Umeå University Medical Dissertations, New Series No 1495

The use of formalin fixed paraffin embedded tissue and global gene expression profiling for increased understanding of squamous cell carcinoma of the tongue

Matilda Rentoft

Department of Medical Biosciences / Pathology
Department of Clinical Sciences / Otorhinolaryngology
Umeå University 2012

Responsible publisher under swedish law: the Dean of the Medical Faculty

ISBN: 978-91-7459-413-3
ISSN: 0346-6612
E-version availibal at http://umu.diva-portal.org/
Printed by: Arkitektkopia AB
Umeå, Sweden 2012

Aerodynamically, the bumble bee shouldn't be able to fly,
but the bumble bee doesn't know it so it goes on flying anyway.
Mary Kay Ash

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Abstract

Head and neck cancer is the 6th most common malignancy worldwide, with tumours of the tongue being one of the most prevalent sites. Despite advances in surgery and radiotherapy, the five-year survival has not changed during the last decades and remains at approximately 50%. Identification of novel biomarkers for more personalized treatment is important for increasing survival in these patients. One of the most commonly used methods in the search for new biomarkers is microarray analysis. A substantial limitation with this technique is the requirement for fresh frozen samples from which high quality RNA can be extracted. This becomes particularly problematic when attempting to discover differences associated with individual sub-types or rare cancers. Recent developments, including the DASL microarray platform, have provided the possibility of analysing RNA of poorer quality from formalin fixed paraffin embedded (FFPE) samples. FFPE is the standard way of preserving tissue from patients and millions of samples are stored around the world. In this thesis we have evaluated the use of FFPE samples and global gene expression profiling for increasing basic knowledge in a subgroup of oral cancer patients with tumours of the tongue.

As confirmation of microarray results using qPCR is of outmost importance for conclusive data evaluation, we first aimed at finding a housekeeping gene stably expressed across malignant and non-malignant FFPE oral tissue. TUBA6, which belongs to the tubulin family was detected as being the most stable out of eight possible genes and was thus used for qPCR normalization throughout the following studies.

We have performed three separate microarray experiments. Initially only a focused DASL array covering 502 cancer related genes was available and we used it to analyze a smaller cohort of patients and controls (n=36). A similar cohort (n=29) was also analyzed for expression of 836 micoRNAs. In 2009 a whole genome DASL array was launched, covering over 20,000 genes, and all tongue tumour samples available between 1997 and 2010 (n=87) were analysed using this array.

Similar to other research groups we observed very high replicate reproducibility using both DASL arrays. When using the microRNA array and the whole genome DASL array an effect of sample quality on the detected expression level of individual genes was noticed. While the expression of some genes severely decreased with a decrease in sample quality others were not changed. This will impair normalization, leading to a residual non-biological variation within the data. Based on our findings we have presented some recommendations for minimizing the effect of sample quality and maximizing the level of biologically relevant information obtained from these experiments, e.g. ensuring that samples in groups to be compared are of the same quality range. For the microRNA data we also introduced an additional normalization step to the standard normalizations. We could show that lists of differentially expressed genes generated when taking these precautions were enriched for genes involved in cancer related processes and contained for tongue carcinoma previously identified changes. A number of differentially expressed genes, novel for tongue carcinoma,

were also confirmed in high quality fresh frozen samples, including BCL2A1 (apoptosis), CXCL10 (immune response), SLC2A6 (energy transport) and miR-424 (angiogenesis).

In conclusion microarrays can be used to analyze FFPE samples but should be performed with care. Standard normalization methods will not remove the variation introduced by samples being of different quality, leading to spurious results. Taking a few precautions, however, led to the identification of differentially expressed genes relevant in tumour development and maintenance. The recommendations we make can facilitate design of future studies using FFPE samples. The genes we identified as being differentially expressed in tumour tissue now need to be further evaluated for their potential as biomarkers in tongue carcinoma.

Abbreviations

General abbreviations

bp	base pair
cDNA	complementary DNA
cPR	complete Pathological Remission
Ct	Cycle threshold
DASL	cDNA mediated Anneling, Selection, extension and Ligation
EBV	Epstein–Barr Virus
FFPE	Formalin Fixed Paraffin Embedded
FF	Fresh frozen
HK	House Keeping
HPV	Human Papilloma Virus
LNA	Locked Nucleic Acid
miRNA	microRNA
MMP	Matrix metalloproteinase
NF	Normalization Factor
PCA	Principal Component Analysis
qPCR	quantitative PCR
r	Pearson correlation coefficient
r^2	Coefficient of determination
SAM	Significance of MicroArray
SCCHN	Squamous Cell Carcinoma of the Head and Neck
WG	Whole Genome

Genes mentioned

ACTB	Actin, beta
BARD1	BRCA1 associated RING domain 1
BCL2A1	BCL2-related protein A1
BLM	Bloom syndrome, RecQ helicase-like
BRCA2	Breast cancer 2, early onset
CCNH	Cyclin H
C-MYC	Myelocytomatosis viral oncogene homolog
CXCL10	Chemokine ligand 10
ER	Estrogen Receptor
ERCC1	Excision repair cross-complementing rodent repair deficiency, complementation group 1
FANCG	Fanconi anemia, complementation groupG
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HER-2	Human Epidermal Growth Factor Receptor2
HPRT1	Hypoxanthine phosphoribosyltransferase1
ITGB4	Integrin, beta 4
OAZ1	Ornithine decarboxylase antizyme1
RECQL	RecQ protein-like
RPL27	Ribosomal protein L27
RPS23	Ribosomal protein S23
TGM3	Transglutaminase 3
TUBA6	Tubulin alpha-6 chain
SLC2A1	Solute carrier family 2, member 1
S100A6	S100 calcium binding protein A6
TP53	Tumour Protein p53
XRCC2	X-ray repair complementing defective repair in Chinese hamster cells 2
XRCC1	X-ray repair complementing defective repair in Chinese hamster cells 1

Original research articles

This thesis is based on the following original research articles

Paper I **Rentoft M**, Hultin S, Coates P, Laurell G, Nylander K: TUBA6 (tubulin alpha-6 chain) is a stably expressed reference gene in normal oral tissue and oral squamous cell carcinoma. Experimental and Therapeutic Medicine 2010, 1(3):419-423.

Paper II **Rentoft M**, Laurell G, Coates PJ, Sjostrom B, Nylander K: Gene expression profiling of archival tongue squamous cell carcinomas provides sub-classification based on DNA repair genes. Int J Oncol 2009, 35(6):1321-1330.

Paper III **Rentoft M**, Coates PJ, Laurell G, Nylander K: Whole genome gene expression profiling of formalin fixed paraffin embedded materials: pitfalls with sample quality and recommendations for identifying biologically significant expression changes. Accepted PLoS ONE, Scheduled publication date 17th of April at http://dx.plos.org/10.1371/journal.pone.0035276

Paper IV **Rentoft M**, Fahlen J, Coates PJ, Laurell G, Sjostrom B, Ryden P, Nylander K: miRNA analysis of formalin-fixed squamous cell carcinomas of the tongue is affected by age of the samples. Int J Oncol 2011, 38(1):61-69

Introduction

Head and neck cancer is a complex disease for which survival is relatively low; the disease is associated with a high incidence of metastasis to regional lymph nodes in the neck, high risk of secondary primary tumours in the region and difficulties to predict response to treatment. A lot of research the last few years has been focused on attempts to try and predict disease and disease behaviour with the help of biomarkers. In the introduction these subjects are presented and achievements in the field of biomarker discovery for head and neck cancer using transcriptomics are summarized.

Head and neck cancer

Survival and risk factors

Head and neck cancers compromise a group of malignant tumours arising in the oral cavity, pharynx, larynx, nose and paranasal sinuses and salivary glands (Figure 1). According to the World Health Organization (WHO) there are around 600 000 new cases and 300 000 deaths of head and neck cancer each year. Tumours in these regions arise in a number of different structures such as craniofacial bones, soft tissue and salivary glands, but the majority (~90%) arise in the mucosal membranes and are denoted squamous cell carcinomas of the head and neck (SCCHN) [1]. The oral cavity is the most prevalent location and the tongue is the most common tumour sub-site within the oral cavity, followed by lip and floor of mouth [2,3]. In Sweden 350 patients with squamous cell carcinomas of the oral cavity were diagnosed in 2008 and of theses 217 were squamous cell carcinomas of the mobile tongue.

The average five year survival for SCCHN in the world is approximately 50% but this varies greatly between tumours of different locations and stage. While for example patients with tumours of the lip have a five year survival of approximately 93%, patients with tumours of the tongue only have an average five year survival of 49% [4]. Additionally the five year survival for patients with tongue tumours decreases from approximately 73% to 43% if there is a spread to regional lymph nodes in the neck [5]. The frequency with which tumours has developed nodal spread also varies depending on tumour location and size with approximately 8% of the patients initially exhibiting a T1 soft palate carcinoma compared to 71% of patients initially exhibiting a T1 tonsil carcinoma [6].

Smoking tobacco and an excessive intake of alcohol are the main risk factors common for all sites and believed to account for over 70% of the cases [1,3]. Also other forms of smokeless tobacco and betel quid are major risk factors which for example is reflected in the high incidence of SCCHN in India where it represents 30-40% of all malignancies [7]. Other risk factors such as exposure to certain viruses are coupled to specific tumour locations. Human papilloma virus (HPV) for example is strongly connected to tonsil

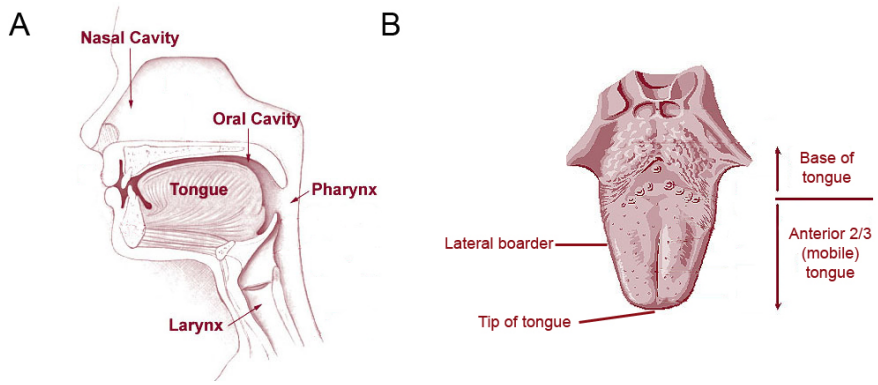

Figure 1 Anatomical picture of the head and neck region. (A) shows the whole head and neck region while (B) is a detailed picture of the tongue. The tongue is divided into base of tongue, which is the posterior one third, and the mobile tongue, which is the anterior two thirds. Base of tongue and mobile tongue are anatomically very different with more lymphoid tissue in base of tongue. Adapted from SEER Training Modules, *Head and neck cancer*. U. S. National Institutes of Health, National Cancer Institute. 13-03-2012

carcinoma and base of tongue carcinoma [8-10] while EBV is associated with nasopharyngeal carcinoma [11]. Carcinoma of the lip is, unlike all other locations, strongly linked to sun exposure and is therefore highly overrepresented in Australia where the protective ozone layer is thinner [2]. Age also strongly increases the risk of developing SCCHN and approximately 90% of people that are diagnosed are over 45 years of age [3].

