New molecular biomarker discovery for diagnosis and prognosis in oral and oropharyngeal cancer
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CHAPTER 10

Summary & general discussion

L.J. Melchers
Summary

Oral & oropharyngeal squamous cell carcinoma (OOSCC) are two of the most common head-neck tumours, with ~400,000 new cases worldwide and ~1,500 new cases in the Netherlands annually. Regional metastases occur in ~50% of all OOSCC patients. The nodal status is the most important factor for treatment choice and prognosis. However, current clinical assessment of the nodal status, using various imaging techniques has only a moderate sensitivity (50-70%), which is mainly due to the inability to detect micrometastases <3 mm in diameter. Because of the inaccurate assessment of the nodal status in OOSCC, a significant number of patients are over- or undertreated. Overtreatment consists of performing an elective neck dissection in a patient clinically suspect for metastases (clinically (c)N+) in which no metastases are found upon histopathological examination (pathological (p)No), resulting in loss of quality of life and increased healthcare costs. Undertreatment consists of undiagnosed metastases (cNo but pN+) which give rise to recurrences or distant metastases, resulting in loss in quality of life, shorter survival and increased healthcare costs when salvage treatment is undertaken. Therefore there is a need for better predictors of nodal status in OOSCC.

Biomarkers in the primary tumour may associate with the biological behaviour of a tumour such as the ability to develop metastases. Histopathological tumour characteristics are associated with nodal status, but suffer from unclear predictive values and observer variability. Single protein biomarkers are not likely to accurately predict the multistep process of metastasis. Genetic signatures, composed of hundreds of genes are too heterogeneous. Methylation is a promising candidate mechanism for the dynamic gene regulation during the multistep metastatic progression in OOSCC.

Biomarkers for the prediction of treatment response (prognostic markers), may aid the clinician in choosing the most optimal treatment for a specific patient and tumour, which is increasingly important now that targeted therapy is becoming available for the treatment of OOSCC. Goal of this thesis was to find new molecular biomarkers in the primary tumour that have predictive value for the nodal status and for prognosis in patients with oral & oropharyngeal squamous cell carcinoma, to improve regional staging and treatment selection which is currently based solely on clinical and histopathological characteristics. To achieve this goal, we constructed a large database with clinicopathological and follow-up data of over 600 patients currently based solely on clinical and histopathological characteristics. To achieve this goal, we constructed a large database with clinicopathological and follow-up data of over 600 patients who developed more than 700 OOSCC, of which tumour tissue was available in the archives of the department of Pathology of the University Medical Centre Groningen.

Patients with an early stage pT1cNo oral squamous cell carcinoma (OSCC) are generally not treated with a neck dissection. However, when these patients are treated by watchful waiting (strict 6-weekly follow-up of the clinical nodal status), ~25% develop clinically detectable nodal metastases during follow-up. The histopathological biomarker, tumour infiltration depth has predictive value for the nodal status in OSCC, however to date no clinically relevant cut-off has been established. In chapter 2 we measured infiltration depth of 212 pT1-2cNo OSCC to recommend a cut-off depth for performing a neck dissection. ‘True N status’ was determined based on neck dissection or at least two years of follow-up in case of watchful waiting. We showed that infiltration depth was not only significantly different between trueN0 and trueN+ cases, but also a predictive factor for trueN+ status, independently from cN status. By ROC-analysis a cut-off at an infiltration depth of 4.59 mm was defined. Using this cut-off on the group of pT1cNo OSCC resulted in a considerable increase of correctly treated patients, from 41% to 76%. Therefore, we recommend an infiltration depth of ≥4 mm (a more practical cut-off with the same predictive value) to be used as a biomarker with an absolute indication for performing an elective neck dissection in pT1cNo OSCC.

Photodynamic therapy (PDT) is a minimally invasive therapy which may be used for the treatment of superficial tumours. A photosensitizer is intravenously injected and activated locally by illuminating the tumour at a specific wavelength, which induces the release of reactive oxygen species causing cell death. Because of limitations of light penetration in tissue, curative treatment is limited to tumours ≤5 mm infiltration depth. No studies have been performed that compare PDT with standard surgical therapy in OOSCC. Therefore, treatment response of 116 OOSCC treated with PDT from a pooled multi-centre database, was compared with treatment response of 91 surgically treated cT1-2No OOSCC ≤5 mm infiltration depth from our database in chapter 3. Complete response rates did not differ between the two groups. However, local disease-free survival after complete response was longer and the need for further treatment was lower in T2 OOSCC treated with surgery. For T1 OOSCC all outcome measures did not significantly differ between surgical and PDT treatment. This study shows that PDT might be an effective modality in the curative treatment of a subgroup of cT1No OOSCC, that was selected based on a histopathological biomarker, paving the way for randomized-controlled trials comparing both modalities.

