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Pään ja kaulan alueen levyepiteelisyöpä on yksi yleisimmistä syöpätyypeistä, muta sillä on silti yhä verrattain huono ennuste muihin yleisiin syöpätyyppeihin verrattuna. Hoitosuunnitelma ja ennuste määräytyvät pääasiassa syövän tarkemman anatomisen sijainnin sekä TNM-luokituksen perusteella. Sädehoito on yksi yleinen hoitomuoto, mutta ongelmana on syöpien vaihteleva herkkyys sädehoidolle. Prekliinisissä tutkimuksissa ennusteen ja sädeherkkyyden on todettu korreloivan syöpäsolujen tiettyjen biokemiallisten markkereiden ilmentymiseen. Näitä ovat muun muassa tuumorisuppressori PP2A:n toimintaan vaikuttavat endogeeniset proteiinit CIP2A, PME-1 sekä SET. Tutkimuksessa selvitettiin näiden kolmen proteiinin ilmentymistä pään ja kaulan alueen syövissä solumikrosirun avulla ja tavoitteena oli löytää uusia hoitovastetta ennustavia tekijöitä.

Solumikrosiru kehitettiin yhdistämällä 30 pään ja kaulan alueen levyepiteelikarsinooman solulinjan solumateriaalia yhteen yksittäiseen parafiiniblokkiin. Solulinjat ovat peräisin eri pään ja kaulan alueilta ja osassa solulinjoista CIP2A:n ilmentyminen oli hiljennetty shRNA:n avulla. Suuri määrä kasvatettuja soluja kerättiin ja fiksoitiin formaliinilla, jonka jälkeen nämä sulautettiin parafiiniin. Kaikkien solulinjojen fiksoidut solulieriöt sisälsivät vain solumateriaalia ja ne liitettiin samaan parafiiniblokkiin niin että muodostui yhtenäinen solumikrosiru. Mikrosiruun liitettiin kontrolliksi lisäksi kuutta eri hiirikudosmateriaalia. Mikrosirusta leikatut leikkeet värjättiin immunohistokemiallisin menetelmin. Värjäyksiin käytettiin CIP2A-, PME-1- sekä SET-vasta-aineita. Värjäykset pisteytettiin kvantitatiivisen pisteytysmenetelmän mukaisesti arvioimalla mikroskoopilla manuaalisesti värjäytyneiden solujen määrää sekä värjäyksen intensiteettiä.

Projektissa kehitimme uuden työkalun, jolla solujen proteiinien ekspressio voidaan selvittää immunohistokemiallisesti tehokkaasti suuresta määrästä eri solulinjoja yksittäisellä värjäyksellä. Tällä menetelmällä immunohistokemiallisia värjäyksiä voidaan suorittaa pelkälle fiksoidulle solumateriaalille ilman solunulkoista komponenttia ja tuottaa luotettavan sekä laadukkaasti tulkittavan värjäystuloksen. Värjäysten perusteella CIP2A:n, PME-1:n sekä SET:n ilmentyminen vaihteli eri solulinjoissa, ja CIP2A-shRNA solulinjoissa CIP2A:n ilmentyminen oli merkittävästi alentunut, mikä viittaa onnistuneeseen CIP2A:n hiljentymiseen sekä metodin kvantitatiivisuuteen. Tutkimuksemme perusteella solumikrosiru on luotettava työkalu proteiinien ilmentymisen määrittämiseksi immunohistokemiallisesti suuresta solunäytemäärästä, ja se voisi tulevaisuudessa olla varteenotettava vaihtoehto kudosmikrosirun käytölle.

Avainsanat: solumikrosiru, ennustetekijä, sädeherkkyys

Contents

1. Introduction

1.1 Development and risk factors of cancer

Cancer cells differ distinctly from the normal cells of the human body. Various features have been identified, which enable cancer growth and metastasis. A succession of random mutations caused by genomic instability enable cells to acquire the characteristic hallmarks of cancer such as replicative immortality, sustaining of proliferative signaling and avoidance of programmed cell death. Sustained angiogenesis is vital for the cancer cells to maintain their increasing metabolism and growth, especially as the tumor keeps on growing in size as the cancer cells multiply. Locally invasive and metastasized cancer carries a poor patient prognosis. Complex changes in protein expressions, for example integrins, have their impact on cells improved tethering to adjacent tissues, promoting the invasive features. Loss of cell-to-cell adhesion molecules, such as E-cadherin, is often witnessed in carcinoma cells. The invasive properties of tumor often require more aggressive treatments and predict poor prognosis. By entering the blood or lymphatic circulation, cells develop their ability to specifically colonize other tissues, enabling metastasization. (Hanahan and Weinberg 2000, Hanahan and Weinberg 2011)

The rate of spontaneous mutations is increased by being repeatedly exposed to mutagenic agents such as radiation, tobacco smoke or alcohol, leading to compromised functioning of the genomic maintenance system. These carcinogens contribute to cancer development by either damaging DNA and thereby inducing mutations or promoting cell proliferation. Some hormones act by the same principle, stimulating the proliferation of target cells, thereby contributing to cancer development. Certain viruses are also oncogenic, such as human papilloma virus (HPV) in cervical carcinoma and, as recently identified, oropharyngeal squamous cell carcinoma (OPSCC). Defects which expose the cells to genomic mutations include flawed caretaker genes or the acquisition of oncogenic abilities. The genomic alterations in cancer cells translate to increase or decrease in the expression of various oncogenic proteins or tumor suppressor mechanisms, respectively, thereby promoting the progression and growth of tumor. (Hanahan and Weinberg 2000, Hanahan and Weinberg 2011)

The role of tumor suppressor genes has recently been recognized as an important focus of cancer research, since impaired tumor suppressor functioning is witnessed in many types of cancer. Protein phosphatase 2A (PP2A) has been identified as one of the tumor suppressors in eukaryotic cells, where it covers for a major amount of the serine-threonine phosphatase activity. PP2A is formed of three variable subunits to form a heterotrimeric complex. Several

of these PP2A subunits have been identified as tumor suppressor proteins. PP2A regulates the activity of various oncogenic signaling pathways, and the PP2A-mediated abnormal cell growth is caused by several mechanisms. Mutations or deletions of PP2A subunits, as well as inhibition of PP2A by endogenic protein inhibitors, may have drastic effects on cells protumor/antitumor balance. PP2A and its functioning has been studied and its association to development of malignancy has been demonstrated in various cancers. (Janssens et al. 2005, Westermarck and Hahn 2008)

1.2 Epidemiology and treatment of head and neck cancer

Head and neck cancer is one the most common cancers worldwide, and squamous cell carcinomas cover over 90 % of all head and neck cancers. Yet, head and neck squamous cell carcinoma (HNSCC) patients have only an approximately 65 % five-year overall survival rate, which is a significantly lower rate compared to other common cancer types such as colorectal and breast cancer (Jemal et al. 2008). HNSCC consists of a heterogeneous group of malignancies, which are further categorized to their exact anatomical location. The incidence and behavior of HNSCC varies substantially according to the anatomical site of the tumor, the most common ones globally being cancer of the larynx, oropharynx and oral cavity. (Gupta et al. 2016) However, the incidence rates vary substantially in different countries. The major risk factors and primary causes of HNSCC are tobacco use and alcohol consumption. Other notable risk factors for HNSCC include older age, male gender and occupational exposures. Low socioeconomical and educational status also correlate with an increased incidence of the disease. There is also a new rising entity of HNSCCs, as majority of oropharyngeal squamous cell carcinomas (OPSCCs) have been found to be related to human papilloma virus (HPV) infection. HPV infection has established its place as a contributing factor at least in OPSCC, whereas the same kind of evidence is still lacking for other types of HNSCC. On the other hand, studies have shown HPV positivity to predict for a more favorable prognosis in OPSCC. (Lewis et al. 2015)

The incidence of HPV -positive HNSCC is on the rise, while the HPV -negative cases are decreasing due to rarification of tobacco use. This increase has particularly been shown in oropharyngeal squamous cell carcinomas and in patients with little exposure to alcohol and tobacco. These cancer types represent a unique cancer entity. (Lewis et al. 2015) There has been shown correlation between HPV infection and overexpression of p16 in HNSCC, while also the tumors overexpressing p16 having a better prognosis (Grønhøj Larsen et al. 2014). Considering the prognosis, HPV positive tumor status predicts a better overall survival and superior therapy response. In the last two decades, the overall incidence and mortality of

HNSSC have been decreasing, while the survival rate remain poor. (Kim et al. 2010, Rettig and D'Souza 2015)

Currently the treatment plan for HNSCC patient is mainly chosen based on the primary anatomical site and extension of tumor assessed by TNM staging. According to these, and patient related factors, the chosen treatment method is either surgery, radiotherapy (RT), chemosensitization in an individually planned combination (Grégoire et al. 2010). Major advances have been made considering RT, mostly enabled by significant technical progress and increased knowledge of the molecular mechanisms behind tumors and normal tissues sensitivity to radiation. Intensity-modulated radiotherapy (IMRT) is one the major advancements by reducing radiation side effects and needed radiation doses in certain treatment procedures. IMRT enables extremely precise targeting of the radiation dose to target tumor site by modulating the intensity of radiation beams by applying the total dose as multiple smaller portions while using magnetic resonance (MRI) or 3-D computed tomography (CT) imaging to navigate the dose calculations. With chemo-sensitization on the other hand the tumor cells are made more susceptible for radiation therapy by using specific chemical compounds or drugs. These improvements in RT have led to improved control of locoregional disease and prognosis, while also the unfavorable effects of RT diminishing and patient's quality of life improving (Grégoire et al. 2015). More tailored treatments provided by state-of-the-art imaging and delivery techniques with advancements in surgery techniques lighten up the future perspectives of HNSCC patients. (Hegde et al. 2017) Even better integration of molecular imaging to RT will probably even more enhance the treatment procedures. For these advancements to be implemented to their full potential, the nature of radioresistance in HNSCC tumors needs to be fully understood.