Despite significant advances in radiotherapy and surgery there has been little improvement in five year survival for SCCHN during the last few decades and patients continue to die from metastatic disease, local recurrence and secondary primary tumours [5,12]. The most reliable prognostic marker today is the presence of metastasis in lymph nodes in the neck and it is an important factor in the choice of treatment strategy. Many patients however, present with a late stage disease and in those who do not there is a fairly high rate of occult cervical nodal metastasis (20-40%), especially for tongue carcinoma [5].

Head and neck tumours may historically have been considered a family of tumours but due to differences in outcome and risk factors as presented above it is important that each location is studied individually. The tongue, as focused on in this thesis, is additionally anatomically different from other locations in the oral cavity. It is a highly muscularized structure with a rich lymphatic network and its epithelium is specialized containing a high number of taste buds. This is believed to be one of the reasons for the high nodal spread and the poor survival seen in these patients [5]. Studies on gene expression patterns of different tissues in the oral cavity have additionally shown that carcinomas as well as normal tissue of the tongue are molecularly distinct from tumours and normal tissue from other locations within the oral cavity [13,14].

Tumours in young people

As noted above, most SCCHN cases occur in older people (peak age 60-69 in Sweden) and historically the traditional patient has been a heavily smoking man in his middle age. In the last decades we have experienced a slight shift towards younger patients however, and an increase of SCCHN in the young population has been reported from many countries around the world [15-18]. The increase is predominantly seen in oral and oropharyngeal carcinomas and it is especially apparent for tongue carcinomas [15]. Many of the young patients have not been exposed to the traditional risk factors, smoking and alcohol abuse, at least not to the same extent as older patients, and men are not as strongly overrepresented. In Scandinavia we had a 5-6 fold increase in tongue tumours in the young population (<40 years of age) between 1960 and 1994, while tongue cancer in older people only increased 2-fold during the same time period [15,18].

It was early on suggested that oral cancer in young people was more aggressive and had a poorer outcome [19-21]. Data on this today is, however, conflicting and studies have also shown similar survival or even better survival in young patients compared to older patients [22-24]. It is nevertheless clear that a sub-group of the young patients have a very aggressive disease course and speculation about young patients as a distinct group still remains [25-27].

HPV viruses have been suggested as one cause of the increase in recent tonsil carcinoma and can be detected in 45-100% of cases [8]. Patients with HPV-positive tumours tend to be younger and have a better survival [8,10]. HPV is also present in base of tongue carcinomas but data for the moveable part of the tongue are inconsistent. Some studies have found HPV in as many as 50% of tongue tumours but most report very few or no tongue carcinomas to be HPV-positive [28-31]. Therefore, we also intended to evaluate the presence of HPV in our tongue carcinoma samples. After several attempts we concluded that results were very unstable and difficult to reproduce (unpublished data). This is perhaps one of the reasons for the inconsistent reports.

Genetic susceptibility is another likely explanation for why young adults are affected by a tumour disease so strongly connected to long term exposure to carcinogens. It is true that many people diagnosed with head and neck cancer are heavy smokers but far from all smokers develop the disease, indicating that some are more susceptible. Indeed, Schantz *et al.* have shown that DNA from lymphocytes in patients with head and neck cancer is more sensitive to bleomycin exposure (causing chromosomal breaks) as compared to controls [32,33]. The high incidence of secondary primaries for this family of tumours also argues for susceptibility in the whole region, often referred to as field cancerization [34,35]. A number of studies have additionally shown that clinically normal tissue adjacent to tumour tissue do not show a normal pattern for a number of molecular markers (e.g. Cox-2, EGFR, and TGF-α) [36-38]. However, this susceptibility does not explain the increase in cases observed for the young population.

Few differences between young and old patients have been shown at the molecular level. O'Regan *et al.* demonstrated a lower number of genomic

aberrations in ten young patients compared to ten old patients. They further showed that one of the regions commonly deleted in older patients contained the gene P16 and that this gene instead was commonly methylated in young patients [39,40]. Sorensen *et al.* claimed that TP53 mutations are less common in young non-smoking head and neck cancer patients than in young smokers or in the general population [41]. Jin *et al.* on the other hand did not see any differences between young and old patients when looking at microsatellite markers at commonly altered chromosomal regions and Shantz *et al.* did not find any difference in immunological activity between young and old patients [42,43]. These studies all included fairly low numbers of samples, constituting one of the biggest problems when studying this disease; even though head and neck cancer in young has increased it is still fairly rare, leaving very few samples at single head and neck cancer institutions.

Treatment and response

In Sweden, surgery and radiotherapy are the main choices for curative treatment of SCCHN. The two treatments can be given either alone or in combination. The treatment choice is based on a number of clinical factors such as tumour location, stage and resectability as well as the patient's wishes and general condition. Tongue tumours are generally treated with combined modality treatment, i.e. radiotherapy and surgery, even if some small low stage tumours are initially only surgically removed.

Response to radiotherapy is a suggested prognostic marker in for example tonsil carcinoma and cervical cancer, important for overall survival [44,45]. Response can be evaluated both clinically and pathologically (looking for viable tumour cells in biopsies or surgical specimens after radiotherapy). Several factors are known to influence the response to radiotherapy for SCCHN patients such as tumour characteristics (e.g. location, volume and grade), patient characteristics (e.g. smoking status) and biological factors (e.g. hypoxia and expression of DNA repair genes), but no factors are used today in the clinical setting to evaluate tumour response to treatment [46-49].

"Omics" and biomarker discovery

Biomarkers

The stable mortality rate in SCCHN patients in spite of improvements in available treatments has evoked a rising interest in finding molecular biomarkers to improve prediction of tumour occurrence and behaviour [50]. Cancer cells are normal cells that through genetic changes have evolved properties making them able to divide uncontrollably and survive in an unfavourable milieu avoiding intrinsic and extrinsic death signals [51]. These genetic alterations can many times be translated into gene and protein expression changes and can either be used to segregate normal tissue from cancerous tissue and/or as molecular biomarkers. The theoretical use for good biomarkers are many, including early detection of tumours using

simple to obtain blood, urine or saliva samples, classification of tumours into subgroups and prediction of outcome and treatment response. A large number of single biomarkers have been suggested for many cancer forms including SCCHN, but studies devoted to the same targets are often inconsistent and it has been problematic to translate results into clinical use [52-54]. A lot of hope and expectation was put into the introduction of large scale analysis of the different "omes"; e.g. transcriptome, proteome and metabolome, using different "omics techniques" in combination with bioinformatic strategies. These approaches were believed to be able to quickly and simply deliver a large number of biomarkers for tumour prediction. The techniques also enabled the use of gene-sets rather than single genes for predicting tumour characteristics which better suits the heterogeneous nature of tumours. The enormous effort and money invested in these possibilities has indeed had a very large impact on our basic understanding of cancer biology. For biomarker discovery results have, however, been somewhat disappointing and the number of biomarkers reaching the clinic is far from in proportion to the number of biomarkers suggested in the literature [53,54]. The reasons for this are many, but lack of uniformity between studies concerning techniques used, samples included and approaches for evaluating and reporting data is a large contributor. Additionally, many studies have covered relatively small numbers of heterogeneous samples, especially apparent for diverse diseases as SCCHN [50,53,55]. Ein-Dor *et al.* investigated the causes for the poor overlap between the many microarray studies on breast cancer and presented calculations for deciding the number of samples needed to produce a desirable level of reproducibility. They found that there could be many possible equally good predictive gene lists generated from the same data and that the predictive power of a gene varied significantly depending on the samples chosen. This resulted in the conclusion that many thousands of samples are needed to identify "master gene sets" predictive of prognosis across "all" breast cancer patients [56,57]. The present commonly applied "use what you have" strategy however results in a large number of small studies on heterogeneous samples that are inconclusive.

One of the neoplastic diseases where the most effort in biomarker discovery has been put is breast cancer. Biomarkers have been a part of the treatment decision for breast cancer patients for decades (e.g. ER and HER-2) and gene expression data has been used for increasing the understanding of the complex sub-grouping of these tumours [58,59]. In recent years microarray experiments have also enabled the introduction of the first multi-gene assays for breast cancer into the clinical setting. These assays help predict tumour behaviour and can be part of the treatment decision [60].

In spite of previous setbacks, biomarkers and more personalized treatment are still believed by many to be the future for improving cancer survival and "omics" the technique for identifying them. To make this happen we need to learn from the past and standardize data handling and techniques, reduce heterogeneity in sample selection and increase the number of samples included in each study to obtain more conclusive results [53,58].

Transcriptomics in head and neck cancer

Analysis of the transcriptome (to what extent genes are expressed) gives a snapshot of what is going on in a tissue at a given time point. Compared to proteomics and metabolomics for which only a fraction of the proteins and metabolites can be detected you have the opportunity to either analyse a selection of genes or the whole genome (WG). qPCR has been the golden standard for gene expression studies but even though it has been made fairly high throughput, microarray is the only method allowing investigation of expression of tens of thousands of genes simultaneously using small amounts of samples. Microarray is today a method which is widely available and large numbers of samples can fairly easily be analysed in a cost effective manner. A coming method that might replace microarrays in the future is next generation sequencing. It is based on sequencing instead of hybridization and removes many of the limitations with microarrays. But like all techniques it comes with new issues that need to be resolved [61].