In chapter 4, the biological role of the epithelial cell adhesion molecule (EpCAM) in carcinogenesis, tumour progression, metastasis and patient survival of human carcinomas was reviewed. EpCAM is a membrane protein that is overexpressed in most carcinomas. In HNSCC EpCAM is expressed de novo. Because of its cell adhesion properties, EpCAM expression has been studied for associations with metastasis in HNSCC. However, no clear associations have been reported. In other tumour types EpCAM is clearly associated with either a better outcome, or a worse outcome. This might be because the role of EpCAM depends on a specific cell environment. Indeed, various biological functions of EpCAM have been described in vitro: EpCAM is a cell adhesion protein and able to disrupt E-cadherin-mediated cell adhesion, but EpCAM also associates with claudin-7 in a complex, which interferes with its adhesion function and which promotes proliferation, cell motility and metastasis. The co-expression pattern of these three closely related proteins might therefore provide a better predictive marker for nodal status than each marker separately.
In chapter 5 the co-expression patterns of EpCAM, E-cadherin and claudin-7 was investigated in a group of 227 OOSCC to determine whether these patterns might provide a better predictor for the presence of nodal metastases and regional recurrence in OOSCC than each of these molecules individually. Expression was investigated in both tumour centre and tumour front. The co-expression patterns of E-cadherin, EpCAM and claudin-7 did not provide a better predictive value for the pN status compared to each marker separately. Individually, lack of E-cadherin and presence of cytoplasmic EpCAM were predictive biomarkers for nodal status, however not independent from current clinical assessment. Lack of claudin-7 in the tumour centre is an independent predictive biomarker for regional recurrence. These data suggest that there is no clinically relevant modulating effect of these three markers in OOSCC.

Amplification of the chromosomal 11q13.3 region is a frequent event in HNSCC. Of the genes located in this amplicon, the expression of the gene encoding the Fas-associated death domain (FADD) protein correlates best with amplification status. Overexpression of FADD may be beneficial for HNSCC and therefore drive this amplification (FADD). All genes reported in more than one of the four studies were selected as well as the five highest ranking genes from each of the two genome-wide studies. Secondly, genes from the HNSCC microarray studies were selected when they showed functional methylation (increased expression after treatment with dac/TSA) in vitro and an association with lymph node metastasis in cervical squamous cell carcinoma, in a previous study performed in our lab. Additionally, four genes were selected that have been associated with lymph node metastasis in literature. Thus, 24 genes were selected that had not been reported previously to be regulated by methylation. Another four genes that show frequent methylation in HNSCC literature were also included in the analysis. We investigated these 28 genes for their predictive value for the nodal status in a group of 70 OOSCC. After optimization and screening, only 2/28 (7%) markers showed predictive value for the nodal status. MGMT methylation was associated with pNo status (p=0.02). DAPKI methylation was associated with pN+ status (p=0.008). The two markers combined had a sensitivity (89%) and specificity (41%) comparable to gene expression signatures for the detection of nodal status. Both markers have been described previously as methylated in HNSCC. The expression of genes that are the most differentially expressed between No and N+ cases, as identified in the four expression array studies, are not regulated by methylation. To efficiently identify additional new methylation markers, use of a high throughput, genome-wide methylation detection method is needed.

Presence of high-risk human papilloma virus (hrHPV) in OpSCC is a marker for longer disease-free and overall survival. A growing but widely varying incidence of HPV-associated OpSCC has been reported in several countries. Recently, a validated triple detection algorithm, consisting of p16 immunochemistry, hrHPV-ISH and hrHPV-PCR became available, which detects clinically relevant hrHPV infection in the tumour tissue. However, no complete cohorts have been tested with this algorithm. In chapter 7 we determined the prevalence and predictive values of hrHPV using the triple algorithm in a complete historic cohort of OpSCC diagnosed during a 16-year period (1997-2012) in our hospital (n=193) as well as in a group of 176 OSCC. In this study, hrHPV-positivity was an independent predictor for longer disease-specific survival and loco-regional disease-free survival. 24% of OpSCC and 11% of OSCC were hrHPV-positive. A clear increase during the study period 1997-2012 was seen, with 31% hrHPV-positive cases during the first half (1997-2004) increasing to 30% hrHPV-positive during the second half (2005-2012). hrHPV may be used as prognostic marker in OpSCC, but not in OSCC. Detection technique, population selection criteria and type of survival analysis may influence both reported prevalence and prognostic value of hrHPV in OpSCC.

