOMY	0	0	60	84	0	0	0	0	0	0	0	0

*FISH: fluorescence in situ hybridization, LSS: locus-specific signal, UT-SCC: University of Turku – Squamous Cell Carcinoma.

5.1.2 Fifty-two patient TMA

The 52 patient TMA was used for the evaluation of prognostic performance of CIP2A and DPPA4 copy number alterations. In the 52 patient TMA, CIP2A copy number increase was observed in 54% of patients and DPPA4 copy number increase in 34% of patients (Table 7). There was a significant, moderate correlation between the presence of CIP2A and DPPA4 copy number increase ($r=0.46$, $p=0.001$). The

copy number increase of CIP2A was associated with a non-significant trend for poor OS, while DPPA4 was not associated with OS in HNSCC patients (Figure 9A-B).

Table 7. Copy number alterations in the 52 patient TMA (adapted from Study I).

FISH STATUS	***CIP2A***					
DPPA4	normal	deletion	gain	amplification	bad*	total
normal	12	0	9	0	0	21
deletion	1	1	0	0	0	2
gain	3	0	13	1	0	17
amplification	0	0	0	2	0	2
bad*	4	0	1	2	3	10
total	20	1	23	5	3	52

*bad: unsuccessful hybridization result.

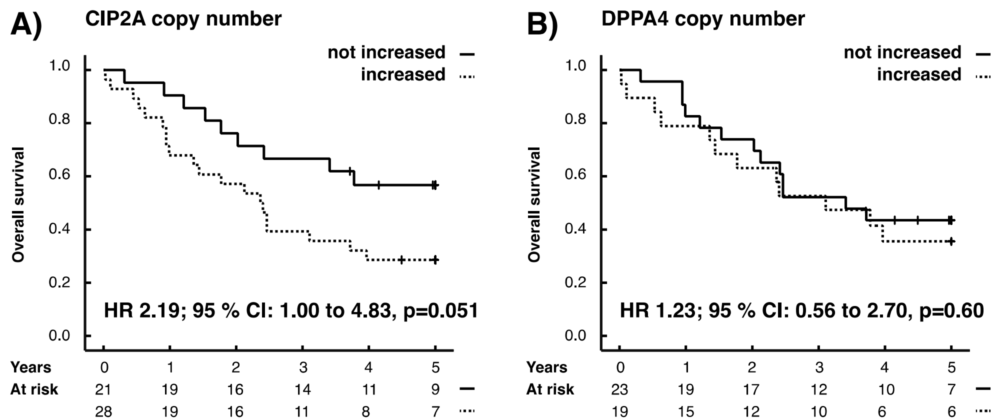


Figure 9. Survival analysis of CIP2A and DPPA4 copy number alterations. (A) CIP2A copy number increase is associated with a trend for impaired overall survival. (B) DPPA4 copy number increase has no effect on survival. Modified from Study I.

Next, FISH results were compared against immunohistochemical stainings of CIP2A and DPPA4 (Table 8). There was no significant correlation between either CIP2A or DPPA4 immunoreactivity and copy number increase (for CIP2A, r=-0.036, p=0.40; and for DPPA4, r=-0.13, p=0.20).

Table 8. CIP2A and DPPA4 FISH status and staining intensities. (Adapted from Study I)

CIP2A	***FISH* status**					
staining	normal	deletion	gain	amplification	bad*	total
negative	6	0	3	1	0	10
moderate	8	0	14	4	2	28
strong	6	1	6	0	1	14
total	20	1	23	5	3	52
DPPA4	***FISH status***					
staining	normal	deletion	gain	amplification	bad	total
negative	6	1	5	1	3	16
moderate	11	0	11	1	5	28
strong	4	1	1	0	2	8
total	21	2	17	2	10	52

*bad: unsuccessful hybridization result.

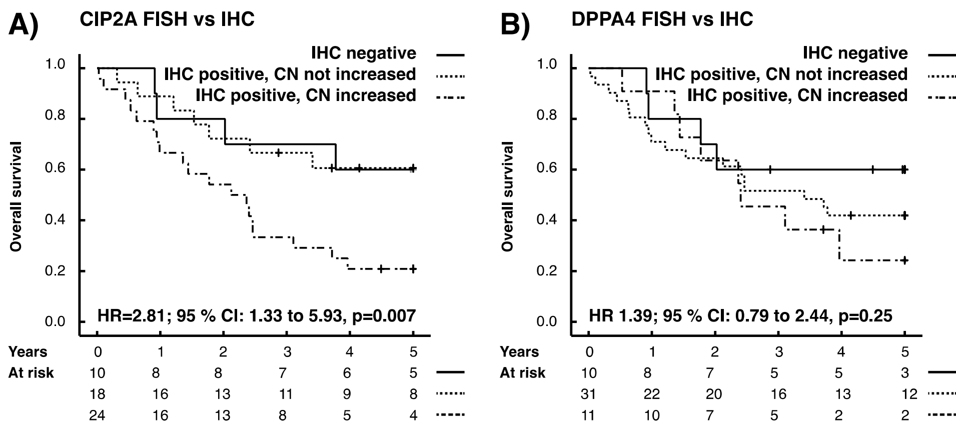


Figure 10. Analysis of the prognostic value of FISH-IHC combinations. A) Addition of CIP2A FISH analysis to IHC significantly improves the prognostic value of CIP2A positivity, while B) the prognostic value of DPPA4 remained insignificant.

Finally, the combined survival impact of CIP2A copy number increase and CIP2A immunoreactivity was analysed. A poor survival was associated with the presence of both CIP2A copy number increase and moderate to strong immunoreactivity, while no survival difference was demonstrated between patients with either low or high CIP2A immunoreactivity in the absence of CIP2A copy number increase (Figure 10A). The use of DPPA FISH and immunohistochemistry

in a similar prognostication strategy did not provide significant prognostic resolution (Figure 10B).

5.2 Discovery of radioresistance biomarkers using a cell microarray (II)

Using the CMA consisting of 26 UT-SCC cell lines, immunohistochemical stains for putative radioresistance biomarkers (p53, EGFR, OCT4, CIP2A, and NDFIP1) were analysed. Association of the biomarkers with intrinsic radioresistance are shown in Figure 11. Expression of p53, EGFR, and CIP2A were not associated with radioresistance. Nuclear NDFIP1 and OCT4 expressions were associated with a significant increase in radioresistance. A significant interaction effect between p53 and OCT4 was observed, while the interaction trend between p53 and high EGFR expression did not reach significance.

5.2.1 CIP2A-shRNA silenced cell lines

Three CIP2A shRNA-silenced cell lines were included in the CMA. Western blot was used to confirm CIP2A shRNA-silencing. Immunohistochemical staining demonstrates a low immunoreactivity of the silenced cell lines in comparison to the parental non-silenced lines (Figure 12).

CIP2A silencing was not related to changes in the expression of the other putative radioresistance biomarkers. However, the expression of two other endogenous inhibitors of PP2A, PME-1 and SET, was also reduced in all three silenced cell lines (Table 9). The expression of CIP2A, PME-1, and SET were not correlated across the CMA.

