Chapter 9

Summary & Discussion
Nederlandse samenvatting
SUMMARY AND DISCUSSION

Even though population-based screening has been very successful in the prevention of cervical cancer in developed countries, cervical cancer still remains the second most common cancer in women worldwide. Current cervical screening programs are based on cyto-morphological examination of exfoliated cervical cells, using the so-called Pap-smears. Despite its great success in the reduction of cervical cancer cases, results of this test are subjective, resulting in a limited sensitivity and specificity.

A persistent, transforming infection with hrHPV is causally related to the development of cervical cancer, which is reflected by the fact that the virus can be detected in virtually all cervical carcinomas. As a consequence, hrHPV testing was shown to have a superior sensitivity for ≥CIN2 compared to cytology. The use of hrHPV testing as a primary screening tool therefore permits less frequent screening. However, only a small proportion of all hrHPV-positive women will ultimately develop an invasive carcinoma if left untreated. Implementation of hrHPV DNA testing as a primary screening tool in cervical screening programs will therefore result in lower specificity for high-grade disease compared to cytology. High-grade premalignant cervical lesions (CIN2/3) can develop within 2-3 years after infection, whereas it can take one or more decades for an invasive carcinoma to develop from these lesions.

Together, these findings indicate that besides hrHPV additional (epi) genetic changes in the host cell are indispensable for the development of cervical cancer. Previous studies using various techniques on both in vitro cell line models of HPV-induced carcinogenesis and primary cervical tissues have already identified a number of alterations, as summarised in Chapter 1.

More insight into the necessary (epi) genetic alterations will not only yield a better understanding of HPV-associated carcinogenesis, but may also provide triage markers for hrHPV-positive women.

In this thesis molecular profiling of HPV-mediated transformation was performed using advanced high-resolution approaches to further complete our understanding of this process as well as to identify potential novel biomarkers for (cervical) cancer screening.

Common chromosomal alterations in cervical carcinogenesis

In Chapter 2 Array CGH was used to determine the chromosomal signatures in cervical SCCs and AdCAs in greater detail than was possible before. As had already been shown in SCCs from other origins high numbers of chromosomal
alterations were observed, with a maximum of 22 alterations per carcinoma. AdCAs showed on average fewer alterations, and the alteration pattern also differed between these 2 histotypes. Especially, gains of chromosome 3q, one of the most common alterations described in cervical cancer to date, appeared more specific for SCCs. Gains at 20q were also more frequent in SCCs, although this was not statistically significant. Subsequent MLPA analysis showed that increased copy numbers of individual genes at 20q could be detected in AdCAs as well. Gains at chromosome 1 and losses at 8q, 11q and 13q were frequent in both SCCs and AdCAs. Results of this study indicated that partially different HPV-mediated carcinogenic pathways may exist in distinct histotypes of cervical cancer, which should be taken into consideration in the search for novel biomarkers.

It is apparent that, in order to improve cervical screening, markers should also detect premalignant lesions with invasive potential. Chromosomal profiles of HPV-immortalised keratinocyte cell lines, which are reminiscent of high-grade premalignant lesions, already showed gains at 3q and 20q and losses at 13q, indicating their potential involvement in progression to invasive cancer. To fully answer the question to what extent the frequent alterations found in carcinomas contributed to the development of invasive cancer from a precursor lesion, a well-defined group of CIN2/3 lesions was chromosomally profiled in Chapter 3. To ensure that all lesions studied would represent true precursor lesions of cervical cancer, only CIN2/3 lesions containing hrHPV DNA and showing p16INK4a overexpression were included. As explained in Chapter 1, overexpression of the tumour suppressor p16INK4a reflects the presence of a transforming hrHPV infection. Within these CIN2/3 lesions, two distinct subsets were identified based on their chromosomal profiles. Whereas 70% of lesions showed relatively few alterations, about 30% of lesions showed chromosomal profiles similar to those of invasive carcinomas, characterised by gains at chromosomes 1, 3q and 20. This finding suggests that within histologically similar CIN2/3 lesions harbouring a transforming hrHPV infection, early and more advanced lesions are present. In support of this, low-grade CIN1 lesions showed little to no chromosomal alterations. CADM1 promoter methylation, a marker for CIN lesions with invasive potential, was significantly higher in CIN2/3 lesions with chromosomal profiles similar to invasive carcinomas. It is important to distinguish advanced lesions with a high short-term risk of progression to invasive carcinoma, because they need immediate treatment. Conversely, since treatment of high-grade CIN lesions can
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give rise to pre-term delivery, a more flexible regime may be considered in terms of the moment of therapeutic intervention in case of early CIN2/3 lesions in women of reproductive age 13.