Copious attempts have been made to clarify the cause of HNSCC's aggressiveness and poor patient survival. A major problem concerning the recurrence of HNSCC is that currently no factor with sufficient prognostic or predictive resolution exist to assess patient response to treatment. The importance of potential prognostic factors has been noted and numerous attempts have been made to correlate different molecular markers and the prognosis of HNSCC patients. (Dahiya and Dhankhar 2016, Dunkel et al. 2013, Heß et al. 2016, Lampri et al. 2015, Routila et al. 2015) Molecular markers could potentially be used to aid the treatment process, by serving as prognostic or predictive tools. By choosing a specific treatment for the individual patient according to the genetic and molecular profile of the tumor, potentially harmful operations could be avoided, while improving treatment response and survival outcomes.

1.3 Structure and function of Protein phosphatase 2A (PP2A)

Tumor suppressor proteins and oncoproteins have been studied and several different candidate genes and proteins have been identified as being related to cancers aggressiveness, poor patient survival and capability of resisting cancer treatment. Kinases and phosphatases are vital for signal transduction in human cells, transmitting information through reversible phosphorylation of various proteins. Aberration of this system may lead to various pathological conditions, including development of malignancies or various other diseases. PP2A covers for a family of serine-threonine phosphatases, consisting of 3 different subunits, some of which act as tumor suppressors by inhibiting cancer initiation and progression. These subunits have at least two different isoforms, which leads to multiple possible holoenzyme compositions, allowing extremely flexible and dynamic functioning of PP2A. (Janssens et al. 2005)

With PP1, PP2A accounts for 90% of all serine/threonine activity in cells. PP2A acts in a variety of crucial cell signaling pathways including those that regulate, in addition to cell cycle, metabolism, survival and migration of cells. By losing the specific and vital PP2A holoenzymes, cells are driven into imminent pathogenic transformation. This may occur through destabilization of tumor suppressors, upregulation of protein kinases taking part in mitogenic and survival signaling, loss of proapoptotic signaling pathways or the stabilization of protoncogenes. (Eichhorn et al. 2009)

The subunits of PP2A are vital for its unique properties. The canonical heterotrimeric complex is composed of structural subunit A (PR65/PPP2R1), catalytic subunit C (PP2AC/PPP2C) and one of various possible regulatory B subunits. Since each subunit has several possible isoforms, there are multiple possible holoenzyme compositions. The core structure of PP2A involves the A-C core dimer complex, which may exist independently or by binding to the B subunit, being the third variable subunit. B subunits originate from 4 distinct genetic families, named as: the B/PR55 family, the B'/PR61 family, the B"/PR72 family and the PTP/PR53 family. At least 26 alternative splicing forms and transcripts have been identified, which are encoded by at least 15 different genes. The substrate specificity and localization of PP2A complex is determined by the suitable B, Aα/ Aβ and Cα/ Cβ in various circumstances, depending on the context and tissue specificity of PP2As needed function. Multiple components of PP2A complex act as tumor suppressors including PR65 Aα and Aβ as well as some of the B subunits. (Eichhorn et al. 2009, Janssens et al. 2005, Westermarck and Hahn 2008) The tumor suppressor activities of PP2A A subunits vary. The two different

encoded PP2A A isoforms (Aα and Aβ) are structurally similar, yet cancer-associated mutations in Aα lead to haploinsufficiency and increased phosphorylation of Akt, whereas mutations in Aβ typically involve both alleles and lead to a total loss of function of the Aβ-formed PP2A complexes. The B subunit also seems to be in a vital role considering the cancer promoting transformation of PP2A activity. All the B subunits have a major part in dysregulating intracellular pathways, emphasizing the importance of these enzyme components. All the B subunit families have a major part in PP2A function in vital cellular pathways, which are virtually always dysregulated in cancer.

PP2A acts as tumor suppressor in various signaling pathways. Specific holoenzymes negatively regulate MAP kinase pathways through ERK, preventing ERK activation. PP2A also suppresses cell survival through decreased AKT activation and cell migration by growth factor and adhesion receptor assembly regulation through RACK1. (Kiely and Kiely 2015) Some viral proteins can form complexes with PP2A core dimer and thereby disrupt the phosphatase function. Simian virus 40 Early Region small t antigen (ST) may cause the displacement of at least B and B' subunits and bind to the core dimer, thereby causing the respective holoenzyme to lose its specific dephosphorylating ability. The effect on the regulation of PP2A is either activating or inhibiting. A major role of PP2A has been proven to be its role in regulating RAF-MEK-ERK pathway, in which PP2A can work either in a positive or negative role regarding the proceeding of signaling into transcriptional activation. Here PP2A acts by diversely inhibiting the proteins participating in the pathway system. The outcome seems to largely depend on stimulus and the designated cell type. Reports also show PP2A having a similar two-way functioning feature in the regulation of WNT signaling, in which it has a crucial role impacting embryogenesis and cellular proliferation regulation in mature tissues. (Eichhorn et al. 2009)

1.4 Endogenous inhibitors of PP2A: CIP2A, PME-1 and SET

Several endogenous proteins have been shown to directly interact with PP2A, most of which are linked to specific B subunits. The mechanism of endogenous inhibitors makes PP2A an especially interesting target for cancer suppression due to the therapeutic potential of restoring PP2A function by therapeutic inhibition of these endogenous inhibitors with antibodies or small molecular interference. It is shown that along with genetic mutations of PP2A, overexpression of these specific endogenous proteins inhibits the cancer prohibitive activity of PP2A (Westermarck and Hahn 2008). CIP2A (cancerous inhibitor of PP2A) was first introduced in 2002, as auto-antibodies of CIP2A were identified and its full-length cDNA

cloned (Soo Hoo 2002). Since then CIP2A has been identified as one of the endogenous suppressors of PP2A activity, by inhibiting the catalytic activity of PP2Ac, and thereby promoting the growth of malignant cells with c-Myc driven gene expression. Data suggests that CIP2A binds to c-Myc directly and stabilizes Myc protein by inhibiting PP2A-mediated dephosphorylation. Findings indicate that one PP2A tumor suppressor mechanism is the ability to dephosphorylate Myc S62.

Recently there has been proposed a link between CIP2A and HPV -infection. HPV oncoprotein E7 has been proposed to regulate CIP2A, thereby mediating cell proliferation. HPV-16E7 protein was shown to upregulate CIP2A mRNA levels and the process to be dependent on retinoblastoma protein pRb, while the regulation of Cyclin-dependent kinases (CDKs) via CIP2A would depend of E2F1. According to these findings, impaired function of CIP2A might downregulate CDKs and thereby decrease cell proliferation through E2F1 in cells expressing E7. (Zhang et al. 2015) It was also demonstrated in cervical cancer cells, that there is a positive feedback between CIP2A and E2F1 regulated by HPV E7, which was shown as increased E2F1 mRNA expression (Wang et al. 2017). Similar findings have alsi been reported in breast cancer cells, where the feedback mechanism between CIP2A and E2F1 was demonstrated to induce cell senescence. (Laine et al. 2013) These results imply that the cross-talk between CIP2A and E2F1 is not a singular finding and that the feedback could be seen in diverse types of cancers. In addition, double-positive co-expression of CIP2A-E2F1 was shown to predict poor prognosis of the cervical cancer patients. (Wang et al. 2017)

In general, PP2A is in charge of regulating multiple specific substrates by dephosphorylation which act as cell transformations suppressors, at least considering the PP2A Aα-C-B56γ complexes. Malfunction of these complexes leads to activation of Akt signaling pathway, implying this pathway being crucial for cell transformation. PP2A is also believed to regulate p53, another important human tumor suppressor, as findings suggest that PP2A inactivation disturbs the activation of p53. Precise underlying mechanisms of PP2A-p53 co-operation is yet unclear. Furthermore, PP2A signaling is associated together also with Ras signaling, as both are often dysfunctional in cell transformation, with shared signaling passages. Due to the stated features, CIP2A has been proposed as a potential therapeutic target in cancer treatment, since by inhibiting CIP2A activity, targeted degradation of c-Myc in cancer cells could be induced. (Junttila et al. 2007, Westermarck and Hahn 2008) Also since CIP2A is not expressed in any normal human tissues in notable levels except in the testicular tissue (Ventelä et al. 2012), there is promising potential of avoiding unwanted side effects with a possible CIP2A -targeting drug.

Prior to CIP2A, other endogenous inhibitors have also been found to regulate PP2A activity, including for example I2PP2A/SET and PME-1. SET works as a non-competitive inhibitor of PP2A and its overexpression has been shown in several types of human cancer, including the tumors of head and neck region. Its characteristics were described in 1995 as a novel heat-stable protein inhibitor alongside with I1PP2A. (Li et al. 1995) Inhibition of PP2A by SET stimulates both BCR-ABL-mediated signaling and MEK-ERK-MAPK pathways activity. In fact, these MAPK (mitogen-activated protein kinase) signaling pathways are essential for the tumor suppressor activity of PP2A. Expression of SET also correlated with tumor growth, as BCR-ABL-expressing cells demonstrated cancer progression and enhanced BCR-ABL stability through SET driven PP2A inhibition. SET was also shown to stimulate c-Jun phosphorylation. (Westermarck and Hahn 2008) The exact role of SET is still unknown, though some studies of its function have been published. The pro-oncogenic role of SET has been shown especially in gynecologic cancers, being manifested as increased expression levels and tumorigenesis as well as cancer progression. SET has been demonstrated to function via both PP2A-dependent and PP2A-independent pathways. Significance of SET has been shown in other diseases as well in addition to human malignancies, such as polycystic ovary syndrome and Alzheimer disease. (Jiang et al. 2017) Relevance of SET has also been studied in HNSCC cell lines by using stable SET knockdown with shRNA. SET was shown to have oncogenic features as promoting cell survival and proliferation, while also regulating invasion and suppressing metastasis. (Sobral et al. 2014)

Protein phosphatase methylesterase-1 (PME-1) has been shown to regulate PP2A through different pathways and modes. It was first described in 1996 as the first eukaryotic carboxylmethylesterase. (Lee et al. 1996) PME-1 acts by various distinct mechanisms. Firstly, PME-1 may act by demethylating the C-terminal leucine, which methylation is induced by leucine carboxyl methyltransferase (LCMT). Reversible methylation appears to be crucial in regulating binding of B subunits to the enzyme complex, and thereby demethylation regulates the substrate specificity of PP2A. In addition, PME-1 may bind directly to PP2As active site and inhibit the catalytic activity of PP2A. PME-1 may also stabilize PP2A, protecting it from proteasome degradation. PME-1 has been shown to promote oncogenic activity, by regulating MAPK/ERK signaling through PP2A in human gliomas. Furthermore, PME-1 inhibiting PP2A promotes ERK and AKT signaling with increased cell proliferation in endometrial cancer cells. Mostly PME-1 has been shown to support anchorage-independent growth. However, in colorectal cancer PME-1 silencing has not shown to inhibit AKT and ERK pathway signaling or having an impact on cancer cell viability. Increased expression of PME-