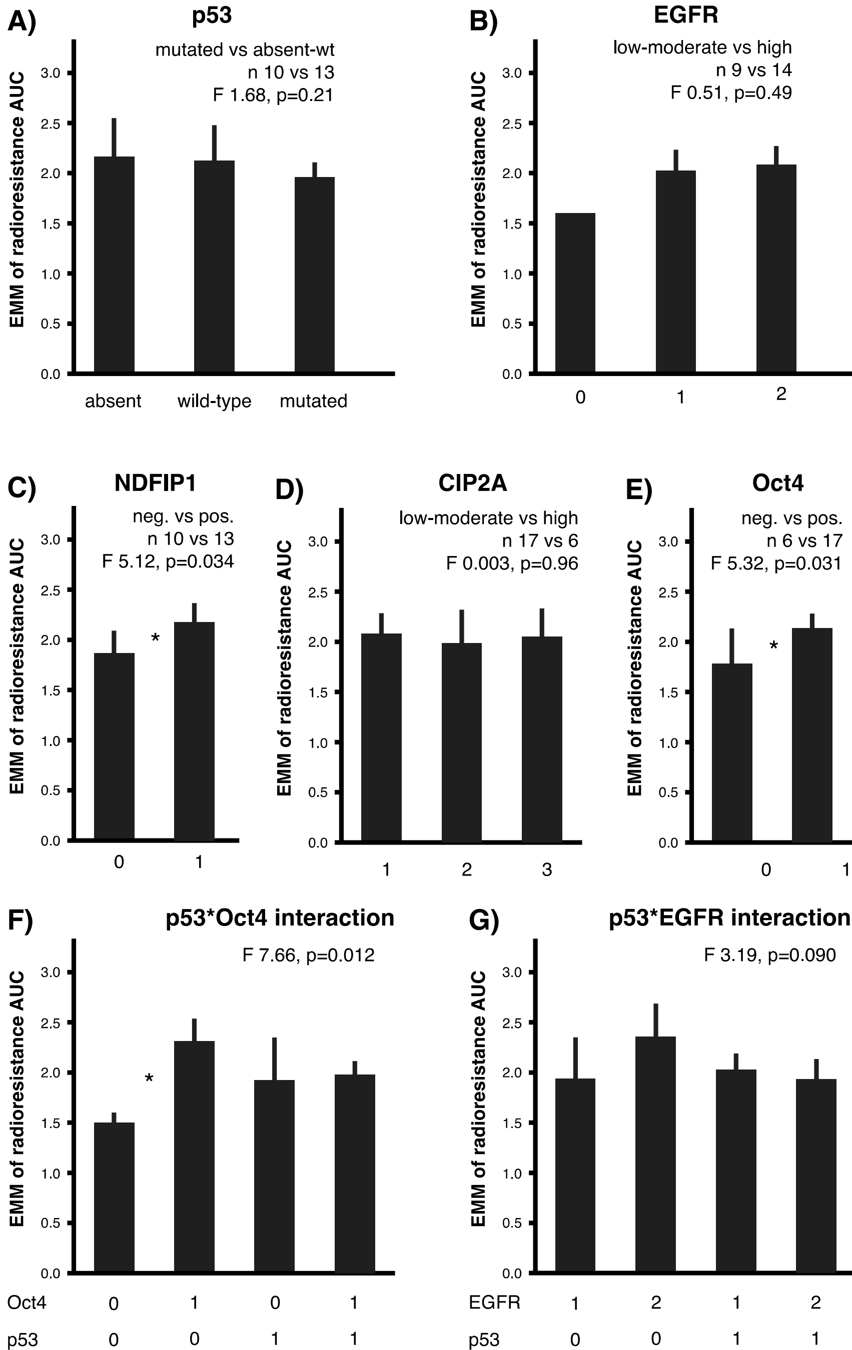


Figure 11. Association of biomarker stains and radioresistance was investigated across 23 cell lines. Bars represent estimated marginal means and error bars indicate 95% confidence intervals determined using bootstrapping. Sample sizes, F values and p values are indicated. Significant ($p<0.05$) results are indicated with an asterisk (*) (Reproduced from Study II)

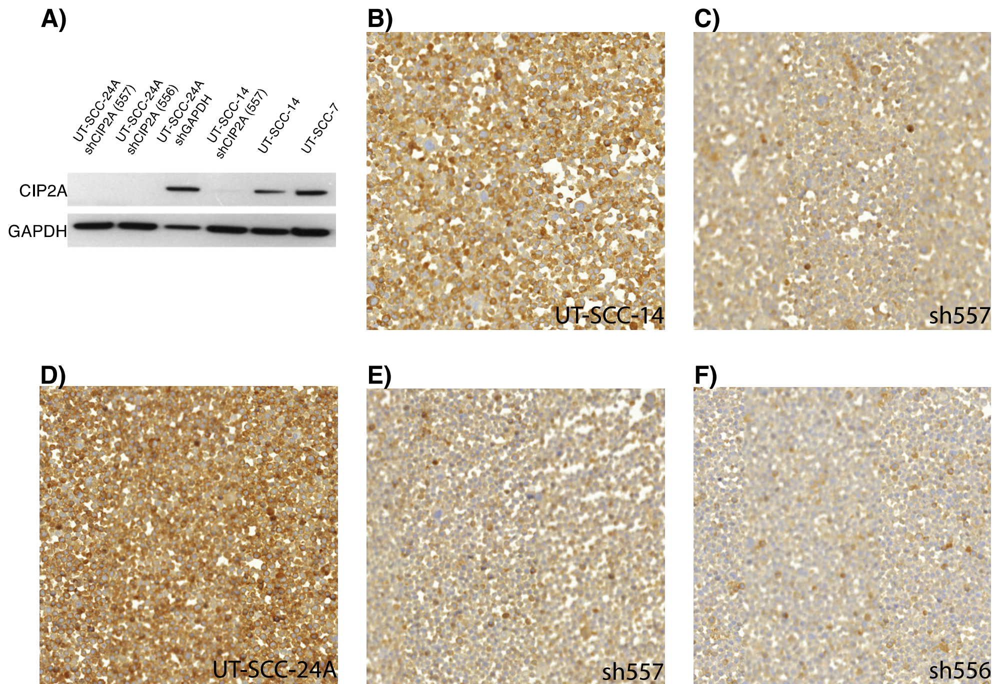


Figure 12. Analysis of CIP2A-shRNA-silenced cell lines. A) Successful silencing was confirmed using Western blot. B) Immunohistochemistry of CIP2A-positive non-silenced UT-SCC-14 cell line, and C) loss of CIP2A immunoreactivity in shRNA-silenced cell line. D) Immunohistochemistry of CIP2A-positive non-silenced UT-SCC-24A, and E-F) loss of CIP2A immunoreactivity in both shRNA-silenced cell line versions of UT-SCC-24A. (reproduced from Study II)

Table 9. Immunohistochemical analysis of the CIP2A-shRNA-silenced cell lines. (Adapted from Study II)

	IMMUNOHISTOCHEMISTRY							
CELL LINE	**CIP2A**	**p53**	**LIMA1**	**EGFR**	**NDFIP1**	**Oct4**	**SET**	**PME-1**
Non-treated UT-SCC-14	2	mutated	1	3	0	1	2	2
CIP2A-shRNA-silenced UT-SCC-14 (plasmid 557)	1	mutated	1	3	0	1	1	1
Non-treated UT-SCC-24A	3	absent	2	3	1	1	1	2
CIP2A-shRNA-silenced UT-SCC-24A (plasmid 556)	1	absent	2	3	0	1	1	1
CIP2A-shRNA-silenced UT-SCC-24A (plasmid 557)	1	absent	2	3	1	1	1	1

5.3 Representativeness of the novel TMA (III-IV)

Altogether 476 patients diagnosed and treated for new HNSCC tumour in 2005-2010 in Southwest Finland region were identified (Table 10). Five-year OS was 53%, and 150 patients died from HNSCC (32%). Distant metastasis was uncommon at diagnosis (n=5, 1.1%), while nodal metastasis was common (n=164, 34 %). A minimal number of 1.3% (6/476) of patients were lost during the first year of follow-up.

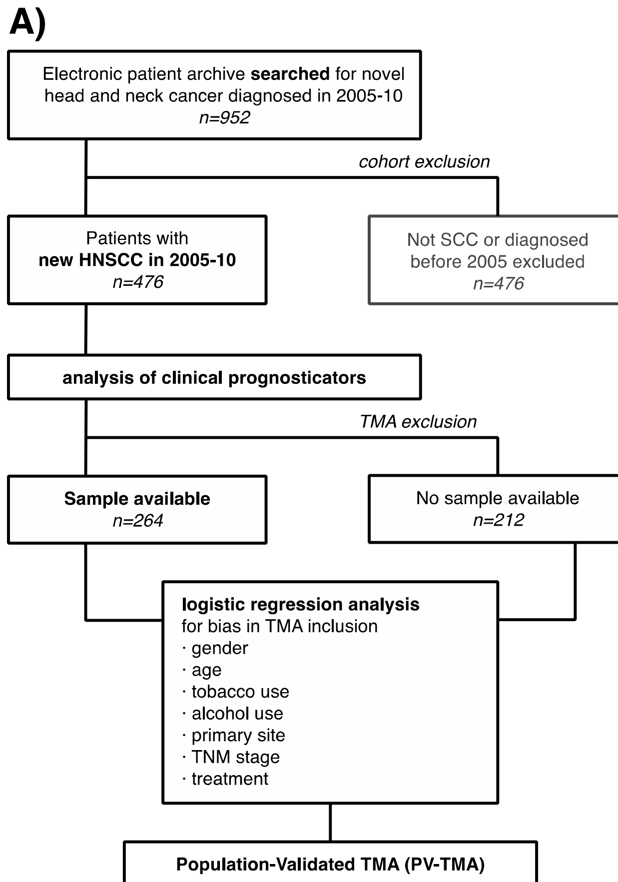
The primary tumour was operated in 282 cases (59%), and neck dissection was carried out in 173 patients (36%). Radiotherapy was used in the treatment of 97 patients (20%), and chemoradiotherapy was used in 191 patients (40%). No treatment was given to 15 patients (3%) and altogether 49 patients' (10%) treatment was not curative. One patient's treatment and follow-up details were not available due to migration. During five years of follow-up, 137 patients developed recurrence (29%).

