Chapter 7

General summary and future aspects
Salivary gland tumors are a heterogeneous class of tumors which despite clinical and technical advancements are difficult to diagnose. One major cause of this is the overlap in histopathology of these tumors. Other important causes are the rarity of salivary gland tumors, and the lack of molecular diagnostic markers that may help in identifying the tumors subtype. The global annual incidence of all salivary gland tumors (malignant and benign combined) ranges from 0.4-13.5 cases per 100,000 population worldwide and 3 cases per 100,000 population in the Netherlands.

Currently, clinical examination, ultrasound scanning with or without fine needle aspiration cytology, preoperative CT-scan and MRI are available for the differential diagnosis in parotid gland swelling. The performance of fine needle aspiration cytology in parotid gland lesions has a specificity of 97% and a sensitivity of 80%. However, the performance variability is relatively large, so improving the accuracy, e.g. by utilization of additional (molecular, non-invasive) biomarkers, is still needed. Therefore, using novel biomarkers as an additional tool can add to the accuracy of diagnoses of salivary gland tumors.

This thesis aimed to find novel, molecular, non-invasive biomarkers for the profiling and diagnosis of salivary gland tumors. Biomarkers can be found at DNA, RNA and protein levels, and all three levels were addressed: (1) on the protein level through investigating the expression of mucins and mucin-associated carbohydrates by immunohistochemistry, (2) on the DNA level by cataloging genomic aberrations using arrayCGH, (3) on the RNA level by determining the miRNA expression levels in whole and parotid saliva by RT-qPCR.

**Altered expression of mucins and mucin-type carbohydrates in mucoepidermoid carcinoma.**

Mucoepidermoid carcinoma belong to the most common malignant salivary gland neoplasms, consisting of mucous, intermediate and epidermoid cells and are classified based on histological characteristics as low, intermediate or high grade tumors. Chapter 2 demonstrates the abnormal expression and localization of mucins and mucin-type carbohydrates in mucoepidermoid carcinomas originating from the parotid gland and from minor salivary glands.

Aberrant expression of membrane bound MUC1 and MUC4, which in normal tissues have an apical membrane localization, resulted in a distribution over the entire membrane highlighting the loss of membrane polarity in MECs. In the parotid gland, MUC1 was expressed in low, intermediate and high grade MECs, while MUC4 was expressed in low and intermediate grade MEC but was absent in high grade MEC. In the minor glands, this difference in MUC1 and MUC4 expression was less pronounced.

The salivary mucin MUC5B and the gastric mucin MUC5AC were expressed in MEC of parotid and minor glands, particularly in low grades. The expression of the gel-forming secretory mucins, MUC5AC and MUC5B derived from the parotid gland is unique. The normal parotid gland is a purely serous gland and therefore cannot express gel-forming secretory mucins. For the minor glands, this is less specific because minor salivary glands are often mixed (serous/mucous acini) and can express MUC5B but not MUC5AC. Intriguingly, MUC5B
and MUC5AC expression was also found in intermediate and epidermoid type cells. These results are in line with previous studies with MECs and other malignancies.4,5

The glycosylation machinery is frequently perturbed in cases of inflammation and in diseases such as cancer, resulting in the expression of truncated carbohydrate chains.6-9 The expression of simple mucin-type Tn and T carbohydrates in low grade MECs was comparable to that in normal tissue, but decreased in high grade MECs. The sialyl-Tn antigen, however, was upregulated in MEC more frequently in the parotid gland than in the minor glands. Sialylation of Tn will terminate the further elongation of Tn to larger oligosaccharides, indicating that in MEC (mucin)glycoproteins are decorated with short, immature oligosaccharide sidechains. It has been suggested that the aberrant expression of mucin-associated antigens is a result of non-functional sugar-transferase such as T-synthase.10 A specific molecular chaperone, COSMC, is required for the correct folding of T-synthase to form an active enzyme.11 When COSMC is lost or mutated, the T-synthase is folded incorrectly and therefore becomes less active or even inactive, resulting in the expression of Tn antigens. The loss of COSMC function is only associated with the loss of T-synthase activity, and no obvious changes in other aspects of protein glycosylation have been observed.11

The expression of mature carbohydrate epitopes Lewis and sulfo-Lewis was found in low grade MEC; Lewis was found in both mucous and non-mucous cells and sulfo-Lewis primarily in mucous cells. Because of the observation of the expression of mature carbohydrate epitopes, Lewis and sulfo-Lewis, we concluded that the COSMC must be mutated and not deleted, otherwise no mature carbohydrate epitopes would be expressed.

