Chapter 6

Summary & Discussion
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Cervical cancer develops over a long period of time as a result of a persistent infection with hrHPV. The main 2 histotypes of cervical cancer i.e. squamous cell carcinoma (SCC) and adenocarcinoma (AdCa) are preceded by precursor lesions. Precursor lesions of SCCs are called Cervical Intraepithelial Neoplasia (CIN). Depending on the degree of atypia CIN can be differentiated in 3 grades i.e. CIN1 (minor atypia), CIN2 (moderate atypia) and CIN3 (severe atypia). Progression of an hrHPV-induced premalignant lesion to invasive cancer is driven by the accumulation of genetic and epigenetic alterations in the host cell genome. Identification of these alterations will not only improve our understanding of cancer biology, but may lead to new screening tools and new (immuno)therapeutic strategies.

Present screening programs are based on the cytomorphological assessment of abnormal cells in cervical scrapes, the so-called Pap-test. Recent randomized-controlled, prospective trials have shown that testing for hrHPV positivity in cervical screening programs results in earlier detection of clinically relevant cervical precursor lesions, enables better detection of AdCA and its precursor lesions and greatly reduces the number of false-negative smears 

However, hrHPV testing also results in the detection of women with transient hrHPV infections who do not have or will develop cervical cancer precursor lesions and for whom follow-up examinations are redundant. Presently, cytology is recommended for risk stratification of hrHPV-positive women, and women with abnormal cytology (threshold BMD or worse) should be referred for colposcopy. Since, however, hrHPV positive women with normal cytology have an increased risk compared to those without hrHPV, additional reliable disease markers are needed as triage tool.

Although prophylactic vaccination against HPV16 and 18 has recently been implemented for pre-pubertal women in many countries, the current vaccines do not achieve full protection against non-HPV16,-18 associated cervical cancer and its precursor lesions. In addition, the vaccines have no effect on existing infections with HPV 16,-18 and consequently, cervical screening remains necessary in the foreseeable future.

Most studies aiming at the identification of cervical disease markers are based on the molecular analysis of cervical carcinomas and its precursor lesions. However, to obtain functional insight in the biologically relevant events and in order to reduce complexity, the study of in vitro models mimicking the multistep process of HPV-induced carcinogenesis is crucial. Previous work by
others and us has revealed that hrHPV types can induce immortalization of primary human keratinocytes in vitro. Primary keratinocytes normally have a limited lifespan due to telomere shortening. Expression of the viral oncogenes in primary cells induces a state of genetic instability from which immortal clones may emerge. These immortal cells display strong telomerase activity, a necessary event for maintenance of telomeres and consequently the immortal phenotype.

In this thesis an in vitro model of hrHPV-transfected keratinocytes was studied, aiming at unravelling molecular events underlying HPV-mediated transformation, with a special focus on the phenotype immortalization. In addition, we performed an integrated analysis of chromosome and expression profiles of cervical carcinomas, as an alternative approach to get more insight into cervical carcinogenesis.

**Mechanisms underlying hTERT deregulation during HPV-mediated immortalization**

In chapter 2 we examined what mechanisms may underlie hTERT deregulation in hrHPV transformed cell lines. Using luciferase reporter constructs we analyzed the transcriptional activity of various hTERT promoter regions as well as proximal exonic/intronic sequences and determined the relationship with epigenetic modifications. Although most telomerase positive cells showing elevated hTERT mRNA expression revealed substantial hTERT core promoter activity, hTERT core promoter activity was also detected in telomerase negative cells with no or strongly reduced hTERT mRNA expression levels. Moreover, regulatory sequences flanking both ends of the core promoter markedly repressed promoter activity. Since this phenomenon was observed in both telomerase positive and negative cells we reasoned that epigenetic events might be involved in blocking this repressive effect in telomerase positive cells.