Data from microarray experiments can be analysed in either an unsupervised or "data driven" manner or in a supervised manner where the investigator states the conditions (Figure 2) [62]. Using unsupervised hierarchical clustering or unsupervised dimension reducing methods, e.g. principal component analysis (PCA), not previously known groupings of samples can be revealed within the data, so called class discovery, and its causes investigated. This was the case with the sub-grouping of breast tumours as mentioned above [59]. On the other hand, supervised analysis by either class comparison or class prediction will provide gene lists most associated or predictive of a predefined outcome, which was the case with the multi-gene assays for breast cancer [60]. Genes from these lists can then be viewed upon as individual genes or lists of genes can be translated into overrepresented pathways and cell functions.

Microarrays have been used in a number of studies trying to predict the behaviour of SCCHN tumours as reviewed in Choi *et al.* and Sahu *et al.* [50,63]. A few examples are O'Donnell *et al.* and Roepman *et al.* who both addressed the question of occult metastasis and tried to build models predicting the presence of nodal spread [64,65]. Hanna *et al.* and Dumur *et al*. identified genes involved in radiation resistance [66,67]. Belbin *et al.* looked at genes associated with more aggressive tumours and noticed that the genes differed depending on tumour location, stressing the importance of analysing tumours of different locations separately [68]. Chung *et al.* and Thurlow *et al*. identified subgroups with differences in disease recurrence free survival while Ginos *et al.* identified genes associated with recurrent disease [69-71]. All these studies vary in design, sample selection and data evaluation. Hanna, Dumur, O'Donell, Belbin and Ginos all included less than 50 samples, with Hanna only using four, whereas Thurlow and Roepman analyzed close to 100 samples. All studies included tumours of mixed locations. O'Donell kept to the oral cavity while all other studies included samples from the whole head and neck region. Choi *et al.* compared gene lists from existing publications on microarray analyses of SCCHN in 2005 and found 84 genes to be common across multiple studies [63]. Many of these were previously known to be changed in SCCHN, such as Matrix

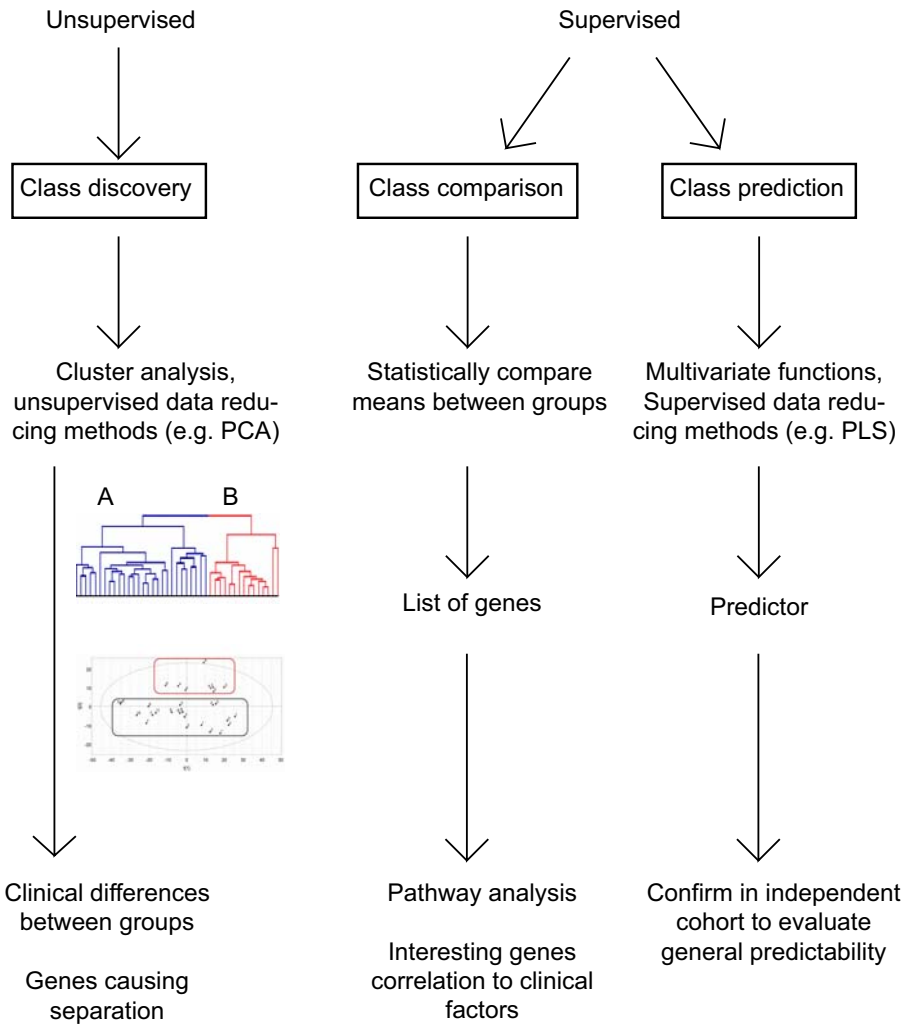

Figure 2 Three common approaches for microarray analysis. Class comparison and class prediction are both supervised methods where differences between predefined groups are of interest. The objective of class discovery is to determine if expression profiles are different between groups and if so which genes are differentially expressed. Overrepresented cell functions among those genes can then be identified or individual genes can be correlated to clinical factors. For Class prediction the emphasis is on developing a predictor/classifier that accurately can predict the group membership of a new sample. These predictors should preferably be confirmed in an independent selection of samples to evaluate its performance outside the samples that helped build it. Class discovery is fundamentally different from the other two. It is unsupervised and novel sub-groups are identified solely based on sample expression profile. The identified sub-groups can then be analyzed for their correlation with clinical factors such as survival and the genes causing them can be identified. Unsupervised methods can also be used to evaluate data; are for example expected groups apparent or do samples form groups based on a non-biologically relevant factor such as for example date of analysis.

metalloproteinases (MMP), integrins, keratins and chemokines. Others had not been well characterized in SCCHN previously, such as transglutaminase3

(TGM3) which was the only gene in seven studies of metastatic status that was common in at least three of them.

There are to our knowledge three previously published studies utilizing large scale microarray techniques investigating mRNA expression and focusing only on tumours of the tongue [72-74]. Ye *et al.* investigated differences in tumours compared to normal tissue while Estilo *et al.* also included analysis of tumours of different stages. Carinci *et al.* looked at genes involved in progression from dysplasia to metastasising tumours. All studies generated gene lists but because of the differences in design these are not comparable. Ye *et al.* generated data for 26 tongue tumours and 12 controls but also included an additional previously published 27 datasets from tongue tumours and ten datasets from controls by downloading them from GEO (database for high through put gene expression data) or asking the authors for them, in this way increasing the number of samples included and the quality of the study significantly.

microRNA and head and neck cancer

In the past the human genome has been hypothesised to contain hundreds of thousands of genes but with the sequencing of the human genome this number has gradually decreased and the consensus today is around 20-25,000 coding genes. This means that the number of genes in humans is less than a factor of two greater than that of many much simpler organisms, such as the roundworm (~18,000) and the fruit fly (~14,000). The coding part of the human genome is, however, just a small fraction of the whole genome (1.5%) while the rest consists of non-coding RNA genes, regulatory sequences, introns and non-coding DNA, adding significant complexity to transcription [75].

MicroRNAs (miRNA) are small non-coding RNAs (ca 22 nt) that in a sequence dependent manner bind to and regulate coding RNA at the posttranscriptional level [76]. Research on these RNAs has exploded since their discovery and in 2011 more than 4300 of the published articles on PubMed concerned miRNA. Each miRNA has the potential to target a number of different mRNAs and miRNAs have been implicated in a number of cellular processes and disease states (e.g. cell development, cell proliferation, apoptosis, and cancer). miRNAs are fewer in number than mRNAs but the number of confirmed miRNAs in mirBase is increasing every day [76-79]. These small RNAs are hypothesised to be highly informative and have been shown to be better predictors of tumours of unknown origin than mRNA in microarray studies [80]. Reasons for this could be that miRNA are closer to their biological function than mRNA which first has to be translated into protein. The higher number of mRNAs present also makes analysis more biased. That some miRNA seem to be highly tissue specific is also beneficial from a prediction point of view. A number of studies have investigated miRNA expression in SCCHN but again the methods, the number of miRNAs included and the selection of patient samples varied and results are therefore difficult to compare. A number of miRNAs have, however, been repeatedly connected to SCCHN, for example miR-21, miR-375, miR-203 and miR-155 [80-88].

Archival formalin fixed samples from patients

RNA analysis in formalin fixed paraffin embedded samples

The low availability of fresh samples from patients is one of the largest limitations for microarray studies today [53,55,57]. Retrospective gene expression profiling of archival samples for which clinical follow up is already available is therefore an attractive alternative. The standard procedure for preserving patient tissue for diagnostic purposes is formalin fixation and paraffin embedding (FFPE) and there are millions of such samples archived around the world. FFPE is an excellent method for preserving the architecture of a sample for immunohistological investigation and for convenient long term storage. RNA and DNA in these samples are, however, modified and partially degraded making it difficult to analyse them in downstream application. Gene expression studies have therefore been restricted to the sparsely available fresh frozen (FF) samples from which high quality RNA can be extracted [89].

Rupp *et al.* first reported on northern hybridization of formalin fixed samples in 1988 and a few years later it was shown that endogenous mRNA from FFPE samples could be amplified using PCR. In all reports successful amplification was, however, restricted to small fragments and sensitivity much lower than in FF tissue. Since then large efforts have been put into understanding the effect of the fixative and long time storage on samples and optimizing recovery of RNA [89-98].

Three main causes for the low quality of RNA from FFPE samples have been suggested; firstly, RNA degradation, which can occur before, during and after fixation, secondly, difficulties to extract RNA due to cross linking with proteins and thirdly, chemical modifications of nucleotides by formalin rendering RNA resistant to reverse transcription and more sensitive to degradation [89]. A large number of factors have been investigated for their influence on RNA in FFPE samples including specimen size, fixation delay, fixative, storage conditions, fixation time, pH and temperature [94,95]. Fixation at low pH has a strong impact on RNA degradation and most samples are now fixed in formalin buffered to a neutral pH. Preferably samples would also be fixed and stored at 4° Celsius but out of convenience most samples today are fixed and stored at room temperature. The choice of formalin as fixative is mainly because of its low cost and great ability to preserve morphological structures. Additionally it inhibits intrinsic enzymes from degrading proteins and nucleic acids, and also functions as a microbicide. The principal of its actions is cross linking of proteins by forming methylol derivatives, schiffer-bases and methylene bridges mainly between primary amino groups of the amino acid lysine but also between other proximal nitrogen molecules [99].