1 and amplification of PPME-1 gene has been shown in various cancer types. In most cases, high PME-1 expression correlated with worse patient prognosis, whereas in colorectal cancer it had the opposite effect. In glioma tumors, PME-1 expression is associated with tumors histologic grading in grades II-IV. In type I endometrioid adenocarcinoma PME-1 expression was increased in comparison to normal endometrioid tissue. (Janssens et all 2005, Kaur and Westermarck 2016)

In some cancer cells treatment with non-selective PP2A inhibitors have been shown to promote cell apoptosis and increase drug sensitivity (Kiely and Kiely 2015). However, there is evidence that PP2A activity could be reactivated with small molecules, since inhibition of SET and CIP2A in certain cancers have been shown to restore the tumor suppressor activities of PP2A. (Westermarck and Hahn 2008)

1.5 CIP2A as a prognostic factor in cancer

The role of CIP2A has been proven essential in diverse human malignancies. For instance, CIP2A has been shown to be overexpressed in various cancer types and its overexpression correlates to poor patient prognosis. (Cristóbal et al. 2017, Katz et al. 2010, Khanna et al. 2013, Routila et al. 2015) In addition, relevance of CIP2A as a clinical molecular marker has been shown in malignancies of the salivary gland (Routila et al. 2016) and association with malignant features in laryngeal HNSCC cells. (Junttila et al. 2007, Chen et al. 2017) Therapeutic potential of chemical agents which restore PP2A activity via CIP2A has also been demonstrated for example in triple negative breast cancer, through cytotoxicity and inhibition of metastasis caused by novel STAT3 inhibitor. (Huang et al. 2017) In most of studies, majority of CIP2A immunoactivity has been documented in the cytoplasm.

1.6 Radioresistance and the role of biomarkers in head and neck cancer

In this project, we used various UT-SCC cell lines. These squamous cell carcinoma cell lines have been originally derived from head and neck cancer patients (Grenman et al. 1988, Grénman et al. 1991). The various characteristics of established cell lines have been determined since then, including their sensitivity to radiation, this being specified as radioresistance (Grénman et al. 1991). Cancer cells may develop radioresistance through genomic mutations, thereby weakening the treatment response to radiation therapy. Radioresistant HNSCC tumors weaken treatment response and narrow down treatment options, with significantly weakening the patient's prognosis especially in locally advanced HNSCC. This thereby forms a prominent clinical problem in patients with locally advanced

HNSCC, narrowing down possible treatment options, requiring often a more aggressive surgical treatment for optimal locoregional control. Due to the anatomical location of HNSCC, the treatment procedures cause a variety of troublesome symptoms for the patient (Bressan et al. 2016), thereby having a negative effect on the quality of life of patients. This emphasizes the importance in holding on to as minimal treatment methods as possible. Association between radioresistance and various components of intracellular signaling pathways have been described, showing the crucial role of for example wild-type p53 function and inhibition of PI3K/Akt/mTOR signaling (Perri et al. 2015). Biomarkers for radioresistance have been studied, and there has been shown prognostic and predictive features by several products of gene expressions (Akervall et al. 2014).

1.7 CIP2A and Oct4 as prognostic and predictive factors in head and neck cancer

CIP2A coexpression with Oct4 has been shown to relate with increased radioresistance in HNSCC (Ventelä et al. 2015). Oct4 was introduced almost 30 years ago as an important and active protein in early mouse development, named after its function in binding the octamer motif (Schöler et al. 1989). Since then Oct4 has proven its importance as a key stemness marker, acting as an essential transcriptional factor while controlling early mammalian embryogenesis. Oct4 is crucial in many cases to enable pluripotency in immortal cells, therefore it is not utterly surprising that it has role in the development of cancer as well. In many cancer types, overexpression of Oct4 has predicted tumorigenicity, metastasis or distant recurrence after chemoradiotherapy. Oct4 is vital for cancer cells survival, thereby the Oct4 expressing cells tend to persist after chemo- or radiotherapy. Oct4 has also been demonstrated to re-express in cancer stem cells in cancer recidives. (Zeineddine et al. 2014) It was seen that CIP2A and Oct4 were co-expressed in same HNSCC cell lines and data implicated that CIP2A expression is regulated by Oct4. Alone Oct4 positivity was linked to decreased tumor differentiation and increased radioresistance while CIP2A indicates poor patient survival in HNSCC. (Ventelä et al. 2015) It is becoming clear, that association between individual biomarkers and radioresistance is only minor even at its finest, and thereby further screening with widespread characterization of the malignancies cellular environment and gene expression.

By using HNSCC cell lines, individual studies have been done to show the correlation of radioresistance and expression of also cell migration-related proteins, by carrying out the experiments on a small-scale stance (Skvortsov et al. 2011). By using these specific cell lines, we aimed to cover the large variety of quite heterogenous HNSCC, while having the

information of their features, and thereby being able to make the conclusions between various factors based on large-scale analysis.

1.8 Significance of TMA and its new cell-based implementations

Tissue microarray (TMA) has become established as a routine method for high-throughput method screening tissue samples on either DNA, RNA or protein level. TMA methods have evolved rapidly over the years, and they have been proven as excellent tools for profiling tumor samples and thereby allowing efficient screening of the genomic features of cancer cells (Kallioniemi et al. 2001, Kononen et al. 1998). Methods for creating cell-based microarrays have also been introduced, including purely the cell component without the interfering extracellular matrix component (Ferrer et al. 2005, Waterworth et al. 2005). This technique is still highly overlooked compared to TMAs. Fixed forms of cell microarrays are most commonly used, but also living cell microarrays of different types are becoming serious contenders in studying cells microenvironmental processes and cell signaling (Jonczyk et al. 2016). Despite TMAs established role as the characterization method of human malignancies, we believe that the form of CMA could bring a robust effect to tumor characterization as an additional screening method. By using merely cell lines we can practically eliminate the contributing effect of extracellular matrix. By using established cell lines as TMA samples, we also know beforehand the cancer cells characteristics, in contrast to harvesting data from tumor samples, which especially in HNSCC are very heterogenous by nature and vary considering their cells microenvironment. Use of cell lines also opens numerous possibilities for implementing new techniques also to TMA diagnostics. The cell lines can be cultured in various conditions before CMA construction. Cell lines gene product expression may be altered in various different methods, for example shRNA or siRNA treatment to knock-out or reduce gene expression, and then verify alterations to gene expressions of various genes of interest. Gene expression analysis through identification of protein product by different IHC stainings is after all a very cost-efficient, simple and fast way to describe gene expressions. The aim of this project was to create a CMA using head and neck cancer cell lines, which could be used to efficiently find factors which correlate with patient response to treatment. This microarray could be used to identify the prognostic factors, in order to select the relevant treatment method for head and neck cancer patients. There is a demand for high-throughput method for screening the protein expression of a large number of cells to demonstrate the correlation of protein expression in specific cell lines, depending on the characteristics of these cell lines and their unique features.

In this study we constructed a cell microarray (CMA) using established, formalin-fixed and paraffin embedded HNSCC cell lines. As a proof of concept, immunohistochemical (IHC) stainings of three endogenous PP2A inhibitory proteins SET, CIP2A and PME-1 were carried out.

2. Materials and methods

2.1 Head and neck squamous cell carcinoma (UT-SCC) cell lines and mouse tissue samples

The cell line array was composed of 30 different human-derived HNSCC cell lines, including 4 short hairpin silenced cell lines. Cell lines were obtained from our collaboration partners Reidar Grénman and Tove Grönroos. These cell lines were established using tissue culture methods and nude mice from patient samples with various kinds of SCC of the head and neck region. (Krause et al. 1981) Origin of the cell lines vary depending on the location of tumor and samples tumor site being primary or metastasis. Cell lines designated with the same numerical marking followed by A or B represent their origin being from the same patient. The clinical course of every donor patient's disease was monitored and clinical data from patient was gathered (Grenman et al. 1988, Grénman et al. 1991). Previously established FaDU cell line derived from hypopharyngeal grade II squamous cell carcinoma was a kind gift of Tove Grönroos (Rangan 1972). Cell lines have previously been characterized by for example histological evaluations and radioresistance measurements (Grenman et al. 1988). Radioresistance has previously been assessed using 96-well clonogenic cell survival assay and is reported as area under curve (AUC), determined by comparing the cells surviving fraction to the used irradiation dose (Gy) given to plated cells, illustrated by radiation survival curves. AUC was used to measure the mean inactivation dose. The cell lines characteristics are described in Table 1. Cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM), with 10 % FCS, glutamine and antibiotics (penicillin and streptomycin). Cells were constantly being held at a density between (1×10^5 and 10^7) cells/ml. Mouse tumor samples were added to construction of TMA for CIP2A staining specificity analysis. Samples were previously harvested from 3 wild type mice and 3 CIP2A knock-out mice formalin-fixed and paraffin-embedded. Mouse tumor characteristics are described in Table 2.

Table 1. Characteristics of cell lines composed in CMA.