For TMA construction, 264 patients' tumour samples (55%) were available, whereas 212 patients' archived samples containing the tumour were either cut through in diagnostic purposes, were lost in the pathology archives, or were archived outside of Auria Biobank (Figure 13A). Importantly, no sample was discarded due to small tumour size. The TMA construction bias was analysed using logistic regression (Table 11). Age distribution, mutagenic exposures, and TNM classifications of the resulting population-validated PV-TMA were representative compared to clinical data of the background population. However, site distribution was uneven. Importantly, TMA inclusion did not offer prognostic resolution for 5-year OS (Figure 13B).

Table 10. Clinicopathological variables of the patient cohort. Results of a multivariable survival analysis are shown. (Modified from Study III)

		TOTAL		**SURVIVAL EFFECT**	
		n	*%*	*HR (95% CI)*	*p*
GENDER					
	male	325	68	not included	-
	female	151	32	-	-
AGE AT DIAGNOSIS					
	<65	236	50	1.04 (1.02-1.05) / yr	<0.001
	>65	240	50	-	
SMOKER					
	>20 pack yrs	225	47	1.09 (0.76-1.55)	0.65
	<20 pack yrs	223	47	1	-
ALCOHOL STATUS					
	yes	139	29	1.50 (1.06-2.13)	0.023
	no	337	71	1	-
PRIMARY TUMOR SITE					
	oral cavity	226	47	1	-
	oropharynx	89	19	0.70 (0.46-1.06)	0.089
	larynx	105	22	1.03 (0.71-1.49)	0.89
	hypopharynx	20	4	1.66 (0.91-3.03)	0.10
	other	36	8	1.10 (0.65-1.86)	0.73
T CLASS					
	T0-2	311	65	0.27 (0.17-0.44)	<0.001
	T3-4	165	35	1	-
N CLASS					
	N0	312	66	0.54 (0.37-0.79)	0.002
	N+	164	34	1	-
STAGE					
	0-II	232	49	1.41 (0.77-2.58)	0.26
	III-IV	244	51	1	-

OR: odds ratio, CI: confidence interval, p: p value, RT: radiotherapy, CRT: chemoradiotherapy, yr: year.



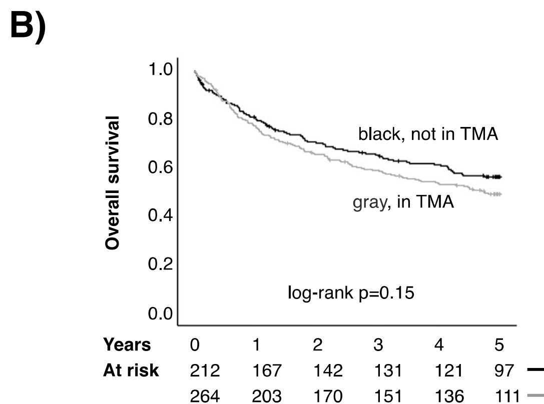


Figure 13. Principle of the population-validated TMA. First, a background population was screened for comprehensive inclusion of all patients treated for HNSCC in Southwest Finland during the period of 2005–2010. This background population was used to assess clinical prognostic factors. All available samples were included in TMA. The representativeness of the TMA was analysed with logistic regression analysis for multiple variables. After the representativeness was confirmed, the TMA is considered a population-validated TMA (PV-TMA). (Modified from Study III)

Table 11. Univariate (left panels) and multivariable (right panels) analysis of TMA inclusion bias. Results from logistic regression modelling. (Adapted from Study III)

N=264	TMA		TMA inclusion		TMA		TMA inclusion	
	n	*%*	*OR (95% CI)*	*p*	*n*	*%*	*OR (95% CI)*	*p*
Gender								
male	164	62	0.52 (0.35-0.78)	0.001	164	62	0.56 (0.36-0.88)	0.011
female	100	38	1	-	100	38	1	-
Age at diagnosis								
<65	137	52	1.23 (0.86-1.77)	0.26	137	52	not included	
≥65	127	48	1	-	127	48		
Smoking history								
≥20 pack yrs	115	44	0.72 (0.50-1.03)	0.071	115	44	NS	
<20 pack yrs	149	56	1	-	149	56		
Alcohol status								
yes	78	30	0.96 (0.65-1.43)	0.85	78	30	not included	
no	186	70	1	-	186	70		
Primary tumour site								
oral cavity	137	52	1	-	137	52	1	-
oropharynx	64	24	1.66 (0.98-2.84)	0.062	64	24	2.34 (1.21-4.54)	0.012
larynx	35	13	0.33 (0.20-0.53)	<0.001	35	13	0.68 (0.37-1.24)	0.21
hypopharynx	11	4	0.79 (0.32-1.99)	0.62	11	4	1.64 (0.58-4.63)	0.35
other	17	6	0.58 (0.29-1.18)	0.13	17	6	0.93 (0.42-2.03)	0.85
T class								
T0-2	173	66	1.02 (0.70-1.49)	0.92	173	66	not included	
T3-4	91	34	1	-	91	34		
N class								
N0	157	59	0.54 (0.37-0.80)	0.002	157	59	NS	
N+	107	41	1	-	107	41		
Stage								
0-II	118	45	0.70 (0.48-1.00)	0.049	118	45	NS	
III-IV	146	55	1	-	146	55		

Recurrence in 5 years								
yes	84	32	1.40 (0.93-2.12)	0.11	84	32	not included	
no	152	58	1	-	152	58		
no curative treatment	28	11	1.20 (0.65-2.21)	0.56	28	11		
Living at 5 years								
yes	131	50	0.72 (0.48-1.08)	0.11	131	50	not included	
no, died of HNSCC	90	34	1	-	90	34		
no, died of other cause	43	16	0.96 (0.54-1.69)	0.88	43	16		
Surgical treatment								
No surgery	*55*	21	-	-	55	21	-	-
Local operation	174	66	1.88 (1.30-2.73)	0.001	174	66	1.75 (1.05-2.94)	0.033
Neck dissection	125	47	3.10 (2.07-4.63)	<0.001	125	47	2.30 (1.49-3.56)	<0,001
Treatment type								
Surgery only	92	35	1	-	92	35	not included	
RT +/- surgery	54	20	1.09 (0.66-1.80)	0.73	54	20		
CRT +/- surgery	112	42	1.23 (0.81-1.87)	0.32	112	42		
no treatment	5	2	0.44 (0.14-1.33)	0.14	5	2		

OR: odds ratio, CI: confidence interval, p: p value, RT: radiotherapy, CRT: chemoradiotherapy, yr: year, NS: not significant.

5.3.1 Representativeness of radiotherapy treated subpopulation (IV)

The radiotherapy (n=97) or chemoradiotherapy (n=191) treated PV-TMA patients were included in the analyses of the fourth study. The representativeness of the radiotherapy treated cohort was separately evaluated. The PV-TMAs analysed in the study IV consisted of samples from 166 patients (Figure 14). This PV-TMA was highly representative of patients treated with radiotherapy or chemoradiotherapy for new HNSCC tumour in 2005-2010 in Southwest Finland region (Table 12).