**Altered gene expression associated with common chromosomal alterations**

As summarized above cervical carcinomas and CIN2/3 lesions have a number of frequent chromosomal alterations in common, indicating involvement of these chromosomal events in cervical carcinogenesis. However, even using array CGH, which has a higher resolution than classical CGH, most regions identified span multiple megabases and contain numerous candidate cancer-related genes. To determine the extent to which expression of genes located within these frequently altered chromosomal regions is affected, in Chapter 4 genome-wide mRNA expression profiles were generated from the same carcinomas that were previously profiled chromosomally (Chapter 2).

Integration of these transcriptional and chromosomal profiles, using two different sophisticated statistical methods, identified a number of chromosomal hotspots within larger chromosomally altered regions in which gene expression was altered as well. We found increased expression of genes located at 1q32.1-32.2, 3q13.32-23, 3q26.32-27.3, and 20q11.21-13.33, as well as decreased expression of genes located at 11q22.3-25. Combining results of the different statistical approaches used in this study highlighted 7 genes, namely FLJ21291 (located at 1q32.1), DTX3L, CCDC14, MCM2, PIK3R4, ATP2C1 and SLC25A36 (all located at 3q13.1-23), as promising putative marker genes. Increased mRNA expression of DTX3L, PIK3R4, ATP2C1, and SLC25A36 was validated in an independent set of SCCs and normal ectocervical epithelial samples. In addition, increased expression of these genes was also found in CIN2/3 lesions (data not shown), indicating their importance in malignant transformation. Of these genes DTX3L and PIK3R4 are especially interesting because they are involved in the Notch and PI3K pathway, respectively, both of which have been indicated to be involved in cervical carcinogenesis 14-18.

**Similarities in chromosomal profiles of HPV-induced SCCs from different anatomical sites**

The causal relation between transforming hrHPV infections and carcinomas of the uterine cervix is well established and supported by strong evidence at both the epidemiological and molecular level 19. However, less is known about the role of hrHPV in a number of other carcinomas, including SCCs of the head and
neck (HNSCC). hrHPV can be detected in 15-35% of HNSCCs, and differences in genetic profiles and clinical outcome between HPV-positive and HPV-negative HNSCCs suggest that hrHPV may play an aetiological role in a subset of HNSCCs as well 20-23. In Chapter 5 genomic profiles of cervical carcinomas were compared to HNSCCs harbouring transcriptionally active hrHPV and HPV-negative HNSCCs. This comparison showed that a number of alterations common to HPV-negative carcinomas were rarely detected in HPV-positive samples, including losses at 3p, 5q, and 8p as well as amplifications at 11q13.3 (CCND1 locus). On the other hand gains of chromosome 20 and losses at 13q were HPV specific, irrespective of the anatomical site. Finally, within the group of HPV-positive carcinomas, a number of organ-specific alterations were found including gains at 8q in HNSCCs and losses at 17p in cervical carcinomas. The high frequency in which HPV-specific alterations were found, suggests that they are crucial in HPV-induced carcinogenesis. Therefore, prophylactic HPV-vaccines may, in addition to cervical cancer, also prevent a subset of HNSCCs characterised by the presence of transcriptionally active hrHPV, arguing for vaccination of both boys and girls. In addition, the fact that HPV-mediated transformation of squamous epithelial cells of different anatomical origins is accompanied by common chromosomal alterations suggests that biomarkers based on HPV-specific alterations may be useful in other cancer screening programs as well.

Identification of additional (epi) genetic alterations
To achieve high sensitivity and specificity a marker panel for cervical cancer preferentially should contain markers reflecting important steps in cervical carcinogenesis. Telomerase activation is an established, important event during carcinogenesis in general. In cervical cancer telomerase activity and hTERT expression (catalytic subunit of telomerase) have been shown in 96% of SCCs and 40% of CIN3 lesions, whereas normal cervix, CIN1 and CIN2 lesions showed no detectable telomerase activation 24. Unfortunately, telomerase activation or hTERT expression cannot be accurately measured in cervical smears 25. In Chapter 6 telomerase-associated transcriptional changes were studied in an in vitro model system of HPV-mediated transformation to identify potential surrogate markers for deregulated telomerase activity. Differential expression associated with telomerase activity was found for 32 genes. Differential expression of two of these potential surrogate markers, AQP3 and
MGP, was confirmed by real-time RT-PCR and immunohistochemistry, respectively, in SCCs and high-grade CIN lesions with hTERT expression.

In Chapter 7 DNA promoter methylation of 29 known tumour suppressor genes was studied in consecutive stages of the same *in vitro* model system for HPV-mediated transformation, ranging from pre-immortal to anchorage-independent phenotypes. An accumulation of frequent methylation events was observed with increasing transformation, resulting in the assignment of 8 recurrent epigenetic alterations (promoter methylation of tumour suppressor genes TP73, ESR1, RARβ, DAPK1, MGMT, CADM1, CDH13 and CHFR) to different stages of HPV-induced transformation (Figure 1). The significance of these 8 events in cervical carcinogenesis is supported by their detection in cervical carcinomas as well.