Cell line	Patient sex	Diagnosis age	Primary tumor site	T	N	M	Specimen site	Type	Grade	Growth in mice	Radioresistance, AUC
UT-SCC-2	m	60	floor of mouth	4	1	0	floor of mouth	pri	2	+	1.8 ± 0.2
UT-SCC-5	m	58	tongue	1	1	0	tongue	pri (per)	2	+	2.3 ± 0.3
UT-SCC-7	m	67	temporal skin	1	0	0	neck	met	2	+	2.0 ± 0.2
UT-SCC-8	m	42	supraglottic	2	0	0	larynx	pri	1	+	1.9 ± 0.1
UT-SCC-9	m	81	glottic	2	1	0	neck	met	1	+	1.4 ± 0.1
UT-SCC-14	m	25	tongue	3	1	0	tongue	pri (per)	2	+	1.7 ± 0.3
sh CIP2A.557 UT-SCC-14	m	25	tongue	3	1	0	tongue	pri (per)	2	+	1.7 ± 0.3
UT-SCC-16A	f	77	tongue	3	0	0	tongue	pri	3	-	1.8 ± 0.1
UT-SCC-17	m	65	supraglottic	2	0	0	sternum	met	3	N/A	1.8. ± 0.1
UT-SCC-19A	m	44	glottic	4	0	0	larynx	pri	2	+	1.7 ± 0.1
UT-SCC-20A	f	58	floor of mouth	1	0	0	floor of mouth	pri (per)	2	N/A	2.1 ± 0.2
sh GAPDH UT-SCC-24A	m	41	tongue	2	0	0	tongue	pri	2	-	2.6 ± 0.3
sh CIP2A.557 UT-SCC-24A	m	41	tongue	2	0	0	tongue	pri	2	-	2.6 ± 0.3
sh CIP2A.556 UT-SCC-24A	m	41	tongue	2	0	0	tongue	pri	2	-	2.6 ± 0.3
UT-SCC-25	m	50	tongue	2	0	0	tongue	rec	1	N/A	N/A
UT-SCC-30	f	77	tongue	3	1	0	tongue	pri	1	N/A	2.0 ± 0.1
UT-SCC-32	m	66	tongue	3	0	0	tongue	pri (per)	2	scid + -	1.7 ± 0.3
UT-SCC-34	m	63	supraglottic	4	0	0	larynx	pri	1	N/A	2.0 ± 0.1
UT-SCC-36	m	46	floor of mouth	4	1	0	floor of mouth	pri	3	-	2.2 ± 0.2
UT-SCC-45	m	76	floor of mouth	3	1	0	floor of mouth	pri	3	+, scid ++	2.0 ± 0.1
UT-SCC-46A	m	62	(retromolar) gingiva (of maxilla)	1	0	0	gingiva	pri	3	(+)	1.6 ± 0.1
UT-SCC-47	m	78	floor of mouth	2	0	0	floor of mouth	pri	3	-	2.0 ± 0.2
UT-SCC-50	m	70	glottic	2	0	0	larynx (rT2N0)	rec	3	scid -	N/A
UT-SCC-60B	m	59	tonsil	4	1	0	neck	met (per)	1	N/A	2.2 ± 0.3
UT-SCC-72	m	50	gingiva (of mandibula)	4	2	0	gingiva	pri	2	N/A	N/A
UT-SCC-74A	m	31	tongue	3	1	0	tongue	pri	2	N/A	N/A
UT-SCC-76A	m	52	tongue	3	0	0	tongue	pri	2	N/A	2.5 ± 0.2
UT-SCC-79A	f	80	parotid (primary was T2N0M0G2)	0	2	0	parotid	met	2	N/A	2.4 ± 0.2
UT-SCC-79B	f	80	parotid (primary was T2N0M0G2)	0	2	0	neck	met	2	N/A	2.5 ± 0.1
FaDU	m	56	hypopharynx	N/A	N/A	N/A	hypopharynx	pri	2	N/A	N/A

Table 2. Mouse mammary tumor samples used in CMA.

Tumor sample number	**CIP2A status**
131	WT
562	WT
573	WT
527	CIP2A KO
541	CIP2A KO
195	CIP2A KO

2.2 Fixation and paraffin embedding of cell lines

A large number of cells was used (2x T-175 $cm^2 \approx 40 \times 10^6$ cells) to acquire a big enough pellet to ease the processing of pellet and to reach tall enough pellet enabling acquisition of plentiful amount of cut sections in the later stage. Cells were harvested by trypsinization and then pelleted. Pellets were washed with PBS and then resuspended in (120-160 microliter) 10% of neutral-buffered formalin. Cells were then added into a microfuge tube containing a base made of 2% agarose in PBS. A 15 ml conical tubes were used as an adaptor, while pelleting cells in microfuge tube by using a swinging bucket rotor centrifuge (1000 rpm x 5 min) therefore keeping the pellet flat. After the spin the supernatant was removed, and buffered formalin was added. Cells were centrifuged again (1000 rpm x 4 min) to ensure pellet being thick and compact. Microfuge tube was emerged into a conical containing 10 ml buffered formalin. Cell pellets were fixed for 48h, after which they were stored in PBS (+4°C). Microfuge tubes were cut open, thereby removing the pellet and moving it into a tissue cassette which was then submitted for paraffin embedding.

2.3 Assembling the cell microarray (CMA)

A microarray was assembled by using the services of Auria Biobank. The formalin-fixed, paraffin-embedded cell pellets and mouse tumor samples were cut to 6 µm sections and hematoxylin-eosin stained. The slides were scanned and annotated for duplicate 0,6 mm cores using Panoramic Viewer software. The annotated cores were combined into duplicate receiver blocks using TMArrayer. Samples from normal human liver were included for orientation.

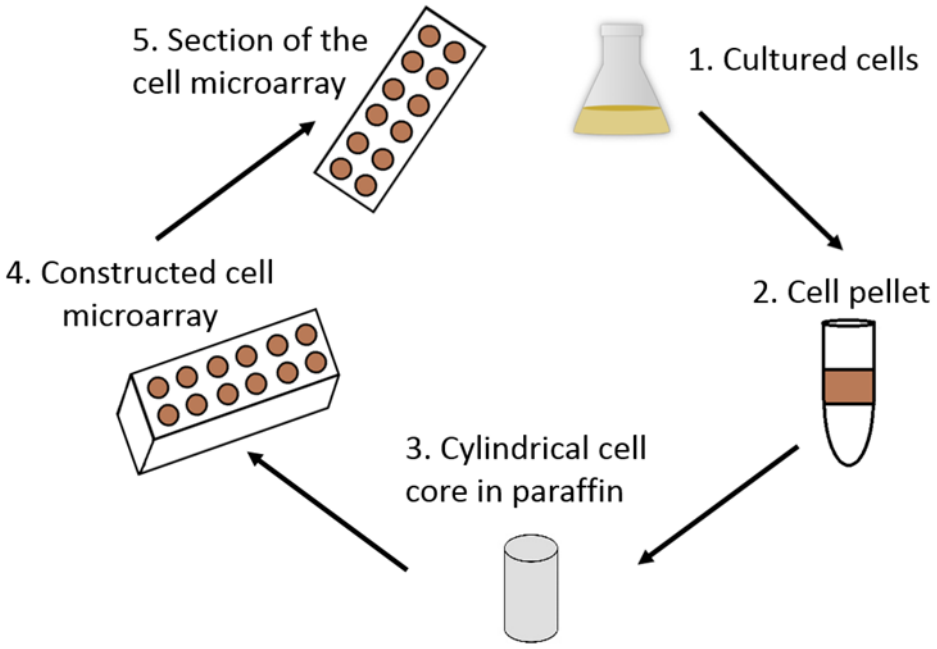

Figure 1. Schematic presentation of the construction of CMA. Cores from each cell line and mice tissue where combined into a single paraffin block by melting them together, forming a single array.

Table 3. CMA map showing the arrangement of all samples in CMA block. Liver samples were added as arrays internal control. CIP2A KO and WT tumor samples are mouse tumor samples described in table 2. Cell line samples (UT-SCC) are described in Table 1.

	X1	X2	X3	X4	X5	X6
Y1		UT-SCC-46A	UT-SCC-16A	UT-SCC-34	UT-SCC-79A	UT-SCC-36
Y2	FaDU	UT-SCC-60B	UT-SCC-72	UT-SCC-45	UT-SCC-32	UT-SCC-76A
Y3	UT-SCC-47	UT-SCC-19A	UT-SCC-5	UT-SCC-17	UT-SCC-8	UT-SCC-74A
Y4	UT-SCC-20A	UT-SCC-79B	UT-SCC-30	UT-SCC-72.2	UT-SCC-24A sh CIP2A.557	UT-SCC-25
Y5	UT-SCC-9	UT-SCC-24A sh CIP2A.556	UT-SCC-2	UT-SCC-7	UT-SCC-50	UT-SCC-14 sh CIP2A.557
Y6	UT-SCC-14	UT-SCC-24A sh GAPDH				
Y7						
Y8	CIP2A KO tumor (527)	WT tumor (562)	WT tumor (573)	CIP2A KO tumor (541)	CIP2A KO tumor (195)	WT tumor (131)
Y9						
Y10				liver	liver	liver

2.4 Immunohistochemical staining (IHC) procedure and scoring

The final CMA -block was cut into 6 µm sections to perform IHC-stainings. IHC-stainings were performed with 2 different CIP2A antibodies, PME-1 antibody and SET antibody.

CIP2A immunohistochemical staining was carried out after protocol optimization in Ventana BenchMark XT staining automate (Ventana etc.) with OptiView DAB kit and with 64-minute CC1 preparation and 32-minute antibody incubation. Mouse monoclonal anti-CIP2A antibody (dilution 1:25, 2G10-3B5, sc-80659, SantaCruz) and rabbit polyclonal anti-CIP2A antibody (dilution 1:100, A301-454A, Bethyl) was used.

For PME-1 and SET stainings, a manual protocol was used. The slides were deparaffinized and subsequently rehydrated with a tapering concentration series of ethanol in distilled water. Heat-induced epitope retrieval was performed in 10 mM Tris-EDTA-buffer (pH 9.0) with a microwave oven or a pressure cooker. After cooling down for 20 min at room temperature, the slides were rinsed with water. For immunohistochemistry, the slides were first treated for 10 min in 3% BSA/PBS to block protein activity and then rinsed in Tris-HCl (pH 7.4). The slides were incubated with rabbit polyclonal anti-SET (H-120) antibody sc-25564 (diluted 1:1000, Santa Cruz Biotechnology), or mouse monoclonal anti-PME-1 (B-12) antibody sc-25278 (diluted 1:1,0 00, Santa Cruz Biotechnology) overnight, rinsed, and treated with the secondary antibody (Dako EnVision anti-rabbit or Dako EnVision anti-mouse K-4001) for 30 min, rinsed, and incubated for 10 min in DAB1 liquid Dako (K3468), and finally rinsed with water. Slides were counterstained with Mayer's haematoxylin, rinsed with tap water, dehydrated, cleared, and mounted

Stainings were scored with semiquantitative scoring method per the number of positive cells and by the intensity of staining. Number of positive cells were scored on scale < 30 %, 30-70 % and > 70 %, whereas intensity of staining was scored on a scale 1 to 3 as weak/negative (+), intermediate (++) or strong (+++). Scorings were performed individually by KS and JR and the contradictory cases were discussed and scoring was decided in consensus. Consensus score was used for final analysis.