None of the factors above can be influenced in a retrospective study and most are also difficult to adjust for. Other factors such as optimization of RNA extraction, RNA amplification and cDNA reactions can however be changed to maximize the yield and quality of the RNA [89,90,96-98]. The use of proteinase K in RNA extractions has for example proven essential for removing protein cross linking, increasing the yield significantly [89,96,100]

and the use of random primers or gene specific primers in the cDNA reaction instead of oligo dT primers which target the often missing and highly modified poly A tail increases efficiency of reverse transcriptase reactions [89,101]. Modification of RNA is also a significant inhibitor of the use of FFPE samples as shown by Masuda and colleagues who found that even when longer RNA could be extracted from FFPE samples they still could only produce short fragments using PCR. They hypothesised that this is mainly due to modifications of the RNA inhibiting reverse transcriptase reactions and further showed that the main modification was methylolation of bases. Interestingly they also saw that the modification was nucleotide specific commonly affecting purines (Adenine and Cytosine) and Adenines to the highest extent (40%) (Cytosine 33%, Guanine 7% and Uracil 4%) [89]. Methylolations are partially reversible but can form methylene bridges with amino groups, which are not easily removed. Formalin also causes apurinic and apyrimidinic sites which means that the N-glycosidic bond between the sugar backbone and the bases is broken leaving a blank space in the sequence which is irreversible. Still today in most cases only short fragments can be amplified from FFPE samples and PCR targets must be kept under 200 bp and preferably below 130 bp [90].

While many early studies focused on factors influencing the ability to amplify RNA from FFPE samples more recent studies have focused on the accuracy of the detected expression levels. A number of studies have either compared reproducibility between paired FF samples and FFPE samples or made use of previously validated changes or sub-groupings from FF samples to confirm the accuracy and reliability of expression levels in FFPE samples. Cronin *et al.* for example used qPCR to compare the expression of 92 genes in 62 FFPE samples stored up to 17 years and saw a 90% decrease in signal in FFPE samples over that time period. However, they showed that normalizing data removed this effect and when comparing one paired FFPE-FF sample they obtained a Pearson correlation (r) of 0.91 [102]. Sanchez-Navarro *et al.* analysed a similar number of genes in 30 matched pairs of FFPE samples and FF samples and saw that approximately 80% of the genes were significantly correlated between the tissues. The average Pearson correlation coefficient between the two tissues was approximately 0.56 but while some genes showed very high correlation (r=0.95) others showed very low correlation between tissues (r=0.098). They concluded that correlation was better in moderately to highly expressed genes and for genes where the variation between samples was large [103]. Gravendell *et al.* reported similar results from comparing 55 paired FFPE-FF glioma samples using large scale microarrays from Affymetrix with Pearson correlation coefficients between 0.24-0.73 depending on selected genes and samples. Gravendell further showed that in spite of the not optimal concordance between tissues, data could accurately separate 87% of the tumours into seven intrinsic subgroups identified using FF samples, demonstrating the biological relevance of the obtained data [104]. Hall *et al.* used FFPE samples to generate gene sets distinguishing between SCC and adenocarcinomas and validated the result on a set of 58 fresh frozen samples. Fifty four of these 58 samples could be correctly classified [105]. For the whole genome (WG) DASL assay, a microarray platform specifically designed to handle partially degraded RNA,

two large studies comparing FFPE and FF samples has recently been published [106,107]. Mittempergher *et al.* analysed 20 paired FFPE and FF samples and showed that samples from the same patient, irrespective of type (FFPE/FF), clustered close together using unsupervised hierarchical clustering. When comparing similarities in genes significantly changed 2-fold or more between low and high grade tumours for FFPE and FF samples a concordance of 53% was seen. This overlap increased when considering higher fold changes (>5 fold 63% and >8 fold 81%). The samples they used were obtained from a single year [107]. On the other hand, Kibrya *et al.* saw a poorer concordance between gene lists from FFPE and FF samples (33%) and therefore more closely examined sources of the variation in their data. They could show that type of sample (FFPE/FF) was the largest source for the variation in the data followed by disease status (tumour/normal) when including all samples. When analyzing data for FFPE and FF samples separately the largest source of variation was disease status for both sample types but age of sample was additionally a very strong contributor to the total variation in data for FFPE samples. Kibrya *et al.* included samples stored for 3-6 years [106]. In spite of a sometimes low correlation between FFPE and FF samples all these studies show that biologically interesting data can be obtained from FFPE samples. The discrepancies between sample types, however, indicate that more can be done in optimizing the analysis of FFPE samples to increase accuracy of detected expression levels.

In theory, detection of correct relative expression levels (target/housekeeping) as is the case when performing qPCR analysis should be possible in FFPE samples as long as it can be assured that degradation and modifications proportionally affect both transcripts similarly. Also, normalization of microarray data relies on the assumption that transcripts have been influenced approximately equally by the quality-limiting factors. von Smolinski *et al.*, however, reported on individual changes in relative expression of different mRNA species in FFPE samples and Godfrey *et al.* showed that the detected relative expression of C-MYC in an FFPE sample compared to a paired FF sample varied 10-fold depending on primers used [108,109]. The studies mentioned above, where individual genes show better or worse correlation between FFPE and FF samples also indicate that the detectable expression for individual transcripts is not equally affected by fixation and storage, making gene expression detection in FFPE samples more complex.

Gene expression studies in FFPE head and neck samples

A few studies of FFPE SCCHN samples have been performed [110-113]. Chung *et al.* analyzed FFPE tissue from 26 SCCHN patients and produced a list of 75 genes dividing samples into high risk and low risk groups with significantly different survival [110]. They noticed a bias correlated to sample age in their data but could remove it by using a statistical method developed to correct for batch effects in microarray experiments (Singular value decomposition, SVD). Sahle *et al.* analysed 31 tumours of the buccal mucosa and 12 non-malignant oral mucosa samples, one of few studies focusing on a single tumour location, for their expression of 502 cancer related genes.

They generated a gene list of 102 differentially expressed genes in tumours. A few genes including BCL2A1, ITGB4 and MMP1 were subsequently validated using qPCR and immunohistochemistry [112]. Loudig *et al.* investigated the reproducibility and sensitivity of the DASL assay using cell lines and then compared five oral cavity squamous cell carcinomas with nonaggressive pattern of invasion and seven oral cavity squamous cell carcinomas with an aggressive pattern of invasion. A large number of genes were found significantly differentially expressed between groups, for example genes involved in cell adhesion, calcium binding proteins with EF-hand motif and signal anchor genes [97]. Comparison of gene lists between these studies is not relevant because of the different questions asked but again all studies confirm that biologically relevant data can be obtained from FFPE samples which can contribute to increased understanding of SCCHN and possibly discovery of new biomarkers for the disease.

microRNA detection in FFPE samples

It has been hypothesised that miRNAs are more resistant than mRNA to the effects of formalin fixation and long-termstorage and therefore more suitable targets for studies in FFPE samples. Their shortness, secondary structure and close association to protein complexes could weaken the degradation and modification effect of formalin. Their similar structure might also advocate a more uniform degradation pattern between separate miRNAs [80,99].

A number of studies on miRNA expression in FFPE material have been performed, confirming the high reproducibility between FFPE and FF samples, many of which could also show a better correlation between miRNA profiles from the two sample types than mRNA profiles [80,114]. Even though many studies have shown very promising results for miRNA profiling in FFPE, several have simultaneously reported a loss of miRNA signals with time in storage [115,116]. Szfranska *et al.* thoroughly investigated reproducibility, sensitivity and accuracy of miRNA profiling in FFPE samples of different tissue origin. They used mouse brain, stomach, small intestine and kidney tissue to evaluate reproducibility between FFPE and FF samples and found Pearson correlation coefficients of 0.82-0.97. When analyzing human samples stored for one, seven or eleven years they observed a loss of detected miRNAs of approximately 22%. The loss was predominantly of low or medium expressed miRNAs while all highly expressed miRNAs were preserved. Interestingly they also saw a very large (>100 fold) increase in expression with storage time for some miRNAs and hypothesised that this could be due to non-specific hybridization of mRNA and/or miRNA degradation products to the miRNA array. Szfranska lastly investigated miRNAs differentially expressed between myometrium and B-cell lymphomas and found a 44% concordance between FFPE and FF tissue [116]. This indicates that formalin fixation and storage might also have a deteriorating effect on miRNA expression analysis, which possibly could be improved by understanding the effect better.

Aims

Since its emergence, expectations on microarray studies for biomarker discovery have been enormous. Through experience we are now beginning to learn the lessons needed to utilize the full potential of these assays. One large limitation in many microarray studies is the low number of samples included. FFPE samples have the potential to change this but need to be thoroughly evaluated because of the degraded and modified nature of the RNA in these samples.

General aim

Evaluate and optimize the use of FFPE samples for detection of biologically relevant gene expression changes in tongue carcinoma, with a special focus on microarray analysis.

Specific aims

Paper I	Identify a proper housekeeping gene for FFPE cancerous and normal oral tissue to be used for normalizing qPCR data
Paper II	Identify if biologically relevant data could be obtained from analysing expression of 502 cancer-related genes in FFPE material from cancerous and normal oral tongue tissue, with a secondary aim of identifying differences between young and old patients
Paper III	Identify if biologically relevant data could be obtained from whole genome array analysis of FFPE material from cancerous and normal oral tongue tissue, and in detail describe the effect of differences in sample quality on detected expression levels.
Paper IV	Identify if biologically relevant miRNA data could be obtained from FFPE material from cancerous and normal oral tongue tissue and investigate if introduction of an additional normalization step on gene level could improve data analysis.

Materials and methods

Materials and methods are briefly described here. For detailed descriptions please see individual papers.

Patient samples

FFPE samples came from patients living in the northern part of Sweden which are all treated at Norrlands University Hospital located in Umeå. Diagnostic samples are however taken at the home hospital and samples were provided by five different hospitals in five cities (Luleå, Umeå, Östersund, Sundsvall and Malmö). In paper I twenty FFPE samples from the oral cavity were analyzed. Ten were non-malignant controls and ten squamous cell carcinomas. The different locations included were gingiva (T=5, C=2), hard palate (C=2), tongue (T=4, C=4) and buccal mucosa (T=1, C=2). Remaining papers focused on tumours of the mobile tongue only and no tumour or control samples from any other location were included. In paper II 36 tongue FFPE samples (T=27, C=9) were analysed for expression of 502 cancer related genes. In this study we had a special focus on young patients and 14 of the tumours came from patients younger than 40 years of age. A similar cohort of patients was used in paper IV when analysing the expression of 836 miRNAs (T=21, C=8). For the whole genome analysis in paper III the study was expanded to include all available tongue carcinoma FFPE samples in the northern part of Sweden between 1997 and 2010 (T=70, C=17). In paper III ten FF tongue carcinoma samples were included for confirmation of results and in paper IV five FF tongue carcinoma samples were included for the same purpose.

