Altered DNA methylation during HPV-induced cervical carcinogenesis

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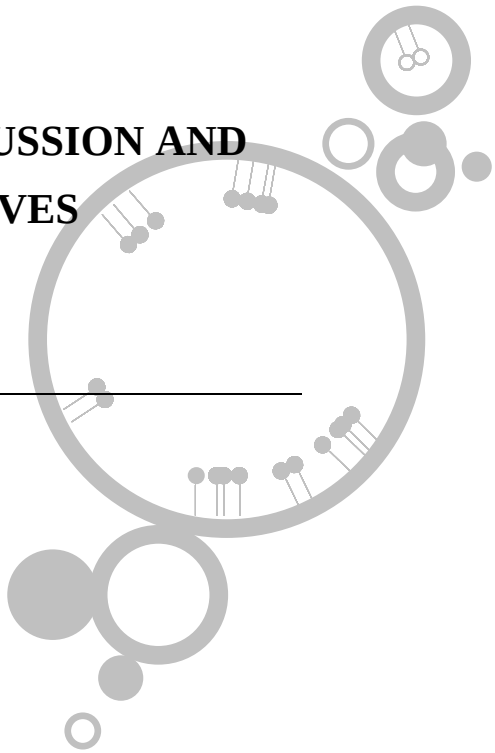
2011

CHAPTER 7

SUMMARY, GENERAL DISCUSSION AND FUTURE PERSPECTIVES



SUMMARY AND GENERAL DISCUSSION

Recent randomised-controlled, prospective trials have shown that testing for hrHPV in cervical screening programs provides a superior protection against high-grade cervical precancerous lesions and cervical cancer compared to cytology, and is therefore an attractive primary cervical screening tool [1-4]. However, the low specificity of hrHPV testing, resulting from the detection of women with transient hrHPV infections who will not develop cervical cancer precursor lesions, necessitates the need for further risk stratification**.** We hypothesised that more insight into the molecular changes that occur during cervical cancer development will not only provide a better understanding of hrHPV-associated carcinogenesis, but may also yield triage markers for hrHPV-positive women at risk of high-grade cervical disease.

In this thesis, the mechanisms underlying hTERT, CADM1 and MAL deregulation in cervical carcinogenesis were established and their consequences in terms of biomarker development for early detection of cervical cancer were considered. Studies were particularly focused on DNA methylation of promoter sequences of these genes. Their function was studied in an *in vitro* HPV-induced transformation model and the potential value of deduced markers as triage tool for hrHPV-positive women was evaluated on well-characterised clinical samples.

Promoter methylation: one of the mechanisms underlying hTERT deregulation during HPV-mediated immortalisation

Activation of telomerase resulting from deregulated *hTERT* expression has been recognised as a key event during hrHPV-induced transformation and cervical carcinogenesis. In **Chapter 2**, hTERT promoter activity and its relation

to DNA methylation in hrHPV-transformed cell lines is described. Using luciferase reporter constructs the transcriptional activity of various hTERT promoter regions as well as proximal exonic/intronic sequences were analysed and the relationship with epigenetic modifications was determined. We showed the existence and position of specific repressive sequences in the hTERT promoter. By successive bisulfite sequencing of these regions, we demonstrated DNA methylation at these regions is correlated with increased *hTERT* expression. This suggests that this methylation event contributes to *hTERT* deregulation in HPV-transformed cells. Upon analysis of cervical tissue specimens, a gradual increase in methylation of the respective hTERT regulatory regions proportional to severity of cervical disease was observed. Therefore, DNA methylation at these regions may provide a biomarker for the early detection of cervical cancer.

CADM1 and MAL promoter methylation in cervical (pre-)malignant lesions

Previous studies have shown that promoter methylation of the tumour suppressor gene CADM1 is highly frequent in cervical squamous cell carcinomas (SCCs) and can even be detected in archival scrapings taken up to seven years prior to cervical cancer diagnosis [5]. A high frequency of CADM1 methylation in cervical carcinomas was confirmed by others [6,7]. In **Chapter 3**, a comprehensive analysis of DNA methylation at three distinct regions in the CADM1 promoter was conducted in HPV-immortalised and cervical cancer cell lines as well as in cervical tissue specimens. The methylation status of the promoter regions was determined by conventional methylation specific PCR (MSP) in combination with a reverse line blot read-out system. Using these assays, the extent of DNA methylation at the three different promoter regions, i.e. density of methylation, was found to be associated with anchorage-independent growth and CADM1 gene silencing *in vitro*. Furthermore, within a spectrum of cervical (pre-)malignant lesions, dense methylation (defined as ≥two methylated regions) increased with the severity of cervical disease from 5% in normal cervical samples to 30% in CIN3 lesions and 83% in SCCs. The

frequency of dense methylation was significantly higher in ≥CIN3 compared with ≤CIN1, as well as in SCCs compared with adenocarcinomas (83% versus 23%). Interestingly, dense promoter methylation was also significantly associated with decreased CADM1 protein expression, as determined by immunohistochemical analysis of CADM1 protein expression in cervical tissue specimens. The results of this study indicate that detection of dense CADM1 promoter methylation may contribute to the assembly of a valuable marker panel for the triage of high-risk HPV-positive women at risk of ≥CIN3.