2.5 Statistical analysis

Statistical tests were performed using SPSS 24 software (SPSS, Chicago, IL). Correlations between clinical data and IHC-scorings regarding PP2A inhibitors were evaluated using Pearsons correlation.

3. Results

3.1 Visualization of IHC-stainings of CIP2A, PME-1 and SET in assembled CMA

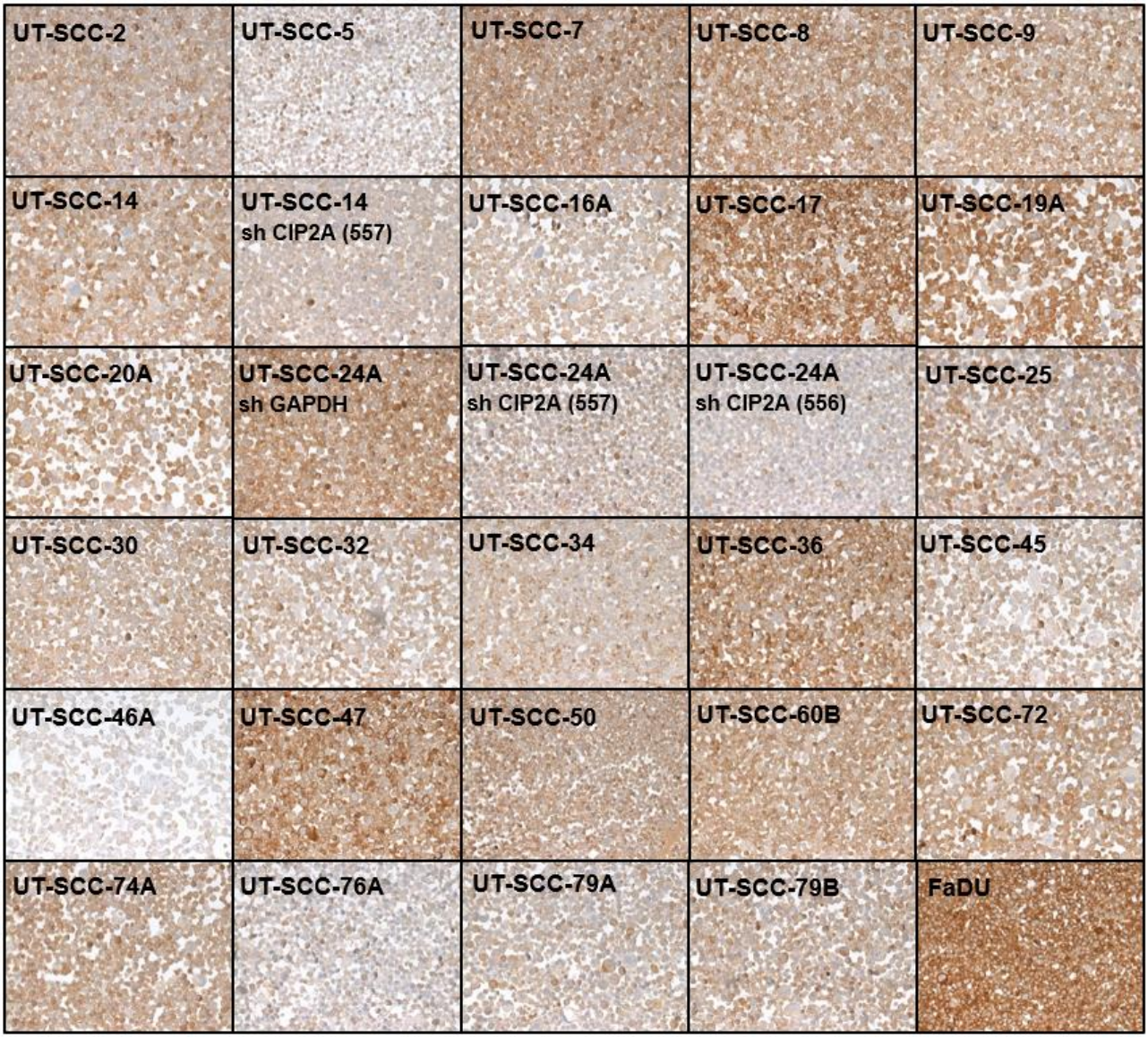

Figure 2. Immunohistochemical CMA stainings with CIP2A SantaCruz (SC) antibody. Samples are placed according to the CMA map (Table 3).

Figure 3. Immunohistochemical CMA stainings with PME-1 antibody. Samples are placed according to the CMA map (Table 3).

Figure 4. Immunohistochemical CMA stainings with SET antibody. Samples are placed according to the CMA map (Table 3).

The cell architecture and morphology were retained in paraffin-embedded cell pellets in assembled CMA (Figures 2, 3 and 4). Head and neck squamous cell carcinoma cells were visualized with IHC methods with antibodies against 3 different PP2A inhibitory proteins: CIP2A, PME-1 and SET. By visualizing the cells with IHC staining, the cells morphology and an overview of the cell architecture may be evaluated by microscopic examination. By this method living cells may be distinguished from the dead cells and from the overall cell architecture the cell nucleus and cytoplasm are visible. The process leading to an appropriate result for staining and microscopic visualization procedures in paraffin-embedded cells may be carried out in a very similar fashion as with paraffin-embedded tissue samples. Visualization of cells with staining procedures allows also general evaluation of cell growth habit, cell growth density and overall cell size between cell lines. Cell growth habit varied across the cell lines, since some cell lines were more colony-forming and required more cell-cell contact, whereas in other cell lines cells grew more separately without as strong cell-cell contact. Cell positivity to specific antibody in question may be evaluated by the IHC staining intensity as well as categorizing staining positivity to either nuclear or cytoplasmic. Since purely cells were used in this method to produce the microarray, only intracellular material is seen with IHC staining. This is the major difference of CMA compared to TMA, where all the tissue components are visualized in IHC staining including all the extracellular material such as the extracellular matrix.

An overview of CIP2A IHC- staining from all cell lines show that the intensity of staining varied substantially between cell lines (Figure 1). Some cell line samples show nearly entirely negative staining result such as UT-SCC-24A sh CIP2A (556) while others show a very strong positive staining result across all the cells such as FaDU. Cells staining positivity did not show as clear differences between cell lines with PME-1 or SET antibody (Figure 2 and 3). In general, nearly all the cells were PME-1 and SET positive in all cell lines. However, there was some variation considering the intensity of the staining result.

3.2 IHC staining scorings and cross-correlations of PP2A inhibitors

Table 4. IHC staining scorings performed on three PP2A inhibitory proteins CIP2A, PME-1 and SET showed notable differences between specific inhibitory proteins and UT-SCC cell lines.

UT-SCC cell line	CIP2A positive cells (%)	Intensity of IHC staining		
		CIP2A SC	PME-1	SET
UT-SCC sh14	<30	1	1	1
UT-SCC 25	30-70	2	1	1
UT-SCC 74A	>70	2	2	2
UT-SCC 76A	<30	1	1	1
UT-SCC 36	>70	3	1	1
UT-SCC 50	<30	2	1	2
UT-SCC 24A sh557	<30	1	1	1
UT-SCC 8	30-70	2	1	2
UT-SCC 32	30-70	1	2	1
UT-SCC 79A	<30	1	2	2
UT-SCC 7	30-70	3	1	1
UT-SCC 17	>70	3	2	1
UT-SCC 45	<30	1	2	2
UT-SCC 34	30-70	1	1	2
UT-SCC 2	30-70	2	1	1
UT-SCC 30	>70	1	2	1
UT-SCC 5	<30	1	2	1
UT-SCC 72	>70	2	1	2
UT-SCC 16A	30-70	1	2	2
UT-SCC 24A shGAPDH	>70	3	2	1
UT-SCC 24A sh556	<30	1	1	1
UT-SCC 79B	30-70	1	1	1
UT-SCC 19A	>70	3	1	2
UT-SCC 60B	>70	2	2	1
UT-SCC 46A	<30	1	1	3
UT-SCC 14	30-70	2	2	2
UT-SCC 9	30-70	2	1	1
UT-SCC 20A	30-70	2	2	3
UT-SCC 47	>70	3	2	2
FaDU	>70	3	1	1