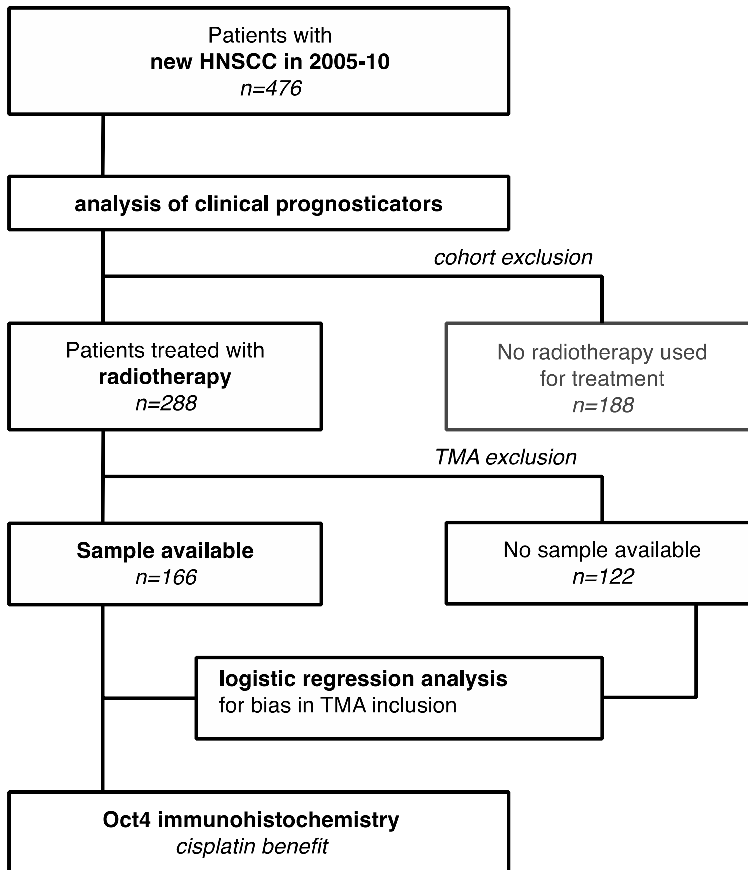


Figure 14. Principle of population-validation in Study IV. (Modified from Study IV)

5.3.2 Comparison with the 52 patient TMA

When the previous 52 patient TMA used in Study I was compared to the patient population included in the PV-TMAs of Studies III and IV (*cf.* Table 4, Table 11, and Table 12), a predilection for higher T class and less frequent use of radiotherapy was observed in the 52 patient TMA. In addition, rates of five-year recurrence were significantly higher in the earlier constructed 52 patient TMA.

Table 12. Clinicopathological variables of the primary HNSCC patient population (left columns) and the population-validated TMA of radiotherapy or chemoradiotherapy treated patients (right columns). (Adapted from Study IV)

	TOTAL		TMA		TMA INCLUSION		MULTIVARIABLE	
	n	%	n	%	OR (95 % CI)	p	OR (95 % CI)	p
Gender								
male	210	73	112	67	1	-	NS	-
female	78	27	54	33	1.97 (1.13–3.42)	0.016	-	-
Age at diagnosis								
<65	180	63	104	63	0.99 (0.97–1.01) / yr	0.35	not included	-
≥65	108	38	62	37	-	-	-	-
Smoking status								
current smoker	91	32	64	39	0.51 (0.29-0.88)	0.017	NS	-
former smoker	47	16	20	12	0.31 (0.15-0.65)	0.002	-	-
non-smoker	150	52	82	49	1	-	-	-
Alcohol status								
no	176	61	105	63	1	-	not included	-
yes	112	39	61	37	0.81 (0.50-1.31)	0.39	-	-
Primary tumour site								
oral cavity	87	30	63	38	1	-	1	-
oropharynx	82	28	59	36	0.98 (0.50-1.92)	0.95	1.65 (0.74-3.66)	0.22
larynx	79	27	23	14	0.16 (0.080-0.31)	<0.001	0.29 (0.13-0.66)	0.003
hypopharynx	15	5	9		0.57 (0.18-1.78)	0.33	1.20 (0.34-4.22)	0.78
other	25	9	12	7	0.35 (0.14-0.88)	0.025	0.61 (0.22-1.71)	0.35
T class								
T0-2	153	53	90	54	1	-	not included	-
T3-4	135	47	76	46	0.90 (0.57-1.44)	0.67	-	-
N class								
N0	137	48	67	40	1	-	NS	-
N+	151	52	99	60	1.99 (1.24-3.20)	0.004	-	-
Stage								
I-II	79	27	39	23	1	-	NS	-
III-IV	209	73	127	77	1.59 (0.94-2.68)	0.082	-	-

Recurrence in 5 years								
yes	85	30	53	32	1.36 (0.80-2.32)	0.25	not included	-
no	175	61	96	58	1	-	-	-
no curative treatment	28	10	17	10	1.27 (0.56-2.87)	0.56	-	-
Living at 5 years								
yes	142	49	78	47	0.74 (0.36-1.55)	0.42	not included	-
no, died of HNSCC	106	37	66	40	1	-	-	-
no, died of other cause	40	14	22	13	0.74 (0.44-1.23)	0.25	-	-
Surgical treatment								
No surgery	126	44	50	30	-	-	-	-
Local operation	110	38	82	49	3.28 (1.95-5.51)	<0.001	2.47 (1.24-4.92)	0.010
Neck dissection	132	46	96	58	3.28 (2.00-5.38)	<0.001	NS	-
Treatment type								
RT only	51	18	20	12	1	-	NS	-
CRT only	75	26	30	18	1.03 (0.50-2.14)	0.93	-	-
RT + surgery	46	16	34	20	4.39 (1.85-10.44)	0.001	-	-
CRT + surgery	116	40	82	49	3.74 (1.88-7.45)	<0.001	-	-

OR: odds ratio, CI: confidence interval, p: p value, RT: radiotherapy, CRT: chemoradiotherapy, yr: year, NS: not significant.

5.4 Prognostic analysis of the PV-TMA (III)

5.4.1 Development of multivariable prognostic model

The prognostic potential of clinical information was thoroughly analysed (Table 10). Patient age was associated with impaired survival (HR 1.02 per year; 95% CI 1.01 to 1.03, p<0.001). Advanced T class and the presence of neck metastasis provided better prognostic resolution than TNM stage in all major subsites of HNSCC (data not shown). The alcohol status of the patients, defined as current problem-level alcohol use or history of severe alcohol-related somatic complications, were also included in the prognostic model (HR 1.45; 95% CI: 1.10 to 1.91, p=0.008), while the survival impact of tobacco history did not remain significant in multivariable analysis. The primary tumour site had no decisive survival impact but was included

in the multivariable models. Applying the multivariable prognostic model, local operation of the primary tumour (HR 0.74; 95% CI: 0.55 to 0.98, p=0.038) and neck dissection (HR 0.73; 95% CI: 0.53 to 1.00, p=0.049) were significantly associated with improved OS.

5.4.2 Prognostic analysis of radioresistance biomarkers (III)

The prognostication capability of p53, EGFR, p16, CIP2A, MET, Oct4, and NDFIP1 was analysed using immunohistochemical stainings, which were dichotomously interpreted (Figure 15). The prognostic information of CIP2A and p16 reached significance in univariate analysis, but none of the biomarkers showed significant prognostic value in multivariable analysis (Table 13).

Table 13. Prognostic performance of the investigated biomarker staining intensities. Adapted from study III.