Interestingly, by extensive bisulfite sequencing analysis of the region spanning nucleotides -442 to +566 (relative to the ATG) a marked increase in methylated CpGs was detected in hTERT positive cells compared to cells with no or strongly reduced hTERT expression. Subsequent analysis of cervical tissue specimens by methylation specific PCR (MSP) for two suppressive regions flanking the core promoter revealed methylation of both regions in 100% of cervical carcinomas and 38% of the high-grade precursor lesions, compared to 9% of low-grade precursor lesions and 5% of normal controls. Hence, the correlation between DNA methylation at these transcriptional repressive regions
and hTERT expression suggests that this methylation event is likely to contribute to hTERT deregulation in HPV-transformed cells. Its association with increasing severity of cervical disease suggests that hTERT methylation is also relevant for telomerase reactivation during cervical carcinogenesis.

**Altered expression of AP-1 complex members and their regulatory genes during HPV-mediated transformation in vitro**

The transcription factor AP-1 represents an important mediator in many cancer-specific alterations in different human cancers. AP-1 does not only activate HPV transcription, but has also been found to interact with sequences 5’ of the hTERT core promoter, which we found to be suppressive in chapter 2. The AP-1 transcription factor is a heterodimer consisting of a member of the Fos gene family, i.e. c-Fos, FosB, Fra-1 and Fra-2, and a member of the Jun family, i.e. c-Jun, JunB or JunD. Depending on its composition this complex is involved in the positive and/or negative regulation of several genes (reviewed by). Alterations in AP-1 complex composition have been associated with tumorigenicity during cervical carcinogenesis in vitro and in vivo. In normal cells Fra-1 is expressed and the AP-1 complex consists primarily of Fra-1/c-Jun heterodimers, while in tumorigenic cells c-Fos is upregulated resulting in a shift to c-Fos/c-Jun heterodimers. In chapter 3 we aimed to obtain further insight in expression alterations of AP-1 family members during HPV-mediated transformation and their relationship to potential regulatory (Notch1, Net) and target (CADM1) genes. mRNA expression levels of all AP-1 complex members (c-Jun, JunB, JunD, c-Fos, FosB, Fra-1, and Fra-2) as well as Notch1, Net and CADM1 were determined by quantitative RT-PCR in primary keratinocytes, early and late passages of non-tumorigenic HPV-immortalized keratinocytes and in tumorigenic cervical cancer cell lines. In a subset of cell lines protein expression and AP-1 complex composition were determined as well. Starting in immortal stages c-Fos, Fra-2 and JunB expression became up regulated towards tumorigenicity, whereas Fra-1, c-Jun, Notch1, Net and CADM1 became down regulated. The onset of deregulated expression varied amongst the AP-1 members and was not directly related to altered Notch1, Net or CADM1 expression. Notwithstanding this, a shift in AP-1 complex composition from Fra-1/c-Jun to c-Fos/c-Jun heterodimers was only observed in tumorigenic cells. Thus, whereas the onset of deregulated expression of various AP-1 family members became already manifest during the immortal state, a shift in AP-1 complex composition appeared a late event associated with tumorigenicity.
Altered gene expression associated with deregulated hTERT in vitro

The study in Chapter 4 aimed at getting more insight into the altered gene expression profiles associated with HPV-mediated immortalization, in particular telomerase activation. Our previous microcell-mediated chromosome transfer studies revealed that introduction of human chromosome 6 in the HPV16 immortalized keratinocyte cell line FK16A and in the HPV16 containing cervical cancer cell line SiHa induced growth arrest, resulting from a repression of hTERT mRNA expression and telomerase activity \(^{16}\). To analyze expression profiles associated with hTERT deregulation in HPV transformed cells we performed microarray expression analysis on 12 FK16A/chromosome 6 hybrids, four of which were negative for endogenous hTERT and 8 of which were positive for endogenous hTERT. This resulted in the identification of 164 differentially expressed genes and differential expression of a selection of 5 genes was verified by quantitative RT-PCR. Of these 164 genes, 32 were also differentially expressed in other HPV transformed cells with deregulated hTERT. For 2 of these genes, encoding AQP3 and MGP, altered expression in hTERT positive cervical carcinomas was confirmed by quantitative RT-PCR and immunohistochemistry, respectively. Moreover, increased MGP protein expression was significantly more frequent in high-grade cervical premalignant lesions with elevated hTERT mRNA expression compared to those without. Thus, we identified 32 candidate surrogate markers for deregulated hTERT mRNA expression, which may enable the identification of cervical premalignant lesions that are at highest risk to progress to invasive cancer.