Response to radiation therapy

In paper II response to radiation therapy was evaluated in patients treated with preoperative radiation followed by surgery. Samples from surgery were investigated by an oral pathologist. If no viable tumour cells were detected in the surgical specimen the patient was said to have complete pathological remission (cPR), whereas if viable tumour cells were detected the patient was said to have non-complete pathological remission (non-cPR).

RNA extraction

FFPE samples were cut in 5 µm sections with 3-20 sections included in every extraction. mRNA from FFPE samples was extracted using the High Pure RNA Paraffin kit (Roche Diagnostics GmbH) according to the manufacturer´s instruction. In brief, sections were deparaffinised using xylene and lysed overnight in proteinase K. Solubilised nucleic acids were washed using filter tubes before DNase treatment and a second round of washing. Purified RNA was eluted in nuclease-free water and stored at -80˚C until use. RNA from FF samples was extracted either using the same protocol (except for incubation in xylene) or using the TRIzol method (Invitrogen). RNA from cell lines was extracted using the TRIzol method. miRNA from FFPE samples was extracted using the High Pure miRNA

isolation kit (Roche Diagnostics GmbH) according to the manufacturer´s instruction. No extra wash step enriching the fraction of small RNA was performed. RNA quantity and quality was measured using a NanoDrop spectrophotometer or a BioAnalyzer system (Agilent).

qPCR reactions

RNA was reverse transcribed either using Cloned AMV First-Strand cDNA Synthesis kit (Invitrogen) or RevertAid H minus first strand cDNA kit (Fermentas) with 200 ng of input RNA and random primers. qPCR reactions were either performed using in house designed primers and the IQ SYBR-green supermix (BioRad) or the Quanti teq primer assay with readily available primers and SYBR green (Qiagen). Primer sequences for in-house designed primers and amplification times and temperatures can be found in corresponding papers. Amplification products were kept short, preferably below 100 bp, and if possible primers were designed to span an intron avoiding amplification of genomic DNA. qPCR reactions for miRNA were performed using the mercury LNA™ universal RT microRNA system (Exiqon) including cDNA reactions.

qPCR reactions were performed to evaluate how well RNA from FFPE samples performed in downstream applications and this was used as quality measurement for the samples. Expression of a stably expressed house-keeping gene was measured both in a small number of FF samples as well as in all FFPE samples and quality was defined as the difference in cycles it took the FFPE samples to reach a threshold value compared to FF samples ($Ct_{diff}=Ct_{FFPE}-Ct_{FF}$). A larger difference meaning a poorer quality.

Software for comparing stability of selected housekeeping genes

Two publicly available software packages, GeNorm and NormFinder were used for comparing gene stability of eight genes (TUBA6, S100A6, ACTB, OAZ1, GAPDH, RPS23, RPL27, HPRT1) across normal and malignant tissue as well as between tissues of different origin in the oral cavity (gingiva, palate, buccal mucosa and tongue) [117,118]. geNorm uses pair-wise comparisons to evaluate the stability of a gene and is based on the assumption that the ratio of two proper housekeeping genes will be approximately the same in all samples. NormFinder is a statistical model based approach taking both the overall expression variability and the variation between subgroups into consideration.

The focused and the whole genome DASL array

Investigation of gene expression levels of coding genes was performed using the DASL assay (cDNA mediated Anneling, Selection, extension and ligation) (Illumina) which is a high throughput method for gene expression profiling of FFPE samples. Initially only a focused array containing 502 cancer related genes was available, but this was later expanded into a whole genome version containing 29,377 probes covering 20,818 genes. In brief, total RNA was converted into cDNA using biotinylated random primers. The cDNA was then annealed to short DASL assay probe sets specifically designed to interrogate each target sequence in the transcripts. Each probe set consists

of an upstream and a downstream oligonucleotide both containing a gene specific sequence and a universal primer landing site. The upstream oligonucleotide was extended and ligated to its corresponding downstream oligoncleotide creating a PCR template that can be amplified using universal primers. PCR products were then washed, precipitated and hybridised to the HumanHT-12 v4 expression bead chip. BeadChips were scanned and images processed using a bead array reader (Illumina), and data was processed and normalized using GenomeStudio (Illumina). All samples were assayed on a single occasion minimizing the day to day variations and variations in execution. Data were normalized using cubiq spline normalization and array performance was evaluated by studying the number of detected genes, background signal, housekeeping gene signal and reproducibility. Genes were considered detected if detection p-values were below 0.01. Normalized data was further evaluated using, Simca-P+ (Umetrics), MultiExperiment Viewer, MeV4.0 (http://www.tm4.org) and Excel software (Microsoft).

microRNA Array

miRNA expression profiling was performed using the miRCURY LNA™ microRNA Array (Exiqon). Locked Nucleic Acid (LNA) bases are structurally constrained by the addition of a methylene bridge to the sugar-phosphate backbone. This results in better stacking and higher stability of the duplex which in turn increases the melting temperature and the specificity of the assay. After RNA extraction all samples were sent to the Exiqon profiling facility where RNA quality check, labelling, hybridisation and scanning of the arrays was performed. Each sample was hybridized together with a common reference consisting of a mixture of all FFPE samples in a two colour set up. Version 11.0 of the array was used, containing all miRNAs in mirBASE version 12.0 in four replicate spots. Raw data and normalized data were obtained from Exiqon and further analyzed using R, Simca-P+ (Umetrics), MultiExperiment Viewer, MeV4.0 (http://www.tm4.org) and Excel software (Microsoft).

Array analysis

Array data was visualized using unsupervised hierarchical clustering and PCA analysis. Gene lists were acquired using three different statistical methods for comparing means, t-test, SAM (significance of microarray, especially adopted for microarray data) and the GenomeStudio accompanying differential gene expression analysis. All calculations were corrected for multiple testing. Gene lists were then further analysed for overrepresented pathways or cell functions using GeneGo.

Statistics

Simple linear regression was used for multiple purposes e.g. when comparing replicate reproducibility and when evaluating the effect of sample quality on array performance and gene expression levels. P-values and/or coefficients of determination (r^2) were presented as the outcome of the analysis. T-test, Mann-Whitney and Wilcoxon tests were used to test differences between means. T-test was used when normal distribution could

be assumed while Mann-Whitney and Wilcoxon are non-parametric tests. Wilcoxon test was used when samples were paired as was the case with the FF samples where normal and tumour sample came from the same patient.

Results and discussion

Results from paper I-IV are collectively presented and discussed below. A visual overview of all four studies can be found in (Figure 3).

Detection of a housekeeping gene for qPCR normalization (Paper I)

Microarray and qPCR are the two most common methods for detection of gene expression changes. While qPCR is used for analysis on a smaller scale, microarrays can be used at the whole genome level. The two methods are also uniformly used to confirm each other by comparing reproducibility between the assays. Normalization is one of the most critical factors for qPCR and selection of a poor housekeeping gene can result in misinterpretation of data [119]. In recent years it has become clear that no single housekeeping gene is stably expressed over all tissues and conditions and instead many of the commonly used reference genes have been shown to vary between tissues and different disease states [120-122]. As RNA from FFPE material in addition is modified and degraded verification of a proper housekeeping gene for the tissue and experimental conditions of interest is essential.

In paper I we investigated the stability of eight potential reference genes in normal and cancerous oral tissue from different locations within the oral cavity. TUBA6 belonging to the tubulin family and the ribosomal protein RPS23 generally had the most stable expression. TUBA6 was slightly more stable both when comparing normal and malignant tissue and tissues of different origin and was therefore used as reference gene in studies II and III. Fairly good reproducibility between array data and qPCR data for selected genes were obtained in these papers. An alternative for increasing accuracy of normalization further could be to take the geometric average of a selection of housekeeping genes, creating a normalization factor (NF). This must be performed with care however, since adding more genes does not directly imply improvement. For example in our data, according to NormFinder, the intergroup variation was oriented towards higher expression in tumours for both TUBA6 and RPS23, and combining the two would thus not be of any major benefit since the intergroup variation would be transferred to the NF. Another gene analysed, ACTB, however had the opposite pattern with slightly lower expression in tumours and combining TUBA6 and ACTB could be beneficial as suggested by NormFinder (Table 1).

Normalization of qPCR data on miRNA expression was done with the commonly used reference gene U48 (SNORD48) coding for a 64 bases long non-coding RNA involved in modification of rRNA. Even if U48 is fairly short it is longer than miRNAs (~22nt) and is thus not the ideal reference gene [123]. Optimally a similar analysis as done for mRNA should be performed to find a stable reference miRNA for oral tissue. At the time of our analysis this would have required a substantial amount of RNA, since only gene specific cDNA reactions were available, and was therefore not feasible. Today a general cDNA kit has been introduced and a stable reference miRNA in oral tissue should be confirmed before performing any future experiments using qPCR.