**Chromosomal copy number aberrations in mucoepidermoid carcinoma.**

Chromosomal DNA changes such as the amplification and/or the deletion of a chromosomal region are frequent occurrences in cancer. Some tumor types can be characterized by chromosomal translocations, in which one specific region of a chromosome is switched with another region of a different chromosome. This is also true for a subset of MEC which have a t(11;19)(q21;p13) translocation. The presence of the translocation in MEC is associated with a more favorable prognosis.12,13

In Chapter 3, a genome wide copy number aberration analysis was conducted by using micro-array comparative genomic hybridization (arrayCGH) technique on 27 MEC samples (10 translocation negative and 17 translocation positive).

Low/intermediate grade MECs had significantly fewer copy number aberrations than high grade MEC samples. Furthermore, translocation positive MEC samples had fewer copy number aberrations compared to translocation negative MEC samples. These results fit the non-aggressive nature of low grade and translocation positive tumors, which generally have a more favorable prognosis than high grade and translocation negative MEC samples.12,13

Within all 27 MEC samples, the most frequently gained regions were 16p11.2 and several regions on 8q, while the loss of region 1q23.3 (RGS4) was the most frequently detected loss. The most frequently detected copy number aberrations in low/intermediate grade MEC
samples were the loss of 1p31.1, 1p31.1-p22.3, 12p13.2, and the gain of 16p11.2. The most frequently detected copy number aberration in high grade MEC samples was the loss of 8p23.3-p21.2. The most frequently observed copy number aberration in translocation negative MEC samples was the deletion of 3p14.1 (FOXP1), which was observed in 4 of the translocation-negative MEC samples. No recurrent copy-number-aberrations were found in translocation-positive MEC samples.

Based on the number of copy number alterations and translocation status, two subgroups could be made: (i) one group in which the majority of the samples were translocation-positive and had 6 or fewer copy number alterations, and (ii) a group in which most of the samples were translocation-negative and harbored multiple (>6) copy number alterations. This could suggest that there are different oncogenic pathways for MEC: one involving a relatively restricted aberration leading to the generation of a fused gene, and one involving more generalized chromosomal aberrations.

Differences in recurrent copy number aberrations have been reported in a number of other studies and underscores the fact that heterogeneity in mucoepidermoid carcinoma is a common phenomenon. Only one region found in this study, the deletion of 9p21.3, was recorded by earlier studies. The loss of 9p21.3, containing CDKN2A/B, is associated with an unfavorable prognosis and is also deleted in other salivary gland tumors such as adenoid cystic carcinoma and salivary duct carcinoma. Furthermore, the deletion of 9p21.3 is a frequent oncogenic event observed in head and neck squamous cell carcinomas and in lung cancer.

The recurrence of some chromosomal aberrations found in chapter 3, may suggest the involvement of certain specific genes. However, the genetic instability of these malignancies seems to be more important than a gain or loss of a specific chromosomal region(s) and/or gene(s).

Taken together, these results suggest that the number of copy number alterations in general, rather than aberrations in specific chromosomal regions may be used for differential diagnosis in MEC.

**Salivary microRNA biomarkers for salivary gland tumors.**

The altered gene expression due to cancer-associated changes in chromosomal DNA may impact the expression of RNA (mRNA and microRNA) and may be used for diagnostic purposes. Most research investigating the diagnostic value of mRNA and miRNAs is tissue-based, but in recent years, salivary diagnostics has gained serious interest. It has evolved from the field of oral diseases to the field of systemic diseases. One of the new players in the salivary diagnostic field is miRNA. Salivary miRNA is both abundant and relatively stable. Taking into account the involvement of miRNAs in developmental processes, it is not surprising that several studies revealed tumor-specific miRNA expression patterns.

In chapter 4, the expression of salivary miRNA was explored to compare miRNA expression levels in whole saliva from patients with a parotid salivary gland tumor and miRNA expression levels in whole saliva from healthy individuals (Chapter 4). A combination of two
miRNAs (hsa-miR-1233 and hsa-miR-211) was able to distinguish patients with a parotid gland tumor from healthy controls (sensitivity of 91%, and a specificity of 86%). Strikingly, the miRNA species that were detected in whole saliva were not present in the secretions from affected parotid glands. The differences in miRNA expression which we observed between unstimulated whole saliva (Table 3) and stimulated parotid saliva (Table 4) suggest that parotid saliva may have a totally different miRNA expression profile. Other studies have shown that tumors express miRNAs which may be involved in intercellular crosstalk. Tumor cells can transport genetic material, including miRNA, encapsulated in micro-vesicles (exosomes) to neighboring and/or distant cells, thereby affecting the miRNA expression of those cells and supporting cell growth and progression\textsuperscript{30,31}.