Altered gene expression and commonly altered chromosomal regions in cervical carcinomas

In chapter 5 we used an alternative approach to discover events that are relevant for cervical carcinogenesis. By integrating genome-wide chromosomal and transcriptional profiles of cervical carcinomas we aimed to detect altered expression patterns related to chromosomal alterations. Previous genomic profiling showed that gains at chromosomes 1q, 3q and 20q as well as losses at 8q, 10q, 11q and 13q were common in cervical carcinomas \(^{17}\). Application of differential gene locus mapping (DIGMAP) analysis and the array CGH expression integration tool (ACE-it) identified hotspots within large chromosomal alterations in which gene expression was altered as well. Chromosomal gains of the long arms of chromosome 1, 3 and 20 resulted in
increased expression of genes located at 1q32.1-32.2, 3q13.32-23, 3q26.32-27.3, and 20q11.21-13.33, whereas a chromosomal loss of 11q22.3-25 was related to decreased expression of genes located there. Overexpression of 4 genes located at chromosome 3q, DTX3L, PIK3R4, ATP2C1, and SLC25A36, as identified by DIGMAP, ACE-it or both, was confirmed in an independent validation set of cervical carcinomas and normal cervical tissues.

So, by studying both an in vitro model system of HPV16 and HPV18-immortalized cell lines and cervical tissue specimens, we gained more insight in the (epi)genetic processes and expression alterations associated with HPV-induced transformation, in particular hTERT deregulation and immortalization. hTERT transcriptional activity was shown to be regulated by both activating and suppressive promoter elements in HPV-immortalized cell lines. The suppressive elements revealed increasing levels of DNA methylation during HPV-induced transformation in vitro. In concordance with the in vitro results hTERT promoter methylation frequencies also increased with the histopathological grade of disease in cervical tissue samples.

Next to the epigenetic modifications, changes in gene expression levels were demonstrated in this thesis. Expression of c-Fos family members, constituting part of the transcription complex AP-1, changed at immortalization of hrHPV-transfected cells, while an altered AP-1 complex composition was restricted to tumorigenic cells. Moreover, based on genome wide expression analysis a number of 32 genes, including MGP and AQ3, was identified that were differentially expressed upon telomerase activation in vitro. mRNA expression of another set of genes, such as DTX3L, PIK3R4, ATP2C1, and SLC25A36, was found to be commonly altered in cervical carcinomas as a result of chromosomal aberrations.

Future perspectives
Following these observations it will be of utmost interest to determine biological relevance of the gene alterations identified in this thesis.

hTERT regulation
hTERT upregulation and concomitant telomerase activation, which are fundamental to immortality, were demonstrated to be correlated to methylation of repressive regulatory hTERT sequences. It remains, however, to be resolved
whether methylation represents one of the most critical events inducing immortalization, or whether other factors, e.g. altered regulation of transcription factors, are the driving force. Moreover, it is not yet clear what determines aberrant hTERT methylation and which methylation sites are most essential. The very heterogeneous methylation patterns seen in individual alleles indicate that hTERT methylation is a very dynamic process. Unravelling the exact regulatory mechanisms will require a more detailed analysis using for example reporter constructs driven by in vitro methylated promoter elements tested in both presence and absence of HPV, as well as the analysis of other chromatin modulators e.g. by ChIP analysis.

It will be interesting to determine whether there is any direct or indirect relation between hTERT upregulation (by these methylation events and/or any of the known hTERT transcriptional regulators) and the genes showing altered expression upon hTERT upregulation, as identified in chapter 4. Hereto both cDNA overexpression and RNA interferences studies using the in vitro model system are warranted.