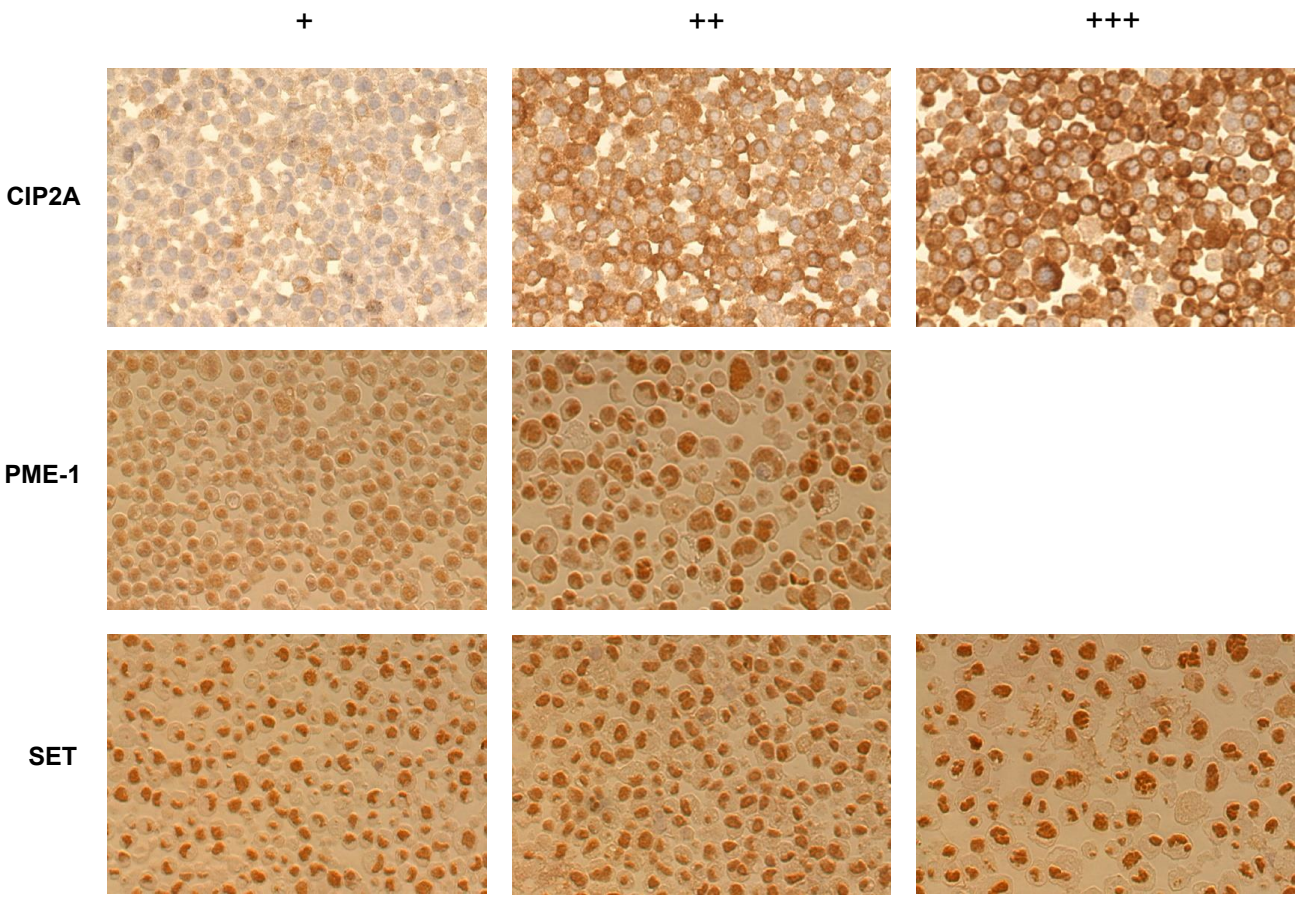

Figure 5. Demonstration of IHC intensity scorings in representative cell line samples.

The intensity of staining positivity was evaluated manually and was reported as strong (+++), intermediate (++) or weak/negative (+) (Figure 5). CIP2A SC antibody showed predominantly cytoplasmic IHC staining positivity, while PME-1 and SET staining positivity was mainly nuclear. IHC staining positivity was distinctly similar in all cells stained with PME-1 and SET antibodies between all the samples (Figures 3 and 4). The staining result was predominantly either positive or negative across all the cells in each sample, thereby only the staining intensity was evaluated in these samples (Table 4). Staining intensity of PME-1 was rated either weak/negative (n=17) or intermediate (n=13) in all cell lines. Similar results were also seen with SET, since only few (n=2) cell lines with strong staining intensity were observed, while most cell lines had weak/negative (n=17) or intermediate (n=11) staining intensity result. CIP2A staining with SantaCruz antibody showed slightly more variation in cell staining positivity between the samples (Figure 3), therefore both the staining intensity and

number of positive cells were evaluated (Table 4). According to the evaluations, under 30% of CIP2A positive cells were observed in 9 cell lines, between 30 to 70% of CIP2A positive cells in 11 cell lines and over 70 % of CIP2A positive cells in 10 cell lines. Majority of CIP2A staining intensity results were rated weak/negative (n=13), only few were rated strong (n=3) and the rest were rated intermediate (n=10).

Table 5. Correlations between CIP2A, PME-1 and SET IHC-scorings and radioresistance or growth in mice in UT-SCC cell lines. ** = Correlation is significant at the 0.01 level (2-tailed).

	CIP2A (Santacruz)	**PME-1**	**SET**	**Radioresistance**	**Growth in mice**
CIP2A (Santacruz)					
Pearson correlation	1	-0,069	-0,133	-0,156	-0,160
Sig. (2-tailed)		0,713	0,477	0,457	0,527
N	30	30	30	25	18
PME-1					
Pearson correlation	-0,069	1	0,227	0,017	-0,065
Sig. (2-tailed)	0,713		0,220	0,936	0,798
N	30	30	30	25	18
SET					
Pearson correlation	-0,133	0,227	1	-0,300	0,095
Sig. (2-tailed)	0,477	0,220		0,146	0,708
N	30	30	30	25	18
Radioresistance					
Pearson correlation	-0,156	0,017	-0,300	1	-,669**
Sig. (2-tailed)	0,457	0,936	0,146		0,003
N	25	25	25	25	17
Growth in mice					
Pearson correlation	-0,160	-0,065	0,095	-,669**	1
Sig. (2-tailed)	0,527	0,798	0,708	0,003	
N	18	18	18	17	18

Correlation between IHC-staining scorings of three PP2A inhibitory proteins (CIP2A, PME-1 and SET) and cell line characteristics was determined with Pearsons correlation (Table 5).

Due to relatively small amount of sample material, no significant correlation was observed between expression of PP2A inhibitory proteins and measured radioresistance or reported growth in mice. In addition, there was no significant cross-correlation observed between expression of PP2A inhibitory proteins.

3.3 Expression of CIP2A in CIP2A-silenced shRNA-UT-SCC cell lines

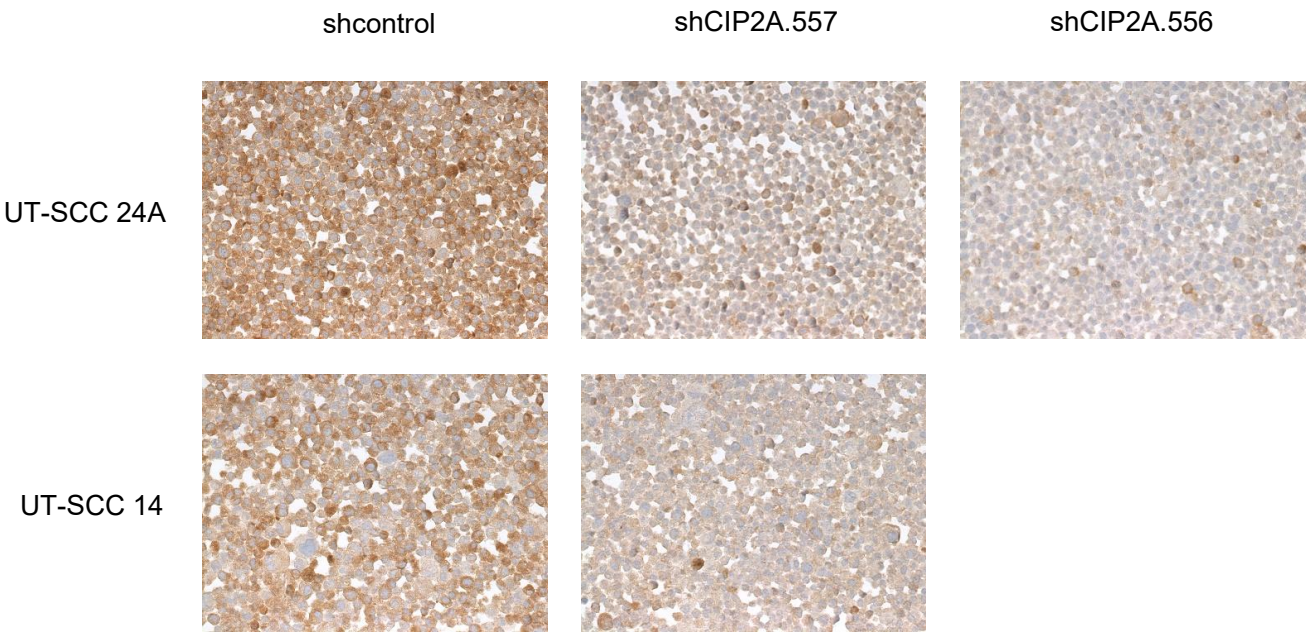

Figure 6. CIP2A IHC-stainings with SC antibody from shCIP2A samples and the correspondent control samples show considerably weaker staining in shCIP2A samples.

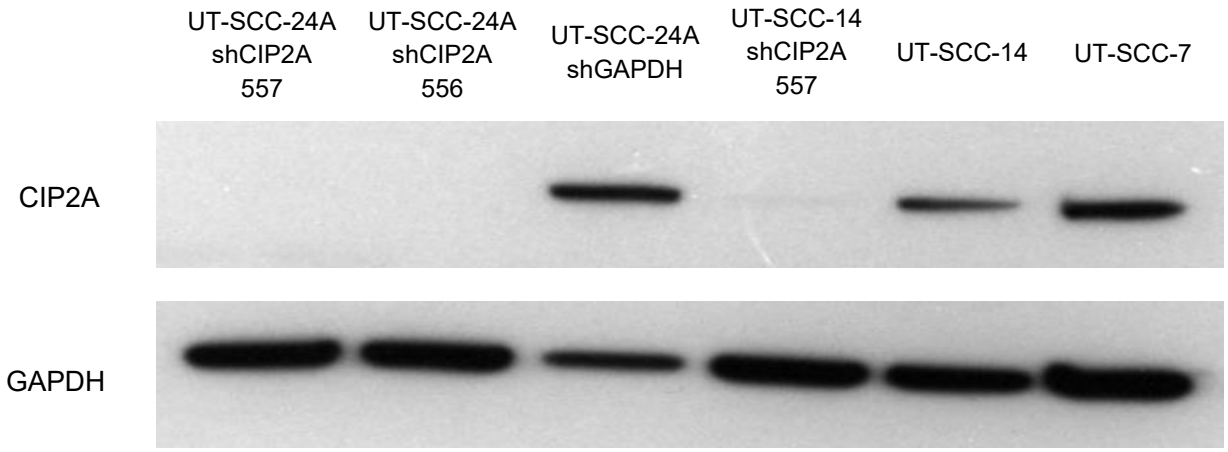

Figure 7. Western blot analysis of CIP2A and GAPDH as control expression levels from 5 different HNSCC cell lines. 3 of the cell lines have been modified with short hairpin CIP2A-RNA transcripts, thereby reducing CIP2A expression in these cell lines.

Table 6. Quantifications from Western blot analysis of CIP2A and GAPDH expression levels from sh-RNA silenced UT-SCC cell lines and their untreated controls. CIP2A (%) and CIP2A/GAPDH (%) have been proportioned against the corresponding wild type cell line.