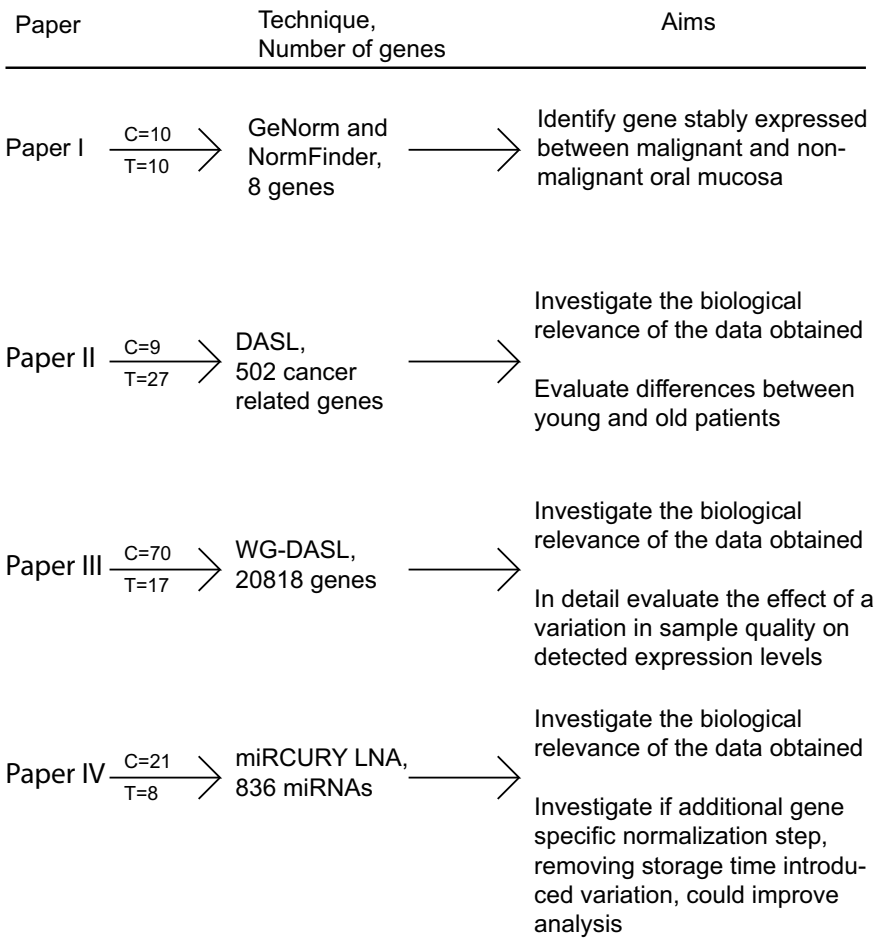

Figure 3 Flowchart summarizing all four papers. Brief description of techniques used, samples included and aims of papers I-IV.

RNA quality and array performance (papers II, III and IV)

It has been known for a long time that RNA from FFPE samples is both modified and partially degraded, two properties making downstream analysis difficult [89]. Recent developments for analyses of RNA from FFPE samples has opened up new opportunities but the techniques are still young and need to be thoroughly evaluated. In paper II we used a focused DASL array to analyze 36 FFPE samples for expression of 502 cancer related genes and in paper III the corresponding whole genome array was used to analyse 87 FFPE samples. In paper IV expression of 836 miRNAs in 29 FFPE samples was studied.

The quality of the RNA extracted from the FFPE samples was investigated using two different methods. In paper II the size of the RNA was evaluated using a Bioanalyzer. The two ribosomal RNAs (28S, 18S) typical

Tab 1. Result from NormFinder on variation between groups and the best combination of two genes

Intergroup variation Group identifier	1	2
RPS13	0.143	-0.143
RPL27	0.143	-0.143
Qaz1	-0.115	0.115
Actin	-0.097	0.097
S100A6	-0.185	0.185
TUBA6	0.072	-0.072
GAPDH	0.039	-0.039

Best gene	TUBA6
Stability value	0.161

Best combination of two genes	Actin and TUBA6
Stability value for best combination of two genes	0.089

for high quality RNA were in most cases not detectable and the majority of the RNA was around or below 200 bp in size. A trend towards higher degradation with time in storage was noticed. In papers II and III a qPCR based method, recommended by Illumina, was used to evaluate RNA performance in downstream applications. This was defined in paper III as the overall quality of the samples and denoted Ct_{diff} (more fully described in material and methods). Samples were of wide quality range but all FFPE samples passed the cut-off, qualifying them for array analysis. Some, but far from all of the variation in sample quality could be explained by sample storage time (r^2=0.18 in paper II and r^2=0.33 in paper III). This is not surprising since modifications rather than RNA size has been shown to be the strongest limiting factor. Fixation time or delay in fixation are thus probably strong factors limiting the quality of RNA from FFPE samples [89].

All samples were analyzed in duplicate in paper II while only two replicate samples were included in paper III. Four arrays in paper II and five arrays in paper III were excluded due to low total signal and reproducibility. For the remaining samples, replicate reproducibility was very high, on average r^2=0.98 for both the focused array and the whole genome array. In paper II two separate biopsies from a single patient were included and also for these samples a very high coefficient of determination (r^2=0.98) was seen. In summary these results show that results from the DASL assay are highly reproducible.

In paper III an effect of sample quality on the expression level of individual genes was noticed. In general samples of poorer quality performed less well on the array and we saw a loss of approximately 800 genes with a one cycle increase in Ct_{diff}. Poorer performance of the array with poorer sample quality is not unexpected, but more worrisome was the fact that individual genes were not influenced equally by sample quality. While the expression of some genes decreased severely with decreased sample quality, others did not show any change. A similar notion was also identified in paper IV for the expression of miRNAs. This gene-specific effect cannot be handled by traditional normalization methods and a non-biological variation caused by a difference in sample quality will remain within the data (Figure 4). A few precautions (as presented in paper III) can be taken to decrease the impact of this bias. Most important when performing class comparison is that samples in groups to be compared should be of the same quality range. The worst scenario would be comparing samples of high quality with samples of low quality where all genes affected by sample quality would be

detected as differentially expressed resulting in a gene list where many genes lack biological relevance (Figure 4). In paper III we could show that when taking this precaution the difference between cancer and control tissue was clearly the largest variation within the data. The list of significantly differentially expressed genes between tumour samples and control samples was of high biological relevance as shown by pathway analysis. The list of genes was further used both for confirming previous results and for making novel findings that also could be confirmed in high quality fresh frozen samples.

Nevertheless the sensitivity of the analysis will still be affected since the standard deviation of the mean expression is one of the factors taken into consideration when statistically comparing groups (Figure 4). Additionally the actual expression value will not be reliable and confirmation using a second method (such as qPCR) is necessary when correlating expression levels to clinical factors. Another approach would be to try and remove the variation introduced by sample quality rather than decreasing its impact. In paper IV we thoroughly evaluated the variation introduced by storage time of sample on the expression level of miRNAs and introduced an additional normalization step with the intention to remove it (described in detail below).

Even though we did not initially notice the effect of sample quality when analyzing the focused array (paper II), going back and taking a second look revealed a similar effect also in this data. For the whole genome array data, expression of approximately half of the detected genes was significantly affected by sample quality whereas only 9% of the genes on the focused array were significantly affected. Because of the large difference in the number of included samples, significance is not the ideal option for comparison between studies. However, a Pearson correlation coefficient (r) between gene expression and sample quality greater than 0.33 always generated a significant correlation in the whole genome array and can thus be used as a cut-off. Using this cut-off, the expression of 35% of the genes in the focused array was affected by sample quality (unpublished data).

Introduction of a novel normalization step for microRNA array data (paper IV)

miRNAs are suggested to be more stable in FFPE samples due to their small size, secondary structure and close interaction with large protein complexes and are therefore a preferable target for analysis. Previous studies have also indicated that they are very informative: not only are miRNAs able to classify tumours into subgroups and identify tumours of unknown origin, but they also do it with higher accuracy than mRNA [80]. In paper IV we analysed expression of 837 miRNAS in 29 tongue FFPE samples using an array containing locked nucleic acid probes (LNA).

Similar to paper III we early on noticed a large non-random variation within the array data that was not of any biological relevance. Using principal component analysis (PCA) we could see that this variation was highly correlated to the time the sample had been stored and that it represented approximately 56% of the total variation within the data.

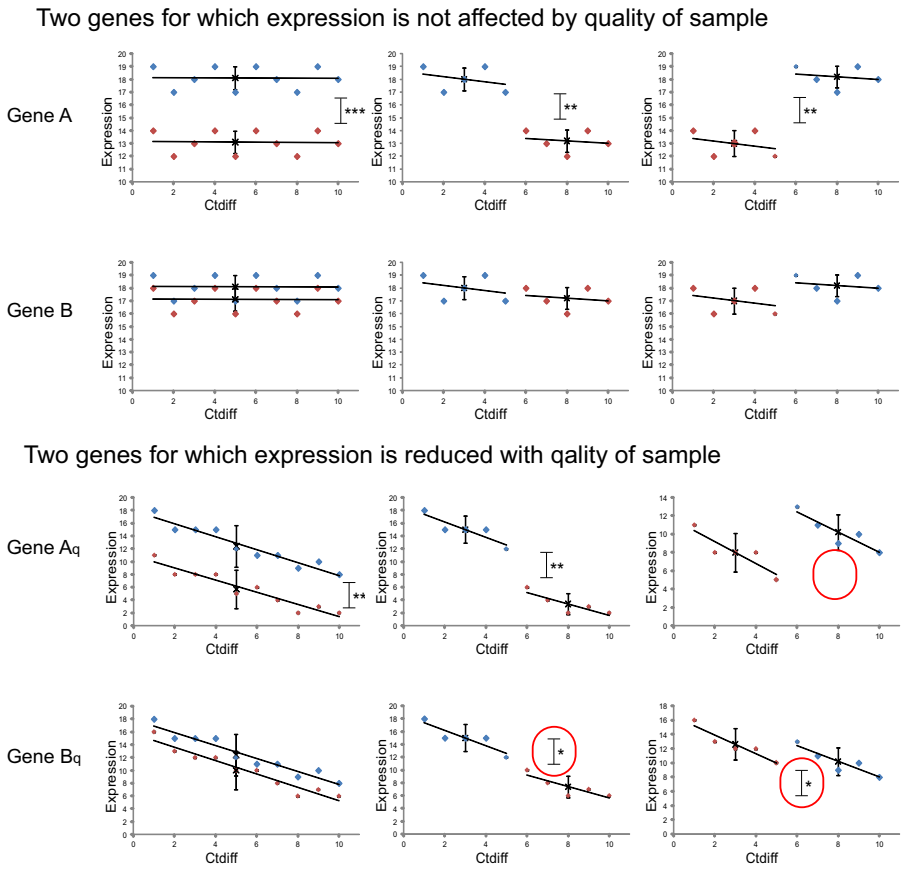

Figure 4 Hypothetical data to illustrate the effect sample quality can have on the analysis. Upper panel illustrates two genes whose expression is not correlated to quality of sample (A and B). Sample quality (Ct_{diff}) is on the x-axis and detected expression level on the y-axis. Gene A shows a highly significant differential expression between cancer (red) and control (blue) samples. This does not change if poor quality tumours are compared to high quality controls or the other way around. Gene B is not significantly differentially expressed between cancer and tumour samples and again this does not change if selecting sample of different quality. In the lower panel you see the same two genes but this time expression level is dependent on sample quality (Aq and Bq). Gene Aq is still differentially expressed but with lower significance level because of the additional variation introduced. The same result is obtained when comparing high quality controls to low quality tumours. Comparing low quality controls to high quality tumours for gene Aq however does not result in any significant difference. Gene Bq is when including sample of similar quality not changed between cancer and control samples. Selecting tumour or control samples of high quality and compare to control or tumour samples of low quality for gene Bq gives a significant p value, however. This is not a true difference but solely caused by the difference in quality of sample. The direction of the change is additionally dependent on what group of sample (tumour/control) is chosen to be of higher quality. Mean value and error bar is shown for both tumour and control samples in each plot. Significance is indicated by stars were one star is $p< 0.05$ two stars is $p< 1.0E-5$ and three stars $p< 1.0E-10$

The separation between tumours and controls which clearly was the second largest variation within the data only represented 13% of the total variation.