**Identifying new tumor suppressor genes**

Similarly, it also remains to be determined whether there are novel tumor suppressor genes amongst the down regulated genes identified in chapter 5. Functional studies on the HPV-transfected cell lines at different stages of transformation will allow us to pinpoint which phenotype the respective genes may impinge on. In fact, one of the down regulated genes identified in chapter 5, i.e., MAL, was in later studies found to act as a tumor suppressor gene in HPV–transformed cells \(^{18}\). However, the exact mechanism by which MAL inactivation promotes tumorigenesis still remains to be resolved.

**Identifying new oncoproteins from upregulated genes**

It is additionally of interest to find out whether identified upregulated genes code for oncoproteins that may actively contribute to HPV-induced carcinogenesis. Some of the upregulated genes identified herein are of particular interest, such as PIK3R4 and DTX3L, which are members for the PI3K-AKT and Notch signalling pathways, respectively. hrHPV E7 has been demonstrated to induce phosphorylation of AKT and increased levels of P-AKT have been described in cervical carcinomas and CIN lesions \(^{19,20}\). Using our in vitro model system we more recently found that the PI3K/AKT pathway becomes activated
during hrHPV-induced transformation in vitro and future studies may tell us whether this is linked to PIK3R4 overexpression. As shown in chapter 3, Notch expression alters during passaging of our hrHPV transfected cell lines. Potentially the altered Notch expression may be related to altered DTX3L expression, encoding a deltex family protein known to regulate Notch degradation. However, present data on the role of Notch signalling in cervical carcinogenesis are inconsistent, necessitating further research.

Clinical implications
As hrHPV testing is now being considered as an alternative for cytology-based cervical cancer screening, there is need for additional triage markers to distinguish hrHPV positive women who are in need of more intensive management because of a high risk for the development of cervical (pre)cancer. As stated above gene alterations associated with HPV-induced transformation as described in this thesis may provide such molecular markers. Recent studies have shown that hypermethylation of gene promoters can be reliably detected in cervical scrapings using quantitative MSP techniques. Presently ongoing studies are exploiting the diagnostic value of methylation markers in clinical practice by studying methylation frequencies in scrapings using both cross sectional studies as well as prospective population based studies, such as the POBASCAM trial. A number of novel methylation markers, such as CADM1, are attractive candidate triage markers. Additional methylation markers, to be deduced from the candidate marker genes identified in this thesis, might contribute to the composition of methylation marker panels displaying high sensitivity and specificity for clinically relevant CIN lesions and cervical cancer, one of which may be hTERT. We identified the MAL gene as the most significantly downregulated gene in cervical carcinomas (chapter 5). In further studies silencing was found to result from promoter methylation, which was detected in 53% of high-grade CIN lesions and 90% of SCC. Similarly, also other down regulated genes identified in this thesis may be located within or near a CpG island, suggesting that decreased expression may result from promoter hypermethylation. As demonstrated for hTERT in chapter 2 we cannot exclude that also part of the upregulated genes may be regulated by DNA methylation. These genes may be either hypermethylated like hTERT, thereby inhibiting binding of methylation sensitive suppressive factors or hypomethylated, as described for several (candidate) oncogenes.
With respect to the upregulated genes it will also be worthwhile to explore whether protein or mRNA overexpression can be measured in scrapings. It has yet been demonstrated that increased protein expression of p16\(^{\text{INK4a}}\) and other cell cycle components such as MCM2, TOP\(\text{II}_{\alpha}\), PCNA and Ki-67 can be detected in cervical scrapings. Recent studies have also described the detection of altered mRNA expression levels in cervical scrapings. For example elevated KIF23 mRNA expression was demonstrated in cervical scrapings of cancer patients, and reduced MAL mRNA expression has been detected in cervical scraping of patients with high-grade CIN disease.

Ultimately, a marker panel consisting of methylation markers, expression markers or a combination thereof is likely to become an attractive triage tool in future screen programs in which HPV testing will play a central role. The urgency of such a molecular marker panel will become reality in case self-collection of cervico-vaginal specimens is more accepted as screening tool, for which cytology is not an attractive option.

References


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