	Western blot volume quantification (Volume (Int))			CIP2A (%)	CIP2A/GAPDH (%)
Cell line	CIP2A	GAPH	CIP2A/GAPDH		
SCC-24A sh CIP2A 557	194×10^2	651×10^5	0,000	0,10	0,07
SCC-24A sh CIP2A 556	548×10^3	701×10^5	0,008	2,77	1,76
SCC-24A sh GAPDH	198×10^5	445×10^5	0,444	100	100
SCC-14 shCIP2A 557	5×10^5	622×10^5	0,008	5,36	5,37
SCC-14	931×10^4	623×10^5	0,149	100	100
SCC-7	176×10^5	658×10^5	0,267	100	100

IHC staining with CIP2A antibody was distinctly weaker in cell lines modified with shRNA transcripts compared to other cell lines (Figure 6). These result show that CIP2A expression is significantly decreased in shRNA cell lines, which are designed to prevent CIP2A transcription. These cell lines (UT-SCC 24A sh557, UT-SCC 24A sh556 and UT-SCC 14 sh557) showed significantly weaker staining intensity and number of positive cells compared to the CIP2A wild type cell lines (UT-SCC 24A shGAPDH and UT-SCC 14) indicating successful silencing of CIP2A transcription. Similar findings were seen in the samples in question with Western blot analysis as much weaker intensity response was seen in UT-SCC 24A sh557, UT-SCC 24A sh556 and UT-SCC 14 sh557 samples (Figure 7.). Western blot data was further analyzed through Western blot intensity volume quantification (Table 6.). After proportioning CIP2A volume intensity to control GAPDH volume intensity in addition to the control wild type cell line in question, CIP2A expression amount was estimated as 0,10 % in UT-SCC 24A sh557, 2,77 % in UT-SCC 24A sh556 and 5,36% in UT-SCC 14 sh557. These findings together point to true CIP2A silencing in these shRNA cell lines and thereby also to SC CIP2A antibody specificity towards CIP2A.

3.4 Variation of CIP2A IHC-staining result between two used commercial antibodies

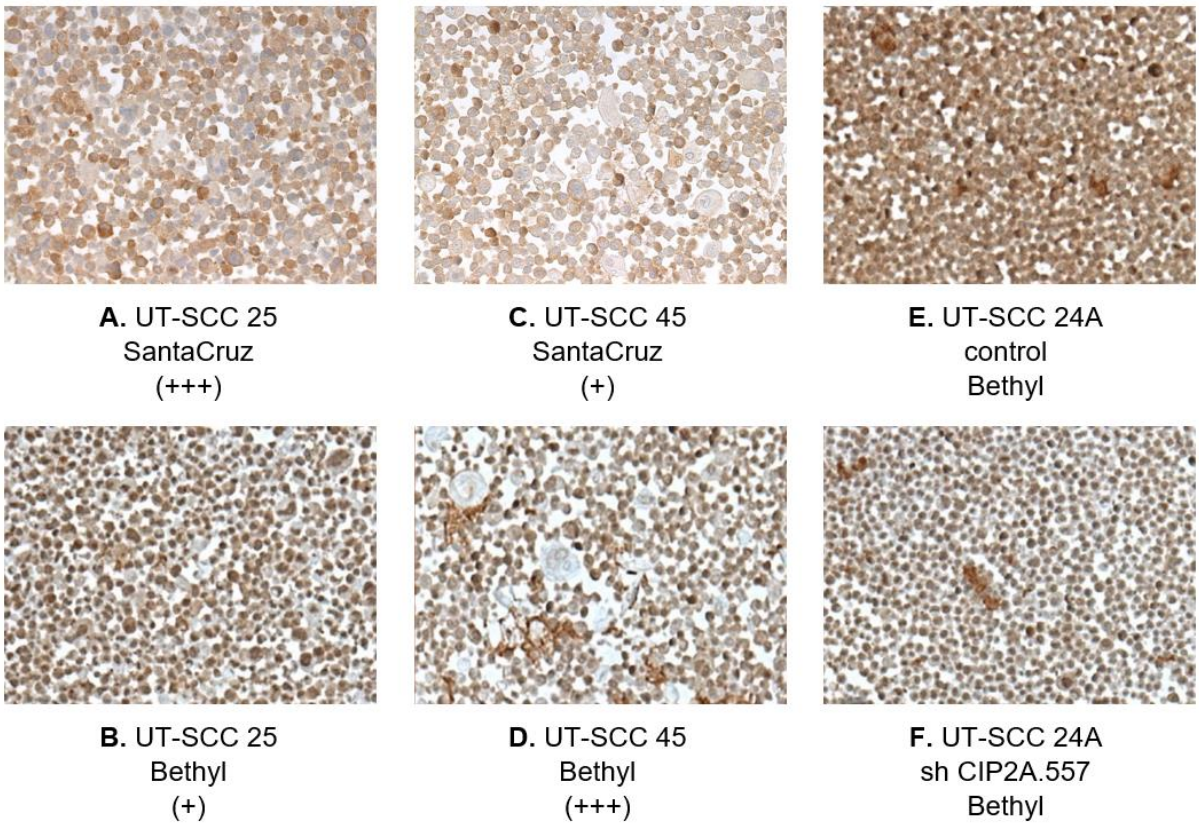

Figure 8. Significant variation in IHC staining intensity between CIP2A SantaCruz and CIP2A Bethyl antibodies was observed in UT-SCC samples.

By comparing the IHC staining results in two different CIP2A antibodies (Bethyl and SantaCruz) a notable difference in staining specificity and intensity was found (Figure 8). Between SantaCruz antibody and cytoplasmic Bethyl staining there was a positive linear correlation ($r= 0{,}737$, $N= 30$, $p <0.01$), whereas between Santacruz antibody and nuclear Bethyl staining there was a negative linear correlation ($r= -0.556$, $N= 30$, $p <0.01$). A negative linear correlation was also observed between nuclear and sytoplasmic Bethyl antibody staining ($r = -0.685$, $N = 30$, $p <0.01$).

Both antibodies are designed to specifically bind to CIP2A, thereby intending to indicate specific CIP2A protein expression in cells. However, the CIP2A Bethyl antibody showed both cytoplasmic and nuclear staining positivity, whereas CIP2A SantaCruz antibody showed exclusively cytoplasmic staining positivity. This leads to different results while determining

the staining positivity of cells and staining intensity based on which commercial antibody was used in the staining process. Indeed, shRNA silencing of CIP2A does not influence nuclear staining intensity as evaluated by the Bethyl antibody (Figure 8E-F.) As the amount of CIP2A nuclear staining intensity is known to be minor, the Bethyl antibody's specificity is put into serious question.

3.5 Visualization of IHC-stainings of PP2A inhibitors in mouse samples

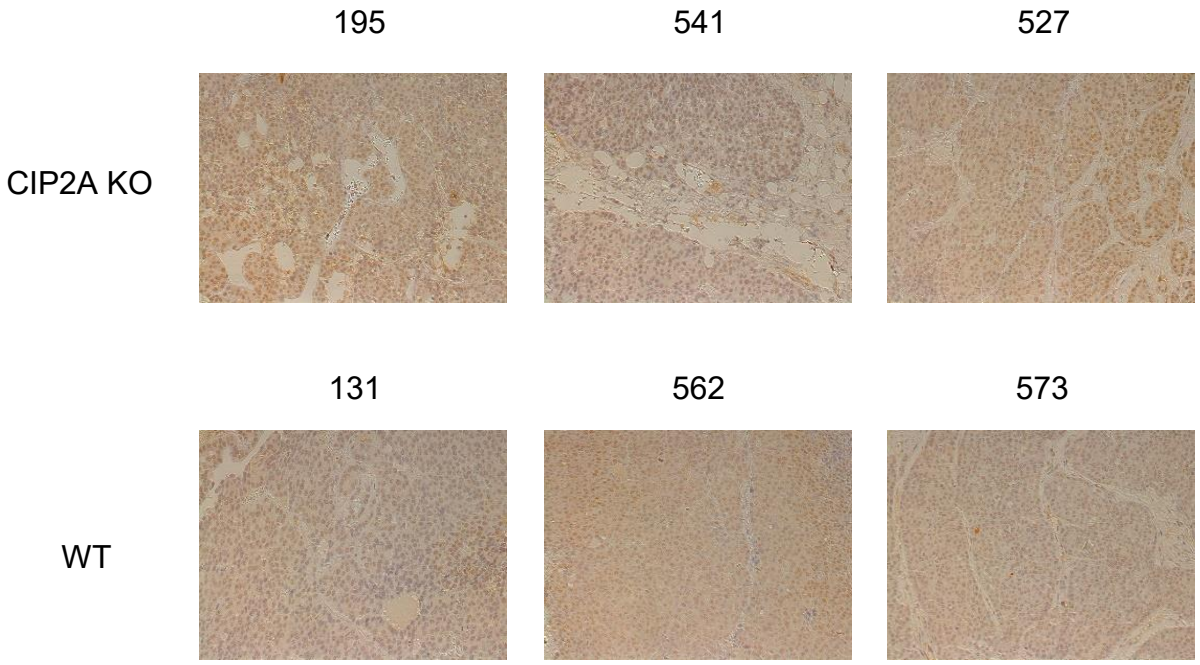

Figure 9. PME-1 stainings from mice tumor samples.