Additionally, differences in miRNA expression levels were determined in whole saliva from patients with a benign versus malignant parotid gland tumor (chapter 5). A combination of four miRNAs (hsa-miR-132, hsa-miR-15b, mmu-miR-140, and hsa-miR-223) could distinguish whole saliva samples from patients with a benign parotid gland and that from patients with a malignant parotid gland tumor (AUC 0.9; specificity of 95%; sensitivity of 69%).

Compared to other types of cancers, such as colon or breast cancer, salivary gland tumors are relatively rare, and only small sample sets are available. Because of the small sample size, the statistical power to make clear conclusions. There may be enough samples for a discovery phase but not enough to be validated in a larger independent sample set. In such cases, machine learning techniques may offer a suitable alternative to standard statistical methods. Although machine learning techniques are not new, they have only recently entered the field of cancer research. They can model high-dimensional and complex biological data which can have background noise and are often heterogeneous\textsuperscript{32}.

By application of machine learning techniques on the data set of chapter 5, an ensemble of seven miRNAs was identified (hsa-miR-374, hsa-miR-411, hsa-miR-599, hsa-miR-1285, hsa-miR-324-5p hsa-miR-449a and hsa-miR-449b) that distinguish between benign vs malignant salivary gland tumors (Chapter 6). These miRNA’s are different form the ones emerging from standard statistical analysis, but had comparable diagnostic properties (AUC 0.9; specificity 86%; sensitivity 91%). Interestingly, four of these miRNAs can be associated with the regulation of the cell cycle, targeting genes that control the speed of cell division (Chapter 6, Figure 3). Hsa-miR-374 and hsa-miR-1285 (increased in saliva from patients with malignant salivary gland tumor) speed-up the cell cycle, acting like gas pedals, while hsa-miR-449a and hsa-miR-449b (which are decreased in saliva from patients with benign tumors) decelerate the cell cycle, acting like brake pedals. One can envisage that increased levels of hsa-miR-374 and hsa-miR-1285 combined with decreased levels of hsa-miR-449a and hsa-miR-449b, may result in uncontrolled cell division.

In chapters 4 and 5, the research design was based on the prospective-specimen-collection, retrospective-blinded-evaluation or PROBE design, which consists of discovery, verification and validation phases\textsuperscript{33}. According to the PROBE design, the discovered miRNAs are validated in an independent sample set\textsuperscript{33}. Validating biomarkers in an independent sample set may prove difficult when the disease is very rare (such as salivary gland tumor).
and/or the number of samples is limited. For these cases, the use of new statistical methods may be a good alternative for ProBE. Unlike the ProBE design, utilizing machine learning techniques require no new independent samples set is needed for the validation of the discovered miRNA. By splitting the sample set into a learning and a test sample set, the miRNAs can be statistically validated without needing new samples. Furthermore, unlike standard methods, statistical learning algorithms are able to capture complex group-based correlations in data which are difficult to detect using standard statistical testing \(^{34-36}\).

**Future perspectives**

The studies compiled in this thesis were conducted with the goal of discovering additional biomarkers in order to increase the accuracy of diagnoses of salivary gland tumors. Our results suggest that saliva and miRNA expression profiling are good alternatives to the usually studied media (blood and tissue) and molecules (protein and mRNA) in cancer research.

An interesting prospect for future research would be the profiling the expression levels of miRNAs in whole saliva of the top 5 salivary gland tumor subtypes separately (pleomorphic adenoma, Whartins tumor, mucoepidermoid carcinoma, acinic cell carcinoma and adenoid cystic carcinoma). This group comprises about 80–90% of all salivary gland tumors. The miRNA data-sets can then be analyzed using machine learning techniques. This gives us predictive biomarkers as well as a more in-depth view of the pathology of salivary gland tumors.

Another interesting point is the fact that the expression level of diagnostic miRNA biomarkers decreases when the tumor is resected\(^ {37} \). When a recurrence of the tumor occurs, does the expression level of the diagnostic miRNA biomarker increase again? Or are other miRNAs aberrantly expressed when the tumor is recurrent? This information could be beneficial for screening during the follow-up visits of patients with a salivary gland tumor.

However, before we can start profiling saliva from different salivary gland tumors, we need a bigger standardized salivary sample database. The Salivary Gland Tumor Biorepository (SGTB) in Houston, TX, USA (https://research.mdacc.tmc.edu/Salivary_DB/index.html) is a good start. In this biorepository, fluids (saliva, NPBL, serum), tissue (FFPE and fresh) and slides of different SGT subtypes are stored. Researchers from all over the world can request samples for use in their research. The SGTB is an initiative of the National Institution of Health (USA), and is a good blueprint for a possible European SGT Biorepository.

While we’re not quite at the level of Tri-corder readings, these results and techniques presented here can serve to expand both the scope and utility of molecular salivary diagnostics.
References