The effect of storage time was not identical for all miRNAs and again could not be removed by any standard normalization method, instead a miRNA specific normalization step was needed. That the effect of storage time on individual miRNAs seemed fairly linear suggested that a linear regression model could be used for that purpose. We therefore introduced a novel normalization procedure. Initially data were normalized using a standard method to overcome common systematic bias seen in microarray experiments. If the effect of storage time had been equal on all miRNAs it would have been removed at this step. Instead we used linear regression modelling to estimate the size of the effect of storage time on each miRNA separately and thereafter subtracted it from the normalized values. By doing this the number of miRNAs detected as being significantly differentially expressed between tumours and control samples increased more than 3-fold and plotting data using PCA no longer separated samples according to storage time (Figure 5). All 16 miRNAs detected as changed before the additional normalization were also among the 56 miRNAs detected as changed after the additional normalization. Several miRNAs now known to be involved in oral cavity cancer e.g. miR-203, miR-21, miR-375, miR-146a, miR-100, let-7 and miR-99a were significantly changed only after the additional normalization step. This indicates that removing the effect of storage time from the measured expression levels of individual miRNAs increased the sensitivity of the analysis.

From our and others previous experience it is known that for FFPE samples storage time only represents part of the problem limiting the use of them. Even better results from the normalization might have been obtained if a more complete measurement of the usability of RNA samples in downstream applications (such as Ct_{diff}) would have been considered.

Before performing the additional normalization step rigorous characterization of the storage time dependent variation was performed. For example the hospital where samples were handled was considered as a possible confounder, since many of the older samples came from other hospitals than Norrland's University Hospital (Umeå). This was, however, not the case. Patient age was also investigated and excluded as a possible confounder. The possibility that the effect of storage time was initiated only after a certain time in storage or by sheer coincidence was also excluded.

Properties of genes influencing the effect of storage and fixation (papers III and IV)

Modifications of bases in FFPE samples affect separate nucleotides with different affinity and how a gene performs in downstream applications could therefore be sequence dependent. In paper III we noticed that probes with a low number of guanines and a high number of cytosines were less affected by sample quality. Mittempergher *et al.* have previously reported a higher GC content in probes with better correlation between FFPE and FF tissue and both our studies suggest that sequence is very important for array performance [107]. For miRNAs the number of consecutive guanines and the number of consecutive adenines showed a significant association with time in storage while the total number of any of the four nucleotides did not

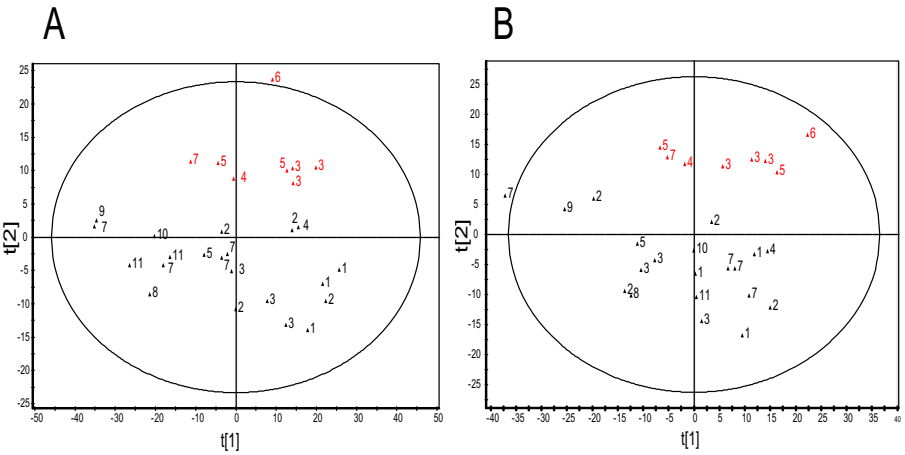

Figure 5 PCA plot of miRNA data. PCA analysis of miRNA data without (A) and with (B) the additional normalization step. Both graphs show first and second components. Controls are marked in red and tumours in black. The number beside each triangle denotes years in storage.

(paper IV). Together these results show that different aspects of the sequence are important to consider when developing new assays or normalization methods for analyzing RNA from FFPE samples in the future. To make this possible a more thorough investigation of the different effects of sequence is needed.

RNA size might also affect the stability and how well a gene can be detected. In paper IV we saw that the number of detected miRNAs did not decrease with time in storage but rather increased instead. Also in paper III we saw an increase in expression of many small RNAs with a decrease in sample quality. This could possibly be explained by a change in the composition of total RNA with an enrichment of the more stable small RNAs in samples of poorer quality. This in turn leads to hybridization of a higher concentration of small RNAs to arrays when using samples of poorer quality. Another possibility is increased non-specific hybridization of mRNA and immature miRNA degradation products to miRNA probes for samples of poorer quality. According to these results short RNAs should therefore be analysed separately and not included in mRNA expression arrays or the other way around.

Gene lists and their reliability (papers II, III and IV)

Lists of significantly differentially expressed genes between cancer and control samples were presented in papers II, III and IV. These lists were evaluated for the presence of genes previously known to be changed in oral cancer and their overall biological relevance. In paper III for example we could confirm 25 of the 35 genes in the gene signature presented by Ye *et al.* for FF tongue carcinoma samples [73]. To confirm the accuracy of genes not previously connected to oral cancer a few FFPE samples were reanalyzed using a second method (qPCR). Some of these genes were also confirmed in a smaller number of FF samples. In general we saw good correlation between

array data and qPCR data for the FFPE samples and all genes analyzed in FF samples were in agreement with the array data. The confirmed genes were involved in a number of different cellular processes including apoptosis (BCL2A1), immune response (CXCL10), energy transport (SLC2A6) and angiogenesis (miR-424) and also potentially interesting for tongue cancer development and maintenance. In these studies we have not further investigated the clinical relevance of any of the genes but rather used them to show that novel findings of potential relevance can be made in FFPE samples and confirmed in FF samples.

A study very similar to our paper II, with the exception that the authors investigated tumours of the buccal mucosa instead of tongue, was published in 2010 [112]. Comparing the lists of genes changed in tumour tissue revealed a surprisingly high concordance between the studies [124]. For example the same set of five MMPs (matrix metalloproteases) was identified in both studies. In total there was 60% similarity between the gene lists from the two studies and seven of the ten top genes were common for both studies. These studies act as independent validations of each other and further confirm the excellent reproducibility of the assay, even when two very different study populations with different living conditions and ethnic background were analyzed. The concordance between these studies also indicates that these genes really are of relevance for oral cancer and should be further studied concerning their clinical importance. Interestingly the same MMPs (MMP1, 3, 7, 9 and 10) were also detected as significantly changed in the WG array with the addition of three more MMPs (MMP11, 12 and 13) not analyzed using the focused array. Exactly the same eight MMPs were also found changed in the study by Ye *et al.* on FF tongue carcinoma samples [73]. MMPs have previously been suggested as possible biomarkers for detecting and monitoring tongue cancer [125] and the consistent detection of these eight MMPs across sample type, irrespective of tumour location and patient population make them interesting targets to analyze.

Comparing results from our own two studies (paper II and paper III) the overall overlap between the focused array and the whole genome array was good. Out of the genes detected as significantly differentially expressed in the WG array and also included in the focused array, 94% were changed in the focused array as well. Comparing the other way around showed that only approximately 40% of genes with significantly changed expression in tumours in the focused array were also found significantly changed in the WG array. This probably reflects a difference in size and sensitivity between experiments. For the WG array you amplify and analyse over 20,000 genes while the focused array contains a restricted number of well investigated genes and therefore is more sensitive. This is further illustrated by the fact that almost 30% of the genes detected on the focused array could not be detected in the whole genome array.

Analysis of young patients (papers II, III and IV)

Patients younger than 40 years of age only constitute a few percent of all tongue cancer patients but an increase in the number of cases, higher than the increase among older patients, has been noticed during the last decades

[15]. This has led to many speculations about similarities and dissimilarities between young and old patients. We tried to identify gene expression differences between tumours in young and old patients, but could not find any genes that were significantly changed in young patients as compared to older patients in either of the studies. This indicates that there are no general differences in developed tongue carcinomas that can be detected at the gene expression level between young and old patients and that a division based simply on age is not beneficial. A few molecular differences in young patients as compared to older patients have been described in the literature before, as reviewed in the introduction. Few samples were used in all these studies which constitutes a general problem when studying this disease. Even though we used samples that had been stored up to 11 years we only had 17 samples from young patients and a further selection based on for example smoking status or treatment response was not possible. This makes groups very heterogeneous which in combination with small sample size makes detection of reliable differences very difficult.

DNA repair genes causing a sub-grouping of patients (paper II)

Unsupervised hierarchical clustering is a method where unknown groupings of data can be discovered. It has to be used with care as a built in characteristic is that samples will always form clusters [126]. Unsupervised hierarchical clustering is also one dimensional and therefore only takes the largest variations in the data into consideration meaning that other separations might be there although not visible. In paper II tumour samples separated into three groups using unsupervised hierarchical clustering. Interestingly, most of this separation was caused by a number of DNA repair genes. Up-regulation of three DNA repair genes (BARD1, CCNH and FANCG) was largely the cause of separation of one group of samples while a down-regulation of four other DNA repair genes (BRCA2, XRCC2, BLM and RECQL) was largely the cause of separation of another group of samples. The fact that patients in the group characterized by lower expression of DNA repair genes seemed to respond better to radiation treatment (all patients treated with preoperative radiation and surgery had complete response to radiotherapy (4/4)) was intriguing, indicating that tumours with a higher expression of DNA repair genes might be more resistant to radiotherapy and therefore could benefit from a combinational treatment also targeting DNA repair. These results mainly indicate that DNA repair as a process might be important for radiation response rather than pinpointing these specific genes.