Methylation of specific genes is being used as biomarker for diagnosis and prognosis in glioma and prostate cancer. DNA methylation is a form of epigenetic gene regulation. Hypermethylation leads to transcriptional repression, and hypomethylation leads to reactivation of gene transcription. Because of its dynamic nature, methylation is a possible candidate mechanism for the dynamic gene regulation during metastatic progression of OOSCC. In chapter 8 we searched for new methylation biomarkers for the prediction of nodal status. Various search strategies were used to select candidate methylation markers (genes with a CpG island and a negative association with nodal metastasis) from four independent microarray expression studies which identified differentially expressed genes in No vs. N+ HNSCC. All genes reported in more than one of the four studies were selected as well as the five highest ranking genes from each of the two genome-wide studies. Secondly, genes from the HNSCC microarray studies were selected when they showed functional methylation (increased expression after treatment with dac/TSA) in vitro and an association with lymph node metastasis in cervical squamous cell carcinoma, in a previous study performed in our lab. Additionally, four genes were selected that have been associated with lymph node metastasis in literature. Thus, 24 genes were selected that had not been reported previously to be regulated by methylation. Another four genes that show frequent methylation in HNSCC literature were also included in the analysis. We investigated these 28 genes for their predictive value for the nodal status in a group of 70 OOSCC. After optimization and screening, only 2/28 (7%) markers showed predictive value for the nodal status. MGMT methylation was associated with pNo status (p=0.02). DAPKI methylation was associated with pN+ status (p=0.008). The two markers combined had a sensitivity (89%) and specificity (41%) comparable to gene expression signatures for the detection of nodal status. Both markers have been described previously as methylated in HNSCC. The expression of genes that are the most differentially expressed between No and N+ cases, as identified in the four expression array studies, are not regulated by methylation. To efficiently identify additional new methylation markers, use of a high throughput, genome-wide methylation detection method is needed.

High expression of Epidermal Growth Factor Receptor (EGFR) is common in many human malignancies and has a strong association with worse prognosis. Despite EGFR expression in >95% of HNSCC, only 10-16% of patients benefit from therapy targeting the EGFR molecule. EGFRvIII is a common mutant of EGFR, harbouring a deletion of exons 2-7. Expression of EGFRvIII is constitutively active and degradation is impaired. The truncated EGFRvIII protein isconstitutively active and degradation is impaired. EGFRvIII expression is associated with a more aggressive phenotype, and resistance to chemotherapy and radiation therapy. Presence of EGFRvIII has been reported in HNSCC and therefore EGFRvIII might be a biomarker for treatment response in HNSCC. To assess the clinical relevance of EGFRvIII as prognostic biomarker, we determined the prevalence of this protein in a large series of 531 HNSCC in chapter 9. Immunohistochemistry for EGFRvIII was performed and compared to EGFR and EGFRvIII RNA expression using a specific rt-PCR. None of the 531 HNSCC expressed the EGFRvIII mutation. Therefore EGFRvIII does not contribute to the malignant phenotype and is not a suitable clinical prognostic marker in HNSCC. The expression that is reported in several small studies is most probably due to immunostaining artefacts, which seems to be associated with the use of a streptavidin-biotin detection method.
General discussion

Predictors for nodal status

In this thesis several biomarkers with predictive value for the nodal status were described. The histopathological biomarker of >4 mm tumour infiltration depth, expression of the protein markers EpCAM, E-cadherin and FADD, and methylation of the genes DAPK1 and MGMT. Although the prediction of nodal status is important for every OOSCC patient, it is often considered the most relevant in the group of T1-2cN0 early stage OOSCC, because in this group the decision to either treat the neck in an elective (staging) neck dissection, or not to treat the neck (watchful waiting) is based on the risk assessment for the presence of occult metastasis combined with a decision cut-off. The cut-off that is most frequently used is 20%, meaning that an elective neck dissection will be performed when the risk of occult metastasis is deemed greater than 20%.

Is Weiss’ cut-off still accurate?