		Total		**5-year survival**		**Survival analysis**	
		n	*%*	*alive, n*	*%*	*HR (95 % CI)*	*p*
p53	*absent*	73	29	34	47	0.91 (0.62-1.36)	0.65
	wt or high	176	71	89	51	1	-
EGFR	*low-moderate*	190	78	93	49	1.27 (0.82-1.97)	0.29
	strong	53	22	26	49	1	-
CIP2A	*low-moderate*	150	66	79	53	1.20 (0.81-1.76)	0.37
	high	78	34	30	38	1	-
OCT4	*negative*	101	39	56	55	0.73 (0.50-1.07)	0.11
	positive	160	61	75	47	1	-
p16	*negative*	181	81	81	45	0.91 (0.68-1.22)	0.54
	positive	43	19	26	60	1	-
NDFIP1	*negative*	137	60	63	46	1.18 (0.81-1.73)	0.40
	positive	90	40	46	51	1	-
MET	*low*	151	66	76	50	0.80 (0.55-1.15)	0.22
	moderate-high	79	34	38	48	1	-

n: number of patients, HR hazard-ratio, CI: confidence interval, p: p value, wt: wild-type

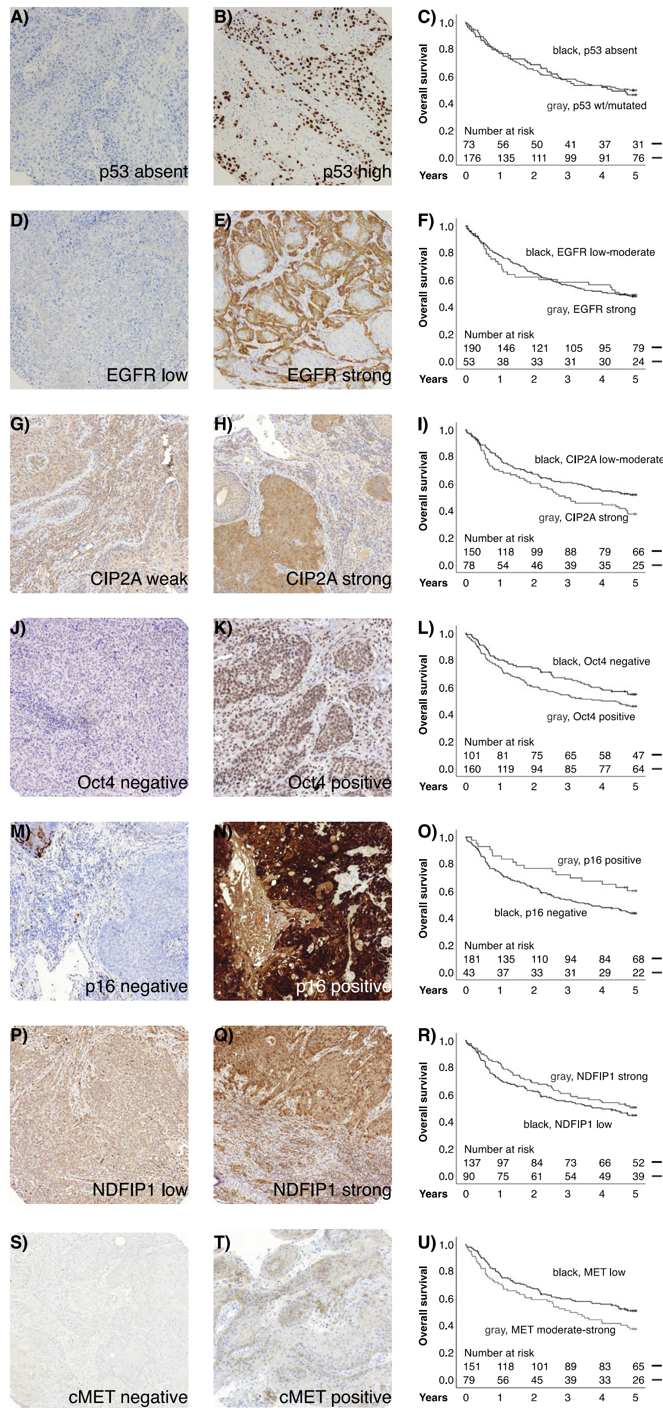


Figure 15. Representative immunohistochemical stains and Kaplan-Meier survival estimates of the investigated biomarkers in HNSCC. A-C) p53, D-F) EGFR, G-I) CIP2A, J-L) Oct4, M-O) p16, P-R) NDFIP1, and S-U) MET. (Reproduced from study III).

Stratified by primary tumour site, none of the investigated biomarkers provided statistically significant prognostic information. Furthermore, after adjustment using the described prognostic model, no combination or interaction of the investigated biomarkers provided significant prognostic potential.

5.5 Radioresponse prediction using OCT4 (IV)

Next, the predictive value of OCT4 immunohistochemical staining for clinical radiotherapy and cisplatin response, indicated by OS after radiotherapy treatment, was analysed in the PV-TMA.

5.5.1 OCT4 antibody validation

Specificity of the OCT4 antibody was evaluated in UT-SCC cell lines and TCAM2 cell line. The UT-SCC cell lines did not show demonstrable endogenous OCT4 expression in Western blot analysis (Figure 16A). Using UT-SCC-36-CRISPRa-OCT4 cell line in which OCT4 promoter is activated by CRISPRa conditionally after doxycycline-trimethoprim incubation, OCT4 expression was induced with high specificity (Figure 16B-C). In tumour samples, nuclear OCT4 positivity or negativity was dichotomously interpreted (Figure 16D-E).

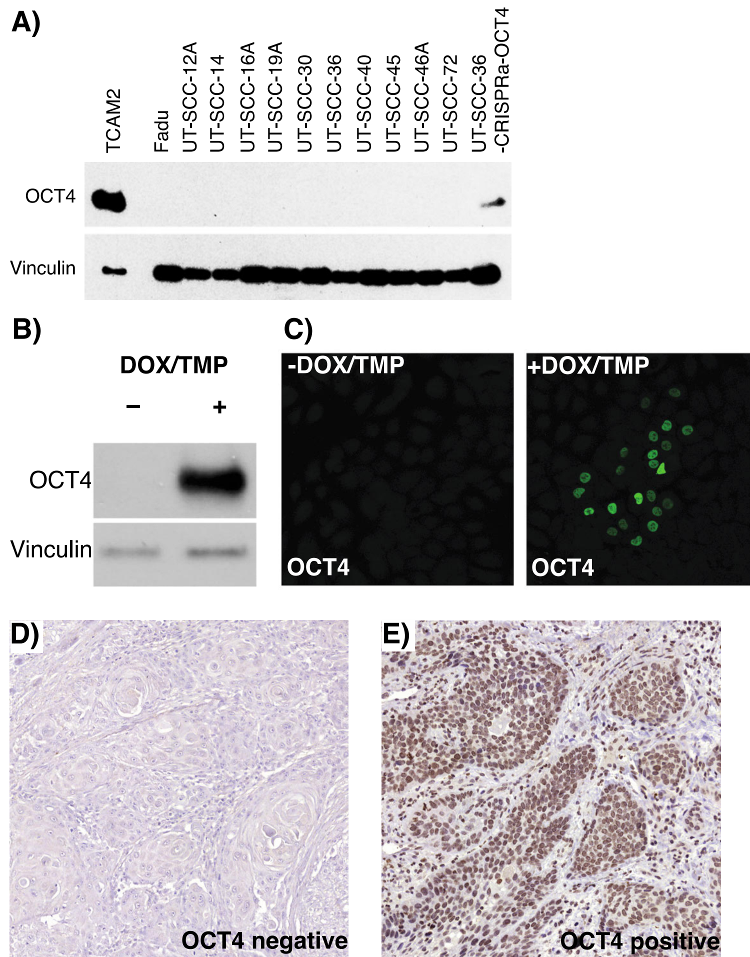


Figure 16. Demonstration of specificity of OCT4 antibody. A) In Western blot analysis, only TCAM2 has strong OCT4 expression, while in UT-SCC-36-CRISPRa-OCT4 cell line, OCT4 expression is induced by doxycycline-trimethoprim treatment. The induction of OCT4 expression is demonstrated using B) Western blot, and C) immunofluorescence staining. In human HNSCC samples, D) OCT4-negative, and E) OCT4-positive samples are readily recognized. Modified from Study IV.

5.5.2 TMA overall survival analysis

The PV-TMA was used to analyse survival effects of OCT4 expression in HNSCC treated with radiotherapy or chemoradiotherapy. There was no difference in OS between patients treated using radiotherapy or chemoradiotherapy in OCT4-negative patients (Figure 17A). In OCT4-positive patients, treatment with radiotherapy without cisplatin was associated with poor prognosis (Figure 17B). The survival of OCT4-positive patients treated with the cisplatin radiosensitization was

indistinguishable from that of OCT4-negative patients treated with radiotherapy alone (Figure 17C).