In search for another methylation marker that could contribute to ≥CIN3 and AdCA detection, we analysed the putative tumour suppressor gene MAL, which we identified as the most down-regulated gene in cervical carcinomas [8]. As described in **Chapter 4**, we investigated the mechanism underlying *MAL* gene silencing and its functional role in HPV-induced transformation *in vitro.* Subsequently, MAL promoter methylation and mRNA expression were analysed in a series of cervical (pre-)malignant lesions as well as in cervical scrapings. *MAL* mRNA was (nearly) undetectable in all HPV-immortalised and cervical cancer cells, but could be up-regulated upon methylation inhibition by 5'-Aza-2'-deoxycytidine (DAC). DNA methylation at two promoter regions (M1 and M2) of MAL, as assessed by quantitative methylation-specific PCR (qMSP), was detected in all HPV-immortalised cells and cancer cells. Ectopic expression of MAL in SiHa cells suppressed proliferation, migration, and anchorage-independent growth. Furthermore, MAL promoter methylation increased with the severity of cervical lesions and was detected in ≥90% of both SCC and AdCA. Additionally, detection of MAL promoter methylation in cervical scrapings was predictive for underlying high-grade lesions. Both in biopsies and in scrapings, MAL promoter methylation was significantly correlated with reduced *MAL* mRNA expression. Taken together, these data indicate that like hTERT and CADM1, MAL promoter methylation may provide a promising molecular marker for the triage of high-risk HPV-positive women at risk of ≥CIN3.

Improved detection of high-grade CIN lesions and cervical carcinomas using panel of CADM1- and MAL-based promoter methylation markers

As described above, both CADM1 and MAL display tumour suppressor activity in cervical cancer cells and are frequently silenced by methylation in cervical carcinomas and a subset of CIN3 lesions. Interestingly, whereas in the HPV-mediated transformation process silencing of MAL by promoter methylation occurred rather early in HPV-immortalised, non-tumourigenic keratinocytes, methylation-mediated silencing of CADM1 was linked to later stages of transformation (i.e. cells with anchorage-independent and tumourigenic phenotypes). Based on their association with distinct stages of transformation, we hypothesised that methylation analysis of CADM1 and MAL may at least be partly complementary in terms of ≥CIN3 detection.

In **Chapter 5** we tested whether combined methylation analysis for CADM1 and MAL could serve as a better triage marker for hrHPV-positive women than either gene alone. Four qMSPs, two for CADM1 (regions M12 and M18) and MAL (regions M1 and M2) each, were applied to 261 cervical tissue specimens ranging from no neoplasia to cervical carcinoma. The highest positivity rates for CIN3 lesions (97%) and squamous cell- and adenocarcinomas (99%) were achieved by combining two qMSPs representing both CADM1 and MAL. Subsequent qMSP analysis of 70 GP5+/6+-PCR hrHPV-positive scrapings revealed that a two-marker panel consisting of CADM1-M18 and MAL-M1 was most discriminative, detecting 90% of women with CIN3 (n=30) whereas it showed a positive result in only 13.5% of women without cervical disease (n=40). Finally, we applied hrHPV GP5+/6+-PCR testing followed by CADM1-M18/MAL-M1 methylation analysis to 79 women visiting the outpatient colposcopy clinic for abnormal cytology. HrHPV testing revealed a sensitivity of 97% and a specificity of 33% for ≥CIN3. Additional CADM1-M18/MAL-M1 methylation analysis on the hrHPV-positive women increased the specificity to 78%. In conclusion, a combined CADM1 and MAL methylation marker panel may serve as an alternative molecular triage tool for hrHPV-positive women.

In a separate study context, MAL promoter methylation was also found to be useful as a prognostic marker for gastric cancer, as described in **Chapter 6**. Methylation of the promoter regions MAL-M1 and MAL-M2 occurred in 71% and 80% of the gastric cancers respectively, but not in normal gastric mucosa tissue. Moreover, methylation of M2, but not M1, was significantly correlated with better disease-free survival and with down-regulation of expression.

In conclusion, for all three genes, hTERT, CADM1 and MAL studied in this thesis, promoter methylation was correlated to altered gene expression, with hTERT expression being upregulated upon promoter methylation and CADM1 and MAL being downregulated following promoter methylation. Particularly, as all three genes are functionally involved in HPV-induced transformation, they are preferred disease markers for malignant progression. Indeed, promoter methylation of these three genes was shown to increase with severity of histological grade of cervical disease, underlining their potential diagnostic significance. Moreover, CADM1 and MAL promoter methylation, as each assessed at two promoter regions, were found to be complementary to each other in terms of ≥CIN3 detection in both biopsies and cervical scrapings derived from hrHPV-positive women. Additional analysis of hTERT methylation did not improve ≥CIN3 detection (data not shown).