For the management of early stage OOSCC, a cut-off of 20% was determined by Weiss and co-workers almost 20 years ago. The authors reported that a neck dissection is beneficial when the risk for occult metastasis is >20%. This was the outcome of a decision analysis comparing observation (watchful waiting) with treatment by radiation therapy or neck dissection. The decision analysis was based on several input parameters: the probability of recurrence, the effectiveness of salvage therapy and utility (desirability) ratings for each outcome. These input parameters were based on reports of large patient series available at that time, dating back as far as 1948. The input parameters have changed since that time, because of an increased accuracy of detection of metastases with modern techniques; the introduction of the selective suprathyroid neck dissection and improved salvage rates. Indeed, in the original article the authors stated that their input parameters were not fixed in stone, and “may be reconfigured (…) to determine optimal therapy based on a different set of underlying assumptions”. More recent reports even encourage clinicians to calculate their own cut-off, based on a specific formula provided and using input parameters based on results obtained in their own institution. In this regard it is interesting to note that the utility values of Weiss’s report are still used to this day, probably because modern studies on neck dissection fail to report data on quality of life. Several studies performed comparable decision analyses, using more recent data to update the input parameters. Reported cut-offs vary widely from 17% to 44% or even 100%. It should be noted that a cut-off of 100% implies that all T1-2cN0 cases should receive watchful waiting. In these studies the included tumour locations differ, as well as several specific analysis details. However, the currently still applied cut-off of 20%, with an actual regional recurrence rate of 20-30% in the watchful waiting group, is in line with most decision analyses.

Does infiltration depth improve the prediction of the nodal status in early stage OSCC?

We report on the validation of infiltration depth as predictor of the nodal status by measuring infiltration depth (figure 10.1) and determining a cut-off to better select the patients that benefit from an elective neck dissection. Using ROC-analysis (determining the point where the combination of sensitivity and specificity for detecting regional metastases is optimal) we determined a cut-off infiltration depth at 24.59 mm. The patients with an infiltration depth 24.59 mm had a ~40% risk for occult metastases. This is far from the 2 mm infiltration depth that, in concordance with Weiss, identifies a group with ~20% risk for occult metastases. A cut-off at 2 mm would consequently lead to 80% overtreatment and associated morbidity, a high increase in healthcare costs because of the large number of extra neck dissections and no increased survival for the treated patients. Although the 20% risk cut-off as determined by Weiss et al. based on data from 1948-1982 is not accurate anymore, it has had major influence on treatment decisions of head-neck oncologists. Determining a new current cut-off for a specific head-neck oncologic centre by using centre-
Can methylation markers act as predictors of the nodal status?
We set out to identify new methylation markers for predicting the nodal status (chapter 8). Of the 28 methylation markers that were selected based mainly on expression array data, only two showed predictive value for the nodal status. Both markers were already known to be frequently methylated in HNSCC. Although both markers have a significant predictive value, the combined negative predictive value is 76%, meaning that still 24% of patients with a negative test outcome do have metastases. These two markers clearly do not outperform current clinical assessment of the nodal status. To efficiently identify new methylation markers a genome-wide methylation analysis of 6 pNo and 6 pN+ OOSCC was performed by our research group using the methylCap-Seq method (figure 10.2) [Clausen et al., in preparation]. In short, tumour DNA is fragmented by sonication. The methylated fragments are bound by methyl-binding domain 2 (MBD2), coupled to nickel coated beads and magnetically separated from non-methylated fragments. Methylated fragments are eluted by increasing salt gradients, and subsequently sequenced by next-gen deep sequencing. Using advanced statistics (in collaboration with the department of Bioinformatics, University of Ghent), a list was composed of the most significantly differentially methylated genes between pN0 and pN+ cases. Pathway analysis showed enrichment of cellular movement, cell death & survival and lipid metabolism, the same pathways as found in expression array metastatic signatures, illustrating that methylation plays an important role in the development of a metastasizing expression profile in OOSCC. Indeed, combination of our methylation data with available expression data, revealed genes that are methylated with an associated transcriptional downregulation. Several of these candidate methylation markers are predictive for nodal status. Some of these genes have been described previously to be associated with metastasis in HNSCC, demonstrating the effectiveness of our approach. Clinical validation of several candidate methylation markers on our well-defined cohort of OOSCC is currently ongoing [Clausen, Melchers et al., in preparation].