![Figure 1: Schematic overview of findings of this thesis in context of the concept of cervical carcinogenesis.](image)

In conclusion, this thesis has identified a number of genetic and epigenetic alterations strongly linked to cervical carcinogenesis. These findings have contributed to our current understanding of the development of cervical cancer (Figure 1). The fact that specimens of invasive carcinomas and well defined high-grade CIN lesions as well as an *in vitro* model system were used, aided in
the identification of alterations relevant to hrHPV-mediated carcinogenesis. Gains of chromosome 3q and 20q were frequently observed in SCCs, CIN2/3 lesions, and HPV-immortalised cell lines, underlining their importance. Gains at chromosome 1 where common to both SCCs and AdCAs and were also frequently found in CIN2/3 lesions. In HPV-immortalised cell lines, on the other hand, gains on chromosome 1 were only found in one out of four cell lines, indicating this alteration may occur later in the transformation process, or alternatively, is not necessary for in vitro immortalisation but is needed for in vivo malignant transformation. Integration of chromosomal alterations with gene expression profiles identified potentially relevant genes within common chromosomal alterations. In addition, surrogate markers were found for telomerase activation, which may enable more reliable determination of telomerase activity in cervical smears. Finally, a sequential order was found in the methylation status of a series of tumour suppressor genes.

The fact that HPV-specific alterations were found in SCCs of different anatomical sites, indicates that the above described concept may apply not only to cervical carcinogenesis, but to HPV-mediated carcinogenesis in general.

**Future perspectives**

This thesis thoroughly studied common chromosomal alterations associated with HPV-mediated malignant transformation and identified potential target genes within these chromosomal regions. Further functional studies using the in vitro model system are needed to discover the biological relevance of the identified genes and corresponding molecular pathways.

To investigate the potential marker value of genes identified in this thesis as triage markers in cervical screening, the most optimal detection system needs to be determined. A pilot study on cervical smears showed that direct detection of altered mRNA expression of genes is difficult due to the presence of large numbers of normal cells. However, preliminary data show that it is possible to detect increased protein expression of DTX3L and PIK3R4 (both located on chromosome 3q) in a subset of CIN2/3 lesions and carcinomas by immunohistochemical staining. Immunocytochemical stainings for these and other targets on cervical smears may facilitate the detection of abnormal cells. Others have already shown the potential value of immunocytochemical stainings for p16\(^{INK4a}\) and cell cycle components, such as MCM2, TOPIIα, PCNA and Ki-67, on cervical smears \(^{26-28}\). Alternatively, increased DNA copy numbers of genes can be directly determined in smears using
Fluorescence in situ hybridisation (FISH) as was shown for the TERC gene on chromosome 3q \(^{29,30}\). Besides genetic alterations, epigenetic events were investigated as well, which resulted in the identification of an additional number of tumour suppressor genes potentially involved in HPV-mediated carcinogenesis. Studies have shown that hypermethylation of gene promoters can be reliably detected in cervical smears with high sensitivity using (quantitative) MSP techniques \(^{31}\).

The linkage study between chromosomal and expression profiles described in this thesis showed that only part of the frequent chromosomal alterations were correlated with altered mRNA expression. For instance, chromosome 1 showed multiple frequently gained regions, which were not reflected by altered mRNA expression profiles of genes residing at these locations. This indicates that the mechanisms driving cervical carcinogenesis are not yet fully understood. To further extend our understanding of HPV-mediated carcinogenesis, an emerging field of players in human carcinogenesis, so-called non-coding RNAs (ncRNAs), needs investigation \(^{32}\). MicroRNAs (miRNAs), which represent an abundant class of ncRNAs, regulate gene activity by mRNA degradation or inhibition of translation. One single miRNA may regulate expression of as many as 200 different gene targets. Altered expression of miRNAs can contribute to malignant transformation by increasing or decreasing expression of oncogenes and tumour suppressor genes, respectively. Interestingly, clusters of miRNAs are located within the frequently gained regions at chromosome 1, which may explain the lack of putative protein-coding oncogenes found in this thesis. At present limited data are available on alterations in ncRNA expression in cervical carcinogenesis. Interestingly, miRNA loci are significantly associated with insertion sites of hrHPV in cervical cancers, suggesting that involvement of miRNAs in HPV-mediated carcinogenesis is likely \(^{33}\). The fact that ncRNAs are small renders them relatively stable and facilitates their detection in cervical scrapes (preliminary data), suggesting they may represent ideal markers for cervical screening.

Ultimately, a panel of markers reflecting different (epi)genetic alterations necessary for cervical carcinogenesis will improve current cervical screening programs by better detection of relevant disease. In addition, since treatment of CIN lesions can have adverse effects on certain aspects of pregnancy, including pre-term delivery, the moment of therapeutic intervention in women of reproductive age should be carefully considered \(^{13}\). A marker panel based on molecular changes in the host cell, which is applicable to cervical scrapes, may
aid in the distinction of early CIN2/3 lesions and advanced CIN2/3 lesions with a high short-term risk for progression that need immediate treatment.

REFERENCES