FUTURE PERSPECTIVES

Clinical validation

To exploit the value of the methylation markers CADM1 and MAL in population-based screening based on primary hrHPV testing, studies are presently ongoing on scrapings collected during prospective population-based studies, i.e. the POBASCAM trial, executed by combined cytology and hrHPV testing [9]. These include efforts for an optimised cut-off definition for the individual markers determined by cross-validation in a training set with subsequent validation in an independent test set.

Similar validation studies are warranted on prospectively collected self-sampled specimens, such as collected in the PROHTECT trial, to which hrHPV testing has been applied effectively [10]. Offering self-sampling to women who do not attend regular screening resulted in a response rate of about 30% [10,11]. Since determination of cytomorphology on self-sampled material is suboptimal and therefore not an attractive option, methylation markers to be applied on the self-sample may confer an attractive triage tool for future self-sampling based screening programs. It can be envisioned that women testing positive for both hrHPV and methylation markers should be referred for colposcopy-directed biopsy immediately, whereas those with a negative methylation test could have a follow-up test after e.g. one year without a substantial risk. This prevents surplus visits to the gynaecologist for colposcopy-directed biopsies and over-treatment. Inclusion of self-sampling may result in an even more patient-friendly and cost-effective referral policy.

Finally, hrHPV testing in combination with methylation markers may also be applied to improve the monitoring of women treated for high-grade cervical disease. Multiple studies have yet shown that testing of HPV improves the effectiveness of detecting post-treatment CIN lesions [12,13], but that the specificity of molecular testing needs further improvement. A multicenter

prospective cohort study has just been initiated to determine whether testing for methylation markers in conjunction with hrHPV testing is more effective in terms of sensitivity and specificity than cytology or a combination of hrHPV testing and cytology in detecting residual/recurrent CIN disease. In addition, it will be investigated whether self-sampling provides a robust and more patient-friendly approach for the detection of hrHPV and CADM1/MAL promoter methylation during post-treatment monitoring.

Assay optimisation and additional marker discovery

Present data indicate that the above described so-called first generation methylation marker panel, based on CADM1 and MAL genes is at least as good as cytology and may serve as a objective, molecular triage marker for hrHPV-positive women. As such, this also holds great promise for the development of next generation methylation marker panels with increased sensitivity and specificity for ≥CIN3.

Presumably, (q)MSPs directed to regions that are critical to gene transcription regulation (as was shown for hTERT in **Chapter 2**) are most sensitive and specific for disease. Although for CADM1 and MAL methylation of the promoter regions analysed by (q)MSP was associated with reduced gene expression, functional proof for a direct transcriptional repressive effect of these DNA methylation events remains elusive. Preferentially, extensive bisulfite sequencing analysis of large promoter regions, flanking the transcription start site, is performed on a series of normal tissue specimens, CIN3 lesions as well as carcinomas. Sequencing of cloned PCR products, as we did for hTERT promoter regions, has been accepted being the gold standard, but leads to high labour intensity. A good alternative, also yielding (semi)quantitative information on percentage of methylated alleles may be pyrosequencing. Subsequent confirmation that methylated CpGs are critical to gene silencing can be obtained by reporter analysis in which various promoter regions, either or not methylated

by *in vitro* methylation using Sss1 CG methylase, can be cloned upstream of a luciferase reporter gene. Besides selecting the relevant promoter regions, the analytic performance of qMSP and the assay specificity may be improved by use of Locked Nucleic Acid (LNA) primers and/or probes, as for example demonstrated for MGMT [14,15] and CADM1 [14]. In analogy to its use for improved DNA mutation analysis (as was reviewed by [16]), peptide nucleic acid (PNA) hybridisation probes, in which the sugar-phosphate backbone of DNA is replaced by a pseudopeptide and very stable heteroduplexes can be formed, may be exploited to further enhance qMSP performance.

Next to CADM1 and MAL, additional methylation markers may still contribute to the composition of a second generation marker panel to improve the sensitivity and specificity for clinically relevant CIN lesions, SCC, ACIS and AdCA. Such markers could include markers described in methylation marker panels before (as summarised in **Chapter 1**), like DAPK1 [6,17,18], TWIST1 [17,19], HS3ST2 [20] and CDH1 [20]. In this respect it is worth mentioning that also miRNA regulatory sequences may represent promising methylation markers, as demonstrated for hsa-miRNA-124 in cervical cancer by Wilting *et al.* [21]. Besides these known markers, a discovery effort specifically for methylated genes in CIN3 lesions and cervical carcinomas may result in identification of yet unknown complementary markers. During the last years, several new platforms (e.g. microarray format, bead array format, massive parallel sequencing format) have been developed that allow for accurate high-throughput genome-wide DNA methylation profiling [22].

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