Predictors for disease outcome
As biomarkers for outcome, in this thesis the protein biomarker claudin-7 (chapter 5) and hr-HPV status (chapter 7) were described. Implementing a biomarker for worse outcome such as claudin-7 seems relatively simple, increasing dose of postoperative radiation therapy or frequency of follow-up visits. However, implementing a biomarker that identifies patients who have a better outcome may be more difficult, lowering treatment intensity whilst avoiding undertreatment. We reported that hr-HPV-positivity is an independent predictor for longer disease-specific survival in a subgroup of OpSCC, with an Odds Ratio comparable to cNo status (OR=0.23; chapter 7). In contrast to our study, most studies suffer from inaccurate detection methods, selection bias and lack of disease specific survival data, making it impossible to generalize the reported findings. The general consensus is that patients with an hr-HPV-positive OpSCC might benefit from de-escalated therapy (less intensive treatment, resulting in less morbidity, while having the same survival rates). However, how much and in what form this should happen is presently not clear. To answer this question, recently several clinical trials comparing standard with de-escalated therapy in hr-HPV-positive OpSCC have been set up. Moreover, because of its viral aetiology specific immunological therapies are being explored for hrHPV-positive OpSCC.

Oropharyngeal tumours of the tonsil and base-of-tongue both arise from tissue, belonging to Waldeyer’s ring (the palatine and lingual tonsils, respectively), consisting of superficial non-keratinizing squamous epithelium, in close relation with underlying lymphoid tissue. Tonsils are unique in that they are not fully encapsulated (like the spleen) but do not have afferent lymphatics (like lymph nodes). The superficial non-keratinized, stratified squamous epithelium shows deep infoldings called crypts (figure 10.3), increasing the epithelial surface of these organs by a factor seven, and is surrounded by lymphoid tissue with germinal centers with lymphocytes infiltrating the epithelium. The bottom of the crypts is lined with a specialized reticular epithelium infiltrated with blood vessels and lymphocytes, called lymphoepithelium. This epithelium is accompanied by disruptions in the basement membrane, which provides direct transepithelial access to antigens, such as HPV, and HPV-associated tumours originate from this area. Infection with an hrHPV type in the oral cavity is common and associated with a high risk of developing extensive lymph node disease. General consensus is that these hrHPV infections are transferred between oral and genital locations, as there is significant concordance in genotypes detected in oral and genital (both cervical and penile) locations. Indeed, cervical cancer patients have been found to be at increased risk for developing an OpSCC. Because of the interrelation of cervical cancer and HPV-positive OpSCC, it will be very interesting to...
observe a possible effect of the population-based prophylactic HPV vaccination programs in various countries on the incidence of OpSCC in the near future. A very recent report shows that vaccination may be effective in reducing the prevalence of HPV in the oral cavity\(^{65}\), although currently hrHPV status does not influence clinical management of OpSCC, this might become reality in the near future. Until that time hrHPV status should be assessed for scientific reasons and for patients who want complete prognostic information.

**Other benefits of biomarker discovery studies**

Tumour biomarkers may not only provide predictive and prognostic information, but may also give clues on biological mechanisms or provide future therapeutic targets. This is illustrated in chapter 5, in which we report on the biological mechanisms of EpCAM regulation, based on immunohistochemical expression in our clinical cohort of OOSCC. In this chapter the co-expression patterns of E-cadherin, EpCAM and claudin-7 were assessed for the first time on a large group of tumour samples. Expression of the complex did not improve the predictive values of the individual markers, but most importantly the complex did not have additional predictive value in sharp contrast with the reported in vitro data\(^{167,168}\). However, comparing expression levels and subcellular localization of EpCAM in both the centre as well as the invasive front of the same tumours, revealed some new clues regarding the possible biological mechanism of EpCAM and its role in OOSCC carcinogenesis. EpCAM can be cleaved by juxtacrine activation, resulting in shedding of the extracellular domain\(^{491}\) and loss of membranous staining of the extracellular domain-specific BerEP4 antibody. The intracellular domain (EpICD) may translocate to the nucleus, inducing transcription of genes such as c-myc and cyclins which promote proliferation and metastasis\(^{492}\). Cytoplasmic EpCAM expression in the tumour front was associated with pN+ status and could reflect increased EpCAM turnover in the endoplasmic reticulum or transport vesicles. Future studies on EpCAM in HNSCC should focus on the nuclear translocation of EpICD as a possible biomarker for nodal status. With current antibodies this is not yet possible. Moreover, because of its tumour-specific overexpression, EpCAM may function as therapeutic target\(^{493}\). EpCAM-targeted therapy has been applied already for several years with some success. Recently a new EpCAM-specific trifunctional antibody (catumaxomab) has been developed and is approved in Europe for the palliative treatment of malignant ascites and being evaluated for other indications\(^{494}\).