As an internal validation, OS was compared between patients, whose cisplatin course was interrupted due to toxicity (n=31). OCT4-positive patients achieved a significant survival benefit from interrupted cisplatin courses in comparison to patients treated with radiotherapy alone, similarly with patients treated with full, uninterrupted cisplatin course (Figure 17D). In patients treated with other chemotherapeutic agents (cetuximab or taxane), OCT4 positivity was associated with poor survival, leaving the survival similar to patients treated with radiotherapy alone (data not shown).

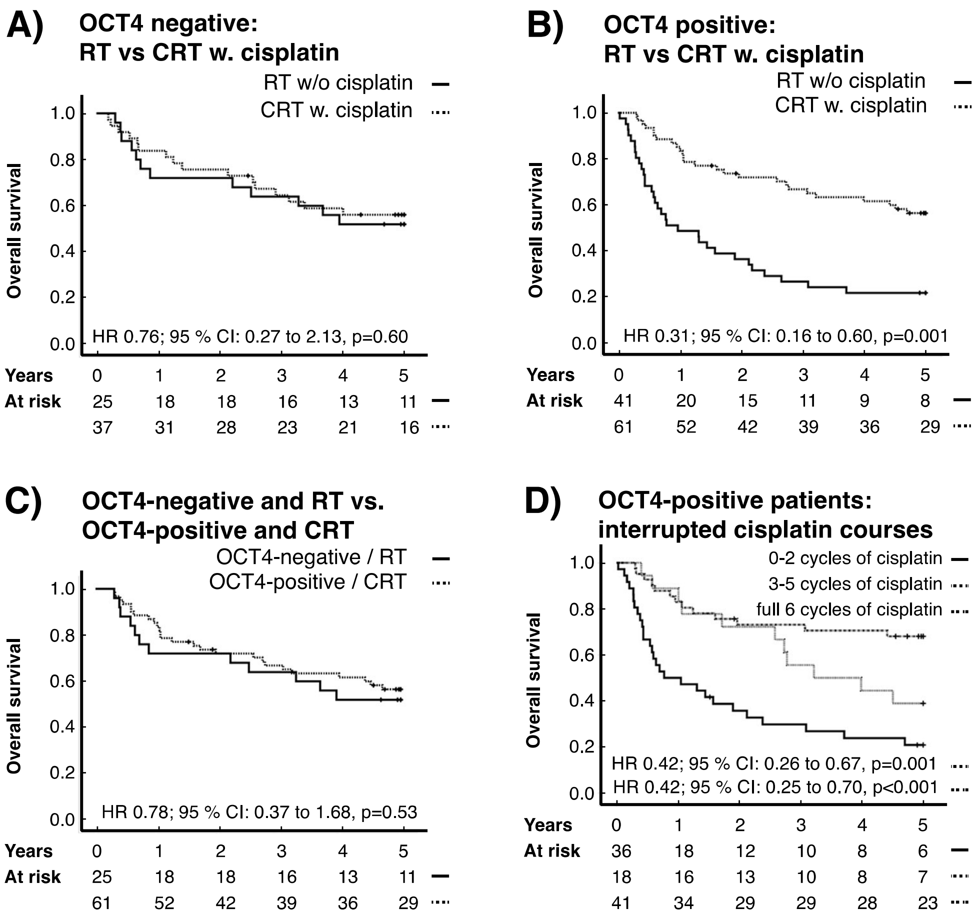


Figure 17. Survival analysis of OCT4 immunohistochemistry in PV-TMA patients treated with radiotherapy or chemoradiotherapy. Modified from study IV.

6 Discussion

6.1 Improving prognostic resolution using genomic methods (Study I)

The main finding of the first study is the improvement in prognostic resolution of CIP2A detection by introduction of copy number analysis using FISH. Despite the genetic proximity of DPPA4, neither expression analysis nor copy number analysis of DPPA4 provided significant prognostic value. In the several years following the publication of the first study, no research group has, to my knowledge, reported results of CIP2A FISH experiments. This highlights the methodological importance of the first study.

Despite the numerous reported associations between patient prognosis and genomic alterations or protein expression changes, no molecular biomarker in addition to p16 immunohistochemistry can offer adequate prognostic resolution (Kim et al. 2014). Importantly, the semiquantitative analysis of immunohistochemical stainings is subjective, and thus, dependant on the experience and expertise of the pathologist. Several relatively laborious approaches can improve the reliability of immunohistochemical analysis, including digital image analysis, consensus-based evaluation by several observers, and scoring systems (Huang et al. 2020; Rizzardi et al. 2012).

Detection of genomic change provides the important advantage of relative objectivity in the analysis of the observation as well as the permanence of the genomic change in comparison with changes in protein expression. Different FISH techniques are routinely used in the prognostic and predictive evaluation of cancers (Hu et al. 2014). FISH detection of HER2 is used in the prediction of response to anti-HER2 medication in breast carcinomas and, to a lesser extent, in salivary duct carcinoma (Takahashi et al. 2019; Wolff et al. 2013). In colorectal adenocarcinoma, EGFR inhibitor treatment is guided by KRAS mutation analysis and EGFR copy number analysis (Ålgars et al. 2011). In HNSCC, several studies have reported associations between patient survival or therapy resistance and copy number increases of *e.g.* ACVR1, EGFR, YWHAZ and GST-π (Ambrosio et al. 2011; Cullen et al. 2003; Lin et al. 2009; Nakata et al. 2011).

The oncogenic role of CIP2A is well-recognised, it is overexpressed in numerous cancers, and is linked to radioresistance of HNSCC and colorectal cancer (Birkman et

al. 2018; Ventelä et al. 2015). CIP2A is located in amplicon 3q13, which is a common site for copy number increase in HNSCC. However, CIP2A and the internal control of study I, neighbouring gene DPPA4, are not reported as commonly altered genes in analyses based on the cancer genome atlas (TCGA) databases. Accordingly, low frequencies of high-level copy number alterations of either of the two genes were observed in the Study I. Low-level copy number alterations remain a contested phenomenon, since they are suggested to occur more due to genomic instability than locus-specific oncogenic driver events (Korkola and Gray 2010).

In the first study, low-level copy number increase of CIP2A and DPPA4 were relatively common, while not significantly correlating with immunohistochemically evaluated protein expression. Notwithstanding due caution, the finding that CIP2A but not DPPA4 copy number increase is associated with poor patient survival suggests that copy number increase of CIP2A is indeed a driver event not caused by the general mutagenic instability of the cancer genome. A passenger increase of DPPA4 was, however, observed, leading to the conclusion, that careful probe design is important when a specific of genomic locus is targeted.

6.2 Cell microarray (CMA) methodology (Study II)

Cell lines offer a powerful tool in the assessment of various cancer phenomena. An important question, however, is the representativeness of tumour-derived cell lines as compared to the original tumours. In genetic studies, cell lines have proven to be relatively well representative of the parental tumours, while important distinctions remain (Domcke et al. 2013; Mouradov et al. 2014). Importantly however, individual cell lines may be contaminated by lengthy cultures and several passages. While cell lines are not able to give information about factors such as patient immunological environment, the measured intrinsic radioresistance of cell lines has been shown to correlate directly with the observed clinical radioresistance (Grénman et al. 1991; Pekkola-Heino et al. 1998; Perri et al. 2015). However, only a part of tumours can give rise to cell lines an in vitro growth of patient-derived tumour cells is a prognostic marker (Pekkola et al. 2004).

The relatively novel methodology applied in the study II, the cell microarray construction, allows for simultaneous assessment of a large number of cell lines with similar staining protocols that are applied in the analysis of clinical tumour samples (Ferrer et al. 2005; Gately et al. 2011; Jonczyk et al. 2016; Wu et al. 2014). Morphology and cellular architecture remain uncompromised and staining results are of high quality. The reliability of immunohistochemical analysis is obviously enhanced by the simultaneous staining of all evaluated samples (Kononen et al. 1998). The CMA of study II is the first large CMA constructed with primarily HNSCC-derived cell lines, and powerfully demonstrates the practical, cost-efficient role that this methodology may play in the analysis of cell lines. This type of FFPE biobank of

cell lines is an important adjunct in cancer research. Importantly, cell lines may be manipulated prior to CMA construction, using silencing or overexpression methods or drug exposures. While the use of only tumour cells, allowing for focus on the IHC staining of cancer cells, the protocols optimized using a CMA may, however, not directly translate into clinical stainings.