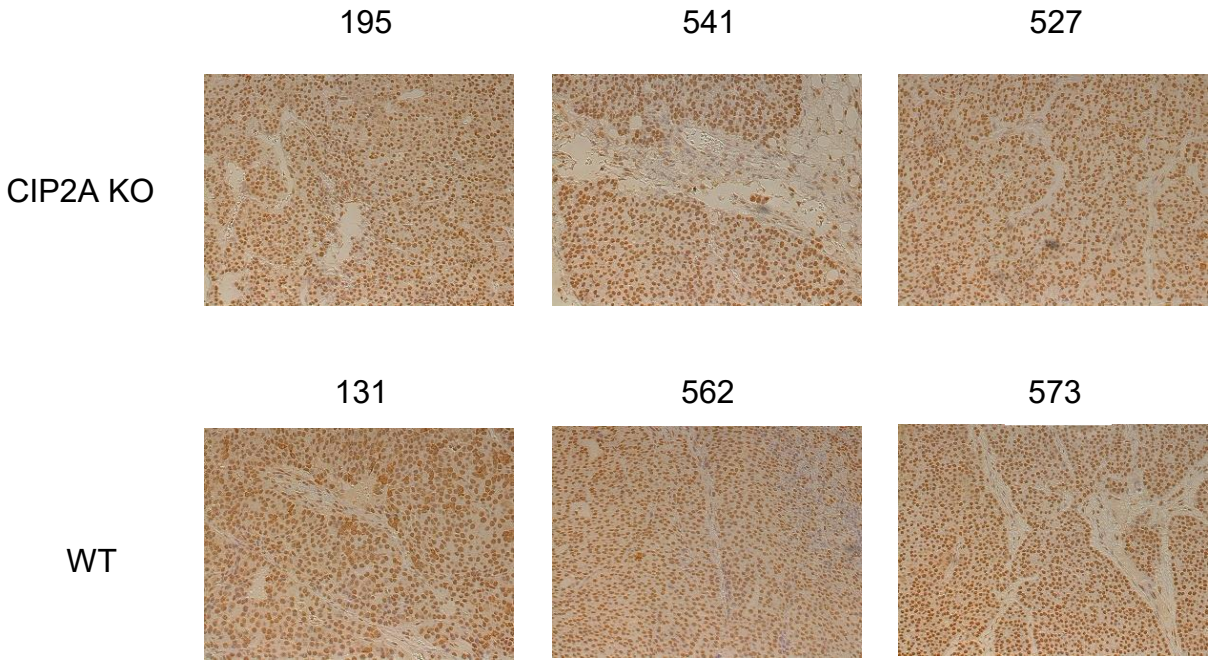

Figure 10. SET stainings from mice tumor samples.

IHC staining intensity was stronger with SET antibody than with PME-1 antibody in both WT and CIP2A KO mouse (Figure 9 and 10). Mouse tissue samples were included into CMA as tissue control samples, which serve as both staining quality and antibody specificity controls. These tissue sample results support the findings from UT-SCC cell lines, as both PME-1 and SET staining show mostly intranuclear positivity. PME-1 and SET staining positivity did not notably vary between WT and CIP2A KO samples, although here the sample size (6) was quite small to find statistically significant results. IHC staining results from mouse tissues with CIP2A antibody were not included in this study, since CIP2A staining protocol was performed with automated Ventana-machine, which leads to an unreliable IHC staining result with mouse tissues.

4. Discussion

In this project we created a new tool for determining protein expression efficiently from large sample material. We combined 30 formalin-fixed and paraffin-embedded HNSCC cell lines into a single array. Normal human liver and mouse tissue samples were also added to the array as quality controls. With this tool a large amount of cell lines may be processed with various staining methods by conducting the staining process only once. This allows for a practical, cost-efficient and time-saving way to determine protein of interest's expression with specific antibody. In this study we show that formalin-fixed, paraffin-embedded cell lines may be handled with IHC staining in a similar fashion as for paraffin-embedded tissue samples. Cells retained their morphology and overall cell architecture, thereby leading to a good quality staining result, which allows for a reliable evaluation of IHC staining positivity.

CMA is a novel method for screening various factors of interest from a broad panel of cell lines. There are some previous studies which present the similar method, however in these studies the panel of cell lines has usually been quite small, and they have been constructed by using other types of cancer cells (Waterworth et al. 2005, Gately et al. 2011, Wu et al. 2014). To our knowledge, this is the first CMA constructed with HNSCC cell lines to this large extent. CMA offers an alternative method for TMA as a tool for screening protein expression in cells and results may be analyzed by using e.g. IHC in the same manner as tissue samples. As the major difference, CMA contains only cell material, thereby excluding the extracellular material and other tissue components from the array. Stainings across the CMA are also more comparable and representative between all the cell lines since staining protocol is conducted simultaneously over the entire CMA. Since cell and tissue material have different features, all the staining methods and procedures may not work properly on both materials. However, IHC staining procedure works appropriately with both cell and tissue samples.

In addition to efficiency, CMA provides other notable benefits as well. Use of exclusively cellular material allows strictly focusing the evaluation of IHC staining positivity on cells and removing majority of the unspecific sources of unwanted IHC signaling positivity. Even though CMA requires some effort to construct, once CMA is ready it can be used multiple times to screen various molecular markers of interest from embedded cell lines by repeatable sectioning. This also provides a fixed, paraffin-embedded biobank of cell lines, which may be stored for a very long time and then use again when needed. Since cultured cell lines are used in CMA, it is possible to manipulate and modify the cell lines before constructing CMA. This allows for example further molecular profiling of cell lines by inhibiting or enhancing

expression or effect of certain genes. This procedure may also be applied to drug development and discovery studies, since cell lines may be exposed to drug of interest in beforehand and then evaluate its impact on cells altered expression profile.

There are also some limitations for construction and use of CMA. First of all, a large amount of cells is needed in order to create large enough cell pellets. This means that a big number of cells needs to be acquired or cells of interest must have sufficient replicative features, such as cancer cells or stem cells do. While using highly proliferative cells, we also must note the specific features of patient derived cell lines. Cell lines which start to grow from cells separated from human tumor samples usually have more aggressive features compared to other tumor samples in order to allow their proliferation in cell culture conditions. These features are also often associated with clinical features such as disease progression and poor patient prognosis. This narrows down and selects only certain types of cancer cells, which often predicts certain common features for these cell lines already in the first place and also may refer to a similar kind of molecular profile and cell signaling network.

Previously various methods have been used to demonstrate specific protein expression in cell lines, such as immunofluorescence (Kaur et al. 2015). However these techniques are not directly comparable with techniques used with certain protein expression analysis in tissue samples. With this technique we can directly compare the staining results with other IHC staining figures, such as tissue IHC stainings. This also enables comparing the antibody specificity between cell lines and tissue samples.

Across the global research field there has been an increasing concern considering antibody specificity, since studies have shown that there is a considerable difference between different manufacturers antibodies, even if they are supposed to bind to and detect the same antigen. Therefore, there is recent call for more attention to antibodies binding specificity and validation (Ogris et al. 2017, Waters et al. 2017). In this study we verified CIP2A expression with Western blot analysis in addition to IHC staining of UT-SCC cell lines with SC CIP2A antibody. Results from both methods showed similar CIP2A expression profile across the used cell line samples, which indicates a reliable demonstration of antibody specificity. These results also include data from shRNA cell lines, which together indicate true silencing of CIP2A in these cell lines.

This procedure allows one antibody validation method to ensure and test antibody specificity in paraffin-embedded cell samples. In addition, CMA may be used as a tool for conveniently defining correlation between protein expression and other factors of interest. In this project

we investigated cross-correlation between expression of PP2A inhibitors CIP2A, PME-1 and SET and the association between expression of PP2A inhibitors and cell line characteristics: radioresistance and growth in mice. Although no significant correlation between these factors was found in this material, these procedures are one of the potential applications of CMA in future studies.

CMA also allows efficient comparing of IHC staining results from different commercial antibodies, which are designed to specifically bind to certain protein of interest. In this study we observed significant variation of the staining result from two commercial CIP2A antibodies. Stainings with Bethyl-antibody showed mostly intranuclear staining positivity, which we categorized as unspecific antibody binding and thereby unreliable staining result, since according to previous research data CIP2A is mostly expressed in the cytoplasm. In fact, Bethyl antibody has proven unreliable in other types of analysis as well. For this reason, stainings made with Bethyl-antibody were excluded from additional analyses. IHC staining results with SantaCruz CIP2A antibody however showed purely cytoplasmic staining positivity suggesting that CIP2A SC antibody is more specific binding directly to CIP2A. These results show the potential of CMA as a reliable and efficient antibody validation method in various antibody validation procedures.

As for PP2A and its endogenous inhibitors, there is evidence that these could be potentially in the future be used with clinical implementations. Especially for CIP2A, there are promising signs of various possible use in clinical practice. CIP2A has become established as one prognostic marker of poor patient prognosis in various cancer types such as HNSCC (Routila et al. 2015, Ventelä et al. 2015, Khanna et al. 2013). In addition, there is evidence that CIP2A could be used as predictive factor, since CIP2A has been shown to contribute to increased radioresistance in HNSCC (Ventelä et al. 2015). In our study we did not detect significant correlation between CIP2A and radioresistance or growth-in-mice. Although in this study our sample size was relatively low, which may explain these results. In the previous study with HNSCC, CIP2A was shown to relate to increased radioresistance while coexpressed with Oct4. However, recently it was shown that reduced CIP2A expression level is associated with both cells radiosensitivity and favorable response to RT in rectal cancer (Birkman et al. 2018).

SET has been shown as a crucial counterpart in diseases such as Alzheimers disease, while considering human malignancies its true nature is yet unclear, although oncogenic features in HNSCC has been reported (Sobral et al. 2014). Connection between PME-1 and various human cancers is undisputed, while its exact role remains unclear. PME-1 seems to have a

varying effect on cancer aggressiveness, as increased PME-1 expression has shown opposite association to patient prognosis in different cancers (Kaur et al. 2016). As for the relationship of CIP2A, PME-1 and SET, connection and synergies of all these three PP2A inhibitors in different cancer types remain elusive. In this study, we did not observe significant cross-correlation between CIP2A, PME-1 and SET. There also was no association with PME-1 or SET and radioresistance or growth-in-mice in this material. However, considering all the results, sample size in this study remained relatively small, leading to potentially some missed existing findings and associations between these factors.

CIP2A has also potential of therapeutic implementations in cancer treatment, as by inhibiting CIP2A activity targeted degradation of c-Myc may be induced in cancer cells, while minimal expression of CIP2A in normal human tissues point to possibility of avoiding side effects from using a potential CIP2A-targeting drug (Junttila et al. 2007, Ventelä et al. 2012). However, more high-quality research is needed to further clarify the true nature of CIP2A and other endogenous PP2A inhibitors in human malignancies in order to provide sufficient data to bring these proteins as tools to the clinics to aid doctors with patient diagnostics, treatment planning and possibly even treating patients with new targeted therapy drugs.

In conclusion, CMA combined from several UT-SCC cell lines in one paraffin block could potentially be used to screen and find new prognostic and predictive factors in head and neck cancer. Correlation between expression of molecular markers, clinical data such as tumor site, and cell line characteristics such as radioresistance and growth-in-mice can be analyzed for each specific cell line. New prognostic and predictive factors could be used to help targeting the cancer treatment in clinics to prevent unnecessary treatment and to secure adequate treatment for head and neck cancer patients. More research work is yet needed in the future to further elucidate the possible role of PP2A and its endogenous inhibitors in human malignancies.

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