The opposite, identification of a prognostic marker without clues on the biological mechanism causing its prognostic value is also possible. In chapter 6, it was shown that FADD expression is a prognostic marker for distant metastasis-free interval in a group of mainly advanced HNSCC. The FADD gene is co-amplified (along with 12 other genes that compose the core of the 11q13.3 amplicon) in ~36% of HNSCC cases\(^{495}\). We can therefore not exclude the possibility that one of the other 12 co-amplified and co-overexpressed 11q13.3 genes is responsible for the association of FADD expression with the presence of nodal metastasis. In order to validate whether increased FADD expression was capable of increasing the invasive and/or migratory potential in vitro, we performed several standard functional assays including scratch assays, transwell assays and spheroid-matrigel assays (figure 10.4). HEK293 cells were transfected with FADD regulated by a ponasterone-inducible promoter. Upon treatment with ponasterone the expression of FADD was induced (figure 10.4A). However, no difference was found between cells overexpressing FADD and the empty vector cell line in any of the in vitro migration and invasion assays that were performed. These findings strongly suggest that FADD does not have a cell migration and invasion promoting function. One explanation is that FADD expression only is not sufficient for the biological effect and that co-activation of one of the other 11q13.3 genes is needed. Yet another explanation for our results is that expression of FADD itself is not directly responsible for the increased metastatic potential of HNSCC, but that its overexpression indicates inactivation of the apoptotic pathway. Overexpression of FADD in normal cells induces apoptosis through the formation of death effector filaments, which are cytoplasmic clusters of death effector domain containing proteins that recruit caspase-8, leading to caspase-dependent, but receptor independent apoptosis (figure 10.4B)\(^{495,496}\). These findings suggest that cells which overexpress FADD specifically inhibit this type of apoptosis. When normal cells detach from their neighbouring cells or from the extracellular matrix, they show a phenomenon called anoikis, a type of apoptosis which is caspase-dependent\(^{495,496}\). Therefore, FADD overexpression may only be possible in cells that are insensitive to anoikis, and thus also have a higher metastatic potential. To establish if this is indeed the case, additional studies should be performed in anoikis resistant cells.
Chapter 10

Data of 6 wells, 4 independent experiments. EV set to 1. A. Spheroid assay.

Scratch assay

Time (h)

EV + 5mM PonA
DADD-S194A + 5mM PonA

Evad vector (EV)). B. Scratch assay. Data of 16 scratches, four independent experiments. C. Transwell migration assays. Functional assays. Data of cell lines with the expression induced for 24h with 5mM PonA (FADD-S194A vs. Empty vector).

Implementation of biomarkers in clinical practice

As stated before, implementation of newly discovered biomarkers in clinical treatment protocols is not an easy process. After initial discovery, validation in independent patient groups is needed, additionally the assessment of the biomarker should be standardized. Biomarker-guided treatment should then be compared to standard care in randomized clinical trials (RCTs). After assessment of efficacy, the adoption of a biomarker in clinical practice is a completely different process. It is dependent on various factors such as the compatibility with current practice, simplicity, the potential contribution to practice, costs, patients’ demand and promotion by commercial parties involved. The speed by which a new prognostic or predictive test is embraced by clinicians and patients is therefore hard to predict. For certain biomarkers that apply to a large patient group and have a large expected effect (eg. HPV) assessment in RCTs may be feasible and desirable. However, as biomarkers identify increasingly smaller subgroups of patients (an inevitable process when moving towards personalized medicine), an RCT becomes both less feasible and less effective. An example of the latter is the implementation of tumour infiltration depth ≥4 mm as an absolute indication to perform an elective neck dissection in early stage OSCC in our institution. Although infiltration depth has been known as predictive factor for nodal status for decades, an RCT has never been performed, probably because this biomarker concerns only 6% of all early stage OSCC. Addressing these issues is not straightforward, but should probably include diagnostic markers to identify the specific subgroup and optimal monitoring of clinical outcome and side-effects in large multicenter databases.