In the study II, the effects of CIP2A silencing were demonstrated using three CIP2A shRNA-silenced cell lines, which were shown to successfully silence CIP2A expression as compared to the parental cell lines. Interestingly, the expression of two other endogenous PP2A inhibitors, PME-1 and SET, were also reduced in the CIP2A-silenced cell lines, while no correlation was observed between the expression levels of these inhibitors across the CMA. Our finding suggests that there exists a CIP2A-mediated circuitry leading to a more universal loss of PP2A inhibitor expression. Importantly, this observation demonstrates the straightforward high-throughput investigation made possible by the CMA.

6.3 Basis for population validation (Study III-IV)

Given the acknowledged risk of recruitment bias in prospective cohorts (Berger 2005; Ellenberg 1994; Rothwell 2005), problems in reproducibility of results between cohorts (Ransohoff 2005, 2013), and the overall need for external validation of results (Pepe et al. 2008), it is surprising, that studies rarely report the quality of their cohorts. One of the most common types of cohort studies is the retrospective registry-based study, which introduces a significant bias in patient inclusion due to missing data or registry exclusion. Similar problems have been encountered in studies with TCGA, the data of which is notoriously problematic (Liu et al. 2018).

Some exclusion of patients is unfortunately unavoidable in retrospective cohorts since patients are not sampled for scientific purposes but for diagnostic aims (Kyzas et al. 2005; Virk et al. 2020). Thus, a small sample of tumour, especially of small tumours with a supposedly superior prognosis, may be used up in the diagnostic process, leaving no sample for scientific study in the biobank or pathology archive. Another important exclusion comes from a portion of patients being diagnosed in other institutes, such as private sector or primary care. Other typical recruitment and inclusion biases such as socioeconomic status and limited insurance coverage are not especially enigmatic for researchers in Nordic countries, due to our inclusive health-care system, at least on the level of tertiary care. Based on the ubiquitousness of bias, the evaluation of inclusion and exclusion is therefore a natural but neglected part of scientific assessment of the representativeness of the sampled cohort.

The typical questions concerning sample studies such as issues of representativeness in comparison to the catchment area, exclusion rates due to loss of samples or due to small sample size, and the risk for convenience sampling are especially important. Such factors highlight the importance of clinical-led

investigation – as opposed to laboratory work – to evaluate the representativeness of the cohort properly, and to provide high-quality clinical background data of the patients. Interaction between clinicians and investigators also makes possible the return to the medical records for data completion when novel approaches to the data are discovered. Importantly, while the samples are reachable through the regional biobanks, it is to be emphasized, that the biobank is a repository of data associated with corresponding samples. Thus, in practice, biobanks are unable to provide data about patients that are not included in their sample cohorts. Therefore, the superficially representative cohort may miss a considerable number of patients that would be eligible for a certain study, indicating the need for clinician-based research for the identification of the complete patient cohort, which forms the background population for population validation.

An important approach for increasing the credibility of cohort studies is the comparison of the recruited cohort with the background population. A similar approach of population-validation should also be a part of retrospective cohort studies, since increasing sample size or the number of independent cohorts will not automatically increase representativeness. However, this kind of population validation is very rare, while it is known, that unrepresentativeness is common and that this type of approach is commonly applied in the analysis of catchment in prospective studies (Sorbye et al. 2015). Thus, in many studies, generalizability issues cannot be properly evaluated. A proper statistical analysis is the backbone of population-validation, while an adequate number of patients is needed for definite conclusions to be made.

The Nordic healthcare system is especially suitable as a retrospective study environment due to several reasons. Importantly, HNSCC patients in need of oncological treatment are referred to regional tertiary care referral centres independent of socioeconomic factors of insurance status. Thus, patients treated at the referral centres represent the real-life cross-section of regional patients with HNSCC. In fact, such a cohort is superior to recruited prospective cohort, which in sample studies, furthermore, often has inclusion bias introduced by the recruitment of patients with large tumours suited for sampling. In addition, loss to follow-up is uncommon due to both a well-reachable public health care system and electronic databases making sure that visits at other institutions and especially disease-related mortality is recorded accurately. (Laugesen et al. 2021.)

In conclusion, all patient samples are enrolled and collected in a prospective manner to pathology archives, where they are reached through the regional biobank. The important retrospective component of sample studies is the evaluation and documentation of patient history. This is most readily attainable when patient records are kept meticulously and in electronic form. Thus, it is imperative, that particular care be taken, when HNSCC patient records are maintained during therapy planning and treatment.

In the third and fourth studies, a PV-TMA was used to evaluate the prognostic potential of several radioresistance related molecular biomarkers. The fundamental principle of population-validation is the assessment of bias in patient inclusion based on a population-based patient cohort, retrospectively identified, and analysed using a specific catchment area.

While important as a scientific pursuit, avoiding the failure of lengthy and laborious prospective drug trials (Burtness et al. 2019; Klinghammer et al. 2019; Mehanna et al. 2019) is an important goal of proper retrospective study of biomarkers. The PV-TMA of Study III is based on the Southwest Finland HNSCC patients from 2005 to 2010. A thorough statistical analysis revealed that this PV-TMA is representative as compared to its background population, allowing for unbiased study of clinical and molecular biomarkers. Further important factor in making this patient population especially rewarding for the study of molecular biomarkers is the congruity in treatment protocols due to the long-standing practice of multidisciplinary tumour board decision-making. Thus, being representative of a real-life patient succession, this patient population is superior to many recruited prospective cohorts.

6.4 Biomarker semiotics

An important emphasis of studies III and IV is the development of a clinical prognostic model and its use in a multivariable analysis. The inclusion of surrounding clinical information in prognostic and predictive evaluation of molecular biomarkers is especially important when a heterogenous cancer such as HNSCC is under investigation. In addition, a focus on overall survival (OS) as opposed to disease-free survival or disease-specific survival allows for a less biased interpretation of biomarker mechanics (Haslam et al. 2019; Mailankody and Prasad 2017).

HNSCC treatment results in Nordic countries are particularly good as compared to other European (Gatta et al. 2015). An important observation of the Study III is that the prognosis of HNSCC patients from Southwest Finland was superior to the reported data from Finland in EUROCARE-5 study. Such a difference may be explained by the wide-spread use of cisplatin radiosensitization. In the analysis of prognostic factors, patient age and nodal positivity at the time of diagnosis are unequivocally recognized to affect HNSCC patient survival. Importantly, however, dichotomous T classification (low T class versus high T class) was a powerful prognostic factor, offering a superior resolution as compared to TNM stage. The fourth significant clinical prognostic factor was patient alcohol use history, which has led to an extended study for the determination of proper cut-offs and interpretation of this finding (Denissoff et al. 2022). Using the PV-TMA and the determined clinical prognostic model, putative biomarkers failed in prognostication of HNSCC. While the failure of these biomarkers was disappointing, it highlights the importance of bias control and the use of high-quality retrospective cohorts in biomarker studies.

A major limitation of studies III and IV is the use of simple prognostic modelling with dichotomous cut-offs for biomarker interpretation. Such an approach was mandated by the relatively low patient number and the use of TMA as opposed to whole sections, although it is also supported by previous experience with these biomarkers and immunohistochemistry. Nonetheless, future studies using more complex scoring systems, digital image analysis, or novel optimization of tumour stainings may provide other interpretations of these biomarkers.

6.5 The role of OCT4 in HNSCC (Study IV)

The second study investigates radiotherapy biomarkers in a microarray of patient-derived UT-SCC cell lines. Radiotherapy plays a major role in the treatment of HNSCC due to a wide genomic insult, the field cancerization phenomenon. Putative potential of OCT4 immunohistochemistry in radioresistance prediction was discovered. Previous studies have linked OCT4-related stemness characteristics to radioresistance in various cancers (Koo et al. 2015; Mishra et al. 2020; Shen et al. 2014; Ventelä et al. 2015; Xing et al. 2015). In addition, OCT4 has a disputed role as a biomarker for cisplatin sensitivity since results in testicular cancer and other solid tumours differ significantly (Gao et al. 2017; Tsai et al. 2011; de Vries et al. 2020). The obvious weakness of the second study is, however, the limited expression of OCT4 and expression in a minority of cells in HNSCC cell lines. Accordingly, analysis in the established PV-TMA was carried out.