Previous result on radiation response and DNA repair in SCCHN include changes in for example expression of DNA-PK (DNA dependent protein kinase) important in the non-homologous end joining (NHEJ) pathway for repair of double stranded DNA breaks [48,127,128]. Microarray studies comparing radiation resistant SCCHN tumours to radiation sensitive SCCHN tumours have also been performed. Hanna *et al.* for example constructed a 60 gene prediction profile that could predict two test tumours accurately. This 60 gene profile, indicative of a poor radiation response, contained among many other interesting changes, two strongly down-regulated DNA

repair genes (XRCC1 and ERCC1) [67]. These studies and our own data show that DNA repair activity is an important cellular function to study when investigating radiation treatment response in SCCHN.

General discussion and future perspectives

Today we have the knowledge to extract an adequate amount of RNA of sufficiently high quality from FFPE samples for analysis using qPCR and microarrays. We have confirmed the excellent reproducibility of these assays in combination with FFPE samples and found that biologically relevant and interesting data can be obtained from them. Now it is time to optimize the analysis and use of FFPE samples so that this invaluable source of material can come to its best use. RNA from these samples is of poor quality, something we cannot change. The choice of probe and gene selection might, however, have a large impact on the analysis and is something we can influence. It has not yet been thoroughly clarified if degradation and modification of nucleotides lead to a change in relative RNA levels in the actual patient sample, an issue difficult to deal with, or if the disagreements between FFPE and FF samples noticed are caused mainly by problems in the analysis of the transcripts. When comparing relative C-MYC levels in one FF sample and its formalin fixed counterpart using three different primer sets, Godfrey *et al.* saw large differences depending on the primers used indicating that location of the primer within the gene and/or primer sequence is of outmost importance for the detection of a gene [108]. The first of their primer pair showed similar relative expression of C-MYC in the paired FF and FFPE sample, the second slightly reduced relative levels of C-MYC in the FFPE sample and using the third primer pair the level of C-MYC was 10-fold lower in the FFPE sample compared to the FF sample. That we as well saw a relationship between the sequence of a probe and how strongly gene expression level of the corresponding gene is affected by sample quality further supports the importance of sequence. A more systematic large scale analysis of genes and primers is needed to clarify the true relationship between primer/probe properties and the detected expression levels, which could aid in future design of arrays.

The design of a study can either reduce or increase the influence of sample quality and affect the accuracy of the retrieved gene lists. One of the most important factors is to use samples of the same quality range when comparing groups. An optimal design for example would be to do paired analysis of tumour and adjacent normal tissue from the same patient obtained at the same occasion and therefore stored and treated similarly. This might be difficult to achieve as normal samples from patients are not routinely collected and a more likely design would be to analyse a larger number of samples for their quality using qPCR and then perform array analysis on a selection of these sample that are quality matched. Because of the large availability of FFPE samples this will in many cases be feasible and still allow for inclusion of large numbers of samples .

The effect of fixation and long-term storage on detected gene expression levels could possibly be removed by including additional normalization steps taking a bias specific for individual genes or groups of genes into consideration. We used a linear regression model to describe and subtract the effect of storage time on detected expression levels of individual miRNAs from the miRNA array. Even though it was a fairly straightforward

normalization step only taking, storage time of sample into consideration we saw substantial improvement of data and after including it we could detect changed expression of a number of miRNAs expected to be altered in these tumours. To improve the regression based normalization step further, a more complete measurement of factors limiting the use of RNA from FFPE sample (possibly Ct_{diff}) could be used, or sequence specific effects could be considered. Also a non-linear relationship could be interesting to investigate but for most of these factors a considerable number of samples are needed.

Microarray studies on samples from patients have the potential to contribute essential information for improving patient treatment and survival, and sub-groups of patients can be discovered. For these assays to be reliable and not only be true for the studied population, large number of samples needs to be included in the primary analysis and results need to be tested on additional samples. This is a substantial problem since archived high quality FF samples are sparse and to do prospective studies takes many years. Therefore the use of FFPE samples for which you often have long term follow up data is extremely attractive. That biologically relevant data can be obtained from these samples has been shown in numerous studies and we have confirmed that this is also true for tongue carcinoma. We have also shown that optimizing experiment design, data handling and array design will further improve the analysis of these samples, which in the future hopefully will lead to detection of new biomarkers improving early detection of disease, individualised treatment and quality of life for patients.

Conclusions

TUBA6 is a stably expressed housekeeping gene in oral malignant and normal tissue (**paper I)**

The reproducibility for large scale gene expression analysis of FFPE samples is very high, proving the assays to be very robust (**Paper II and III**)

RNA from FFPE samples are of very varying quality (Ct_{diff}) which partially (to 30% in our studies) can be explained by a difference in storage time (**Paper II and III**)

Samples of poorer quality perform less well in array experiments and we see a loss in the number of detected genes in samples with higher Ct_{diff} (**Paper III**)

The effect of sample quality on the detected expression levels differs between individual genes. Some genes show a high or moderate decrease in expression with poorer sample quality, while others show no decrease or even, for a small number of genes, an increased expression with poorer quality of sample (mainly small RNA) (**Paper III**)

As long as a variation introduced by difference in sample quality remains in the data, design of the study is crucial for accurate analysis (**Paper III**)

Introducing an additional normalization step on the gene level can drastically improve results from microarray data analysis of FFPE samples (**Paper IV**)

Biologically relevant and important data can be obtained from FFPE samples but there are still a lot of improvements that can be made to array design, experiment design and data handling for further improving the quality of the data that can be obtained from these invaluable samples (**Paper II, III and IV**)

Acknowledgements

Many people have helped, guided and supported me throughout this work. I would like to pay a special THANKS to:

Karin Nylander my supervisor, it has been an unbelievable journey where I have learned far more than I thought I signed up for four years ago. Your positivity and enthusiasm is inspiring. Thanks for giving me chances to face challenges I did not believe I was capable of and the freedom to work independently.

Göran Laurell my co-supervisor, for opening up a whole new world beyond the cell to me. Your clinical expertise has been invaluable.

Philip J Coates my co-supervisor, your incredible knowledge and interest in science is an inspiration to me. Much of my work would not be the same without your expertise.

Bodil Bäcklund and **Astrid Höglund,** our two technicians and **Cathrine Johansson** over at ENT, thanks for always sharing your expertise and for many nice chats. Astrid and Cathrine those sectionings off FFPE samples would have taken me ages I'm very grateful for your help.

To other, former and present members of the Nylander group, thanks for many insightful discussions. **Xiaolian Gu** for introducing me to the lab, you are such an inspiration and a spreader of joy! **Linda Boldrup**, for uncountable discussions covering everything from science to everyday life. Starting taking riding lessons for the first time at an age of 27 was a terrific experience. My time here would not have been the same without you! **Karin Danielsson**, my thesis writing companion. I'm very glad we shared office when all the others were off on maternity leave. Writing all day can be very exhausting and I was lucky I could just turn around and steel a few of your minutes to talk about something totally different to clear my mind. **Sara Hultin**, the first student I supervised and shared first author of paper I. I'm very glad to have gotten to know you and also happy that I did not manage to scare you away from science, good luck with your thesis. **Majid Ebrahimi**, we only shared office for a couple of months but your frenetic writing and happy face at the dissertation party showed me what the goal ahead was. **Björn Sjöström** for your efforts in collecting samples and incredible knowledge and understanding of the patients.

Patrik Rydén and **Jessica Fahlén,** co-authors, for a very inspiring collaboration resulting in paper IV.

A very big thanks to all the administrative personnel here at the department especially **Karin Boden**, **Terry Persson**, **Helena Fousinette, Clara Olsson, Tarja Rantala** and **Åsa Lundsten**. You carry this department and do it excellently.

Former and present **employees of the department of Medical Biosciences** thanks for four terrific years. It is you that has created this inspiring workplace. I have so many happy memories from "Christmas present games", wine tasting evenings, "MedPub" and "Thursday fika". A special thanks to **Vincy Eklöf** who always make me happy with here bubbly personality we really miss you here at the lab. Good luck with your studies and research.

Finally I would also like to thank all the people that have been a part of my life outside of work. You are my inspiration!

Pernilla L, I can't believe it's already 11 years since we walked out of that classroom after our first University lesson in mathematics wondering where this would take us. We have shared so much fun these years I'm so happy that I got the chance to get to know you, you are one of a kind!

Åsa J my only "native" Umeå friend (even though you now have abandoned us for the big city). I'm very happy you still have your roots here and come and visit from time to time. You are a very good friend with a very determined personality and I know it will take you far!

To all the people in the "Pysselgruppen" there is nothing as relaxing as working with your hands and drinking tee after a long day of thinking. You are all such good and caring friends. **Therese E** your paintings are beautiful I'm very happy I have one hanging on my wall. **Mari L** your small animals just as wonderful, I wish you all the best with your future adventure. **Christina S** our weekly "dancing and sauna nights" has really been an inspiration when darkness and tiredness has tried to take over. I'm really looking forward to visiting you in Scotland. **Pernilla L** you have so much creativity in you, now we all know that comics can make beautiful art and toys great jewellery. And to all other pysslare: BIG THANKS for your eminent company.

Ann-Mari N, **Fia J** and **Mari K**, you always make it fun to come home. Sharing childhood/youth is to carry a special piece of someone that no one ells will never really know. I hope to always be a part of your life.

To my closest family mamma **Agneta**, pappa **Göran** bror **Martin**, svägerska **Anna-Karin**, and mormor **Margit** I know I have not been very close in distance to you the past 12 years but you mean the world to me. We love visiting you all and there will never be a place like home!

To the **Kirkwood and Nilsson family** for always welcoming us both in Kiruna, Halland and Scottland. We have such good times wherever we meet.

♥♥♥

At last but absolutely not least all my love to **Michael Hall** the kindest most caring partner and dad, together we can do anything, and to my stubborn most wonderful little **Alexandra,** you are our angel.

♥♥♥

This work was supported by Lion's Cancer Research Foundation, the Swedish Cancer Society, Cancer Research Foundation in northern Sweden, Kempe foundation, Vasterbotten County Council and The National Board of Health and Welfare.

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