Problems in standardizing biomarker use

Before a biomarker may be implemented in clinical practice, testing and assessment of the marker should be standardized, to reduce the variability between subsequent tests and assessment of results (intra- and interobserver). This is especially important in the assessment of immunohistochemical markers, because of the multitude of variables in performing (tissue fixation and age, antigen retrieval, antibody clone and concentration, detection systems, controls etc.) and in assessing (specific tissue sublocalization analyzed, subcellular expression pattern, percentage and intensity of expression etc.) immunohistochemical markers.

In chapter 9 it was shown that the age of tissue sections and the detection system can have major influence on the staining pattern and, when used as single technique may lead to wrong conclusions regarding the presence of EGFRvIII in HNSCC. Assessing a biomarker with an algorithm of a highly sensitive screening technique (eg. immunohistochemistry) followed by a highly specific technique (eg. PCR-based), as was done for EGFRvIII (chapter 9) and for hrHPV (chapter 7) seems a practical way to achieve highly accurate biomarker detection.

Another example of a factor that should be taken into account when analyzing biomarkers is the specific tumour sublocalization in which the marker is assessed. Many biomarkers, especially those related to cellular adhesion and metastasis, are known to be differentially expressed in tumour centre versus tumour front. Most studies using tissue microarrays (TMAs) to study immunoeexpression on a large number of tumours simultaneously do not describe the tumour compartment that has been sampled. Most probably the tumour centre has been used, because it is the easiest to sample and a representation of the average expression in the tumour. Construction of front- or centre-specific TMAs as described in chapter 5 and by
RNA, DNA and epigenetic level, a high level of sensitivity might only be achievable with markers that can be assessed by a PCR-based technique. Combination of such a sensitive technique with a limited panel of very specific biomarkers may allow the detection of tumour cells not only in tissue samples but even in other samples obtained in less invasive ways.

Recently, in our research group, a pilot study was finished on the detection of tumour-specific methylation markers in saliva. All squamous epithelia of the mouth are lubricated by saliva, and shed cells and cellular debris from the mouth are absorbed in saliva. Therefore, saliva can be regarded as a reservoir of (epi)genetic information on the condition of the oral epithelium.

We hypothesized that a tumour will present itself at the cellular level in saliva long before clinical detection is possible. In this pilot study we identified four methylation markers that very accurately distinguish saliva of patients with an oral tumour from that of healthy controls. Currently, a validation study is ongoing in which these four methylation markers are prospectively validated for the early detection of local recurrences. This validation study also includes several methylation markers that have been identified in our genome-wide methylation study as having high methylation in OOSCC compared to normal tissue.

It has been demonstrated that circulating tumour cells (CTCs) can be identified in blood of head-neck cancer patients using markers such as EpCAM or EGFR. The presence of CTCs has even been identified as an independent predictor for nodal status. The main drawback is that with current technology CTCs can be detected only in a minority of patients, and especially in advanced cases with a high tumour load (N2b-N3). Therefore CTCs cannot be used for the detection of local (N0) and early metastasized (N1-2a) tumours, which are the clinically most relevant groups. However, the already high sensitivity of PCR-based techniques may be even increased further with the advent of new methods expanding on the PCR technique such as digital droplet-PCR, which is able to detect one mutant allele in a background of 100,000 wildtype copies.

Very recently, the digital PCR technique has been used to successfully detect cell-free DNA with tumour-specific alterations in serum of breast cancer patients. Circulating tumour DNA was detected in 97% of patients and the number of copies was better correlated with disease progression than CTCs. This report showed that a personal, tumour-specific genomic alteration can be used to identify circulating tumour DNA. Such personalized marker detection in serum or saliva might be the future of predictive and prognostic biomarkers in OOSCC.

Although many studies report on associations of biomarkers with nodal status or prognosis, predictive values and clinical relevance are frequently not assessed. This thesis identified several new predictive and prognostic biomarkers in OOSCC and focussed on the possible relevance of these markers in the clinical setting, to improve diagnosis and outcome for patients with oral and oropharyngeal squamous cell carcinoma.

**Figure 10.5.** Schematic representation of A. FADD overexpression with an inactivated anoikis pathway. B. Normal Fas-mediated anoikis pathway (with normal FADD expression). DEF = Death Effector Filament; DD = Death Domain; DED = Death effector Domain; FasL = Fas ligand; Fas = Fas receptor; DISC = Death-Inducing Signalling Complex. Adapted from Menaker & Jones, 2003.