The fourth study takes a more in-depth approach to the predictive role of OCT4 immunohistochemistry in radiotherapy and chemoradiotherapy stratification. Based on previous studies, including Studies II and III of the present thesis, the hypothesis was forwarded, that OCT4 would be linked to poor radiotherapy response, while a possible association with cisplatin sensitivity was acknowledged. Interestingly, in radiotherapy-treated patients OCT4 positivity was indeed a significant prognostic biomarker, while in patients treated with cisplatin-based chemoradiotherapy, there was no prognostic role with OCT4 immunohistochemistry.

The main finding of the fourth study is that simple OCT4 immunohistochemistry could be used for stratification of HNSCC patients between radiotherapy alone and concurrent cisplatin-based chemoradiotherapy (Figure 21). Since the lack of biomarkers for prediction of clinical benefit from the addition of cisplatin radiosensitization remains a crucial problem, this finding could potentially have a wide impact on the therapy selection in HNSCC. While cisplatin is the most prevalent chemotherapy in HNSCC, enthusiasm is reined in by the many potential toxicities, especially in frail and elderly patients.

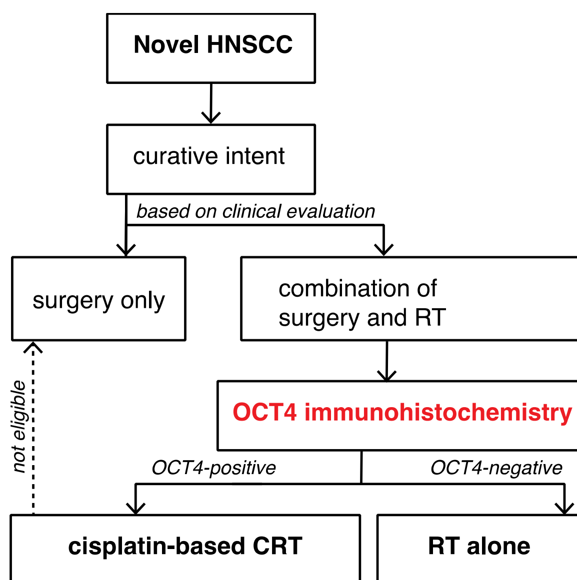


Figure 21. Proposed OCT4-based stratification for HNSCC. The decision for chemosensitization using cisplatin should be based on OCT4 immunohistochemical status, as OCT4-positive tumours benefit from cisplatin-based chemoradiotherapy (CRT). Surgical treatment should be preferred when patient is not eligible to even low-dose cisplatin therapy. Reproduced from study IV.

Regarding cisplatin toxicities, ototoxicity related to the use of cisplatin is a major concern since it is the most prevalent toxicity, and the concurrent occurrence of previous age-related hearing loss is often encountered in elderly patients. Improvements in hearing aid technology and especially cochlear implants have changed the picture of hearing rehabilitation in recent years. Thus, the refusal of cisplatin based on a risk for ototoxic damage must be weighed against the potential improvement in patient prognosis, especially since low-dose strategies are applied to increase cisplatin tolerance. Importantly, implantation can be accomplished even under local anaesthesia and due to improvements in electrode array technology, the traditionally enigmatic high-frequency hearing loss typically associated with cisplatin can well be rehabilitated using a hybrid electroacoustic fitting (Ryu et al. 2015). Despite this optimism, there is, however, one incidental report on cisplatin toxicity in a cochlear implant user, in which implant benefit was reduced by cisplatin (Harris et al. 2011). Mechanism for such damage is currently unknown.

Importantly, based on the findings of the fourth study, de-escalation of radiotherapy in OCT4-positive patients cannot be regarded safe. However, the increased potential for transoral robotic surgery in the treatment of oropharyngeal tumours may provide an option when full cisplatin-based chemoradiotherapy cannot be applied. Furthermore, in the fourth study, OCT4 positivity was associated with a favourable survival even in patients in whom the cisplatin was discontinued due to

toxicity, suggesting that even low-dose strategies might be beneficial in OCT4-positive HNSCC.

In addition to finding novel therapeutic approaches such as immunotherapies targeting PD-1 and PD-L1 checkpoint (*cf.* Section 2.2.2.2), finding biomarkers for identification of patients benefitting from existing therapies is an important focus of HNSCC research. The novel method of drug screening using fresh cancer tissue samples allows for a real-time stratification based on definite observed tumour cell responses (Mäkelä et al. 2020).

While the potential association between OCT4 and radiotherapy response can well be founded on the cancer stem cell hypothesis, previous results with clinical cohorts and in vitro studies have been conflicting (*cf.* Section 2.4.2.2). Thus, the role of OCT4 in radiosensitization and chemoresistance is not established and detection of OCT4 is not used in clinical stratification. The results of the fourth study, together with previous studies, suggest that the association of OCT4 and cisplatin response comes through targeting of OCT4-related DNA repair mechanisms.

6.6 Future perspectives

The clinical decision making in selection of HNSCC therapy cannot be guided by previous molecular biomarkers. In this thesis, the population validation approach was developed in response to the accumulating numbers of suggested molecular biomarkers, which have proven poorly reproducible. To guide the interpretation of molecular biomarkers, both a well-defined clinical context and an unbiased study cohort was deemed necessary. In the PV-TMA analysis, OCT4 immunohistochemistry was shown to be a promising therapy stratification biomarker for the crucial crossroad between radiotherapy and cisplatin-based chemoradiotherapy to improve the survival of HNSCC patients and to limit the harms of unnecessarily escalated therapy. On the other hand, improvements in the clinical prognostication using simple patient characteristics such as the current alcohol use may also prove beneficial in terms of improving the OS of HNSCC patients.

Evaluation of stemness using CIP2A and OCT4 has a promising outlook in the biomarker development of HNSCC. This important potential should be evaluated in rigorously recruited prospective cohorts treated with different radiotherapy regimes and cisplatin-based chemoradiotherapy. CIP2A is deemed a druggable target (De et al. 2014; Khanna et al. 2015), and thus the potential of anti-CIP2A drugs would be especially interesting to see in HNSCC therapy. The potential of OCT4 in predicting cisplatin response should be evaluated in cohorts of laryngeal HNSCC, since radiotherapy is often used without cisplatin in the curative treatment of low-stage primary tumours, while laryngeal HNSCC was almost ubiquitously OCT4-positive in the cohort of this thesis.

One of the most important repercussions of this thesis project have been the establishment of CMA technology at our institute. CMA of HNSCC cell lines is used to screen antibodies and expression levels as well as during the optimization of immunohistochemical staining procedures. Of at least equal importance is the extension of the PV-TMA of this project to an even larger HNSCC patient material, currently spanning from 2005 to 2015.

7 Conclusions

Based on the four studies of this thesis, robust methods, models, and workflows for the validation of biomarkers with a well-defined clinical application in HNSCC were identified. The research paradigm created and applied in this thesis were particularly promising in identification of radio- and chemosensitivity on an individual level. The four studies offer important lessons in terms of biomarker interpretation and emphasize a proper definition of the clinical problem-field prior to investigations.

The following conclusions are drawn from the results of the individual studies:

α. CIP2A copy number analysis improves the prognostic resolution of CIP2A immunohistochemistry in HNSCC.

β. CMA is a powerful method for simultaneous assessment of multiple cell lines and can be used for correlation with intrinsic radioresistance as well as other cell line characteristics.

γ. PV-TMA together with multivariable modelling allows for reliable prognostic analysis assessment in HNSCC.

δ. OCT4 has a definite predictive role in HNSCC radiosensitivity and cisplatin radiosensitization benefit, which could be translated to future patient stratification studies.

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The purpose of this thesis project has been to develop and apply my understanding of head and neck squamous cell carcinoma in the search for molecular biomarkers. This understanding has developed gradually over several years with growing clinical expertise, increasing intimacy with scientific method, and discussions with various colleagues. For this, I wish to express my gratitude for all my clinical and scientific colleagues over the years.

The project was started in 2014, while I was working at the Jukka Westermarck lab at the Centre for Biotechnology, a joint venture of the University of Turku and Åbo Akademi university, during alternative civilian service. Since 2016, I have been affiliated with the Department of Otorhinolaryngology – Head and Neck Surgery at the University of Turku and Turku University Hospital.

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