Clinical implications of (epi)genetic changes in HPV-induced cervical precancerous lesions

Renske D. M. Steenbergen, Peter J. F. Snijders, Daniëlle A. M. Heideman and Chris J. L. M. Meijer

Abstract | Infection of cervical epithelium with high-risk human papilloma virus (hrHPV) might result in productive or transforming cervical intraepithelial neoplasia (CIN) lesions, the morphology of which can overlap. In transforming CIN lesions, aberrations in host cell genes accumulate over time, which is necessary for the ultimate progression to cancer. On the basis of (epi)genetic changes, early and advanced transforming CIN lesions can be distinguished. This paves the way for new molecular tools for cervical screening, diagnosis and management of cervical cancer precursor lesions.

Epigenetic alterations
Changes in DNA methylation and chromatin that do not involve a change in the DNA sequence.

Cervical intraepithelial neoplasia
(CIN; also known as cervical dysplasia). A premalignant condition of the uterine cervix, which can be histologically subdivided into CIN1, CIN2 and CIN3.

Department of Pathology, VU University Medical Center, PO box 7057, 1007 Amsterdam, The Netherlands. Correspondence to R.D.M.S. e-mail: r.steenbergen@vumc.nl
doi:10.1038/nrc3728

With about 530,000 new cases annually, cervical cancer is the third most common cancer in women worldwide and the seventh most common cancer overall. In 2008, cervical cancer was responsible for 275,000 deaths, thereby being the fourth leading cause of cancer death in females worldwide[1,2]. Virtually all cervical cancers result from a persistent infection with certain high-risk types of the human papilloma virus (hrHPV) family[3]. However, cervical cancer is a rare complication of a rather common viral infection; the lifetime risk of an hrHPV infection is estimated to be around 80% (REF. 4) and, fortunately, the large majority of infections are cleared by the host immune system and do not give rise to lesions. Most of the remaining hrHPV infections develop into lesions that are thought to be 'productive' infections that lead to the generation of new viral progeny. Although such infections show no signs of cellular transformation, morphologically, they can show dysplastic features that overlap with those seen in progressive precancers. Only a minority of hrHPV infections become 'transforming' infections, characterized by the altered expression of two viral genes, *E6* and *E7* (discussed below). Such a condition may ultimately lead to cancer if the respective precursor lesion is left untreated. It is still poorly understood which factors determine the malignant fate of an hrHPV infection.

In this Review, we discuss recent advances that shed more light on the development and progression of transforming hrHPV infections. The focus is on cellular genetic and epigenetic alterations that underlie the progression to cancer. Their implications for the development of new molecular diagnostic tools for cervical screening, diagnosis and management of patients with cervical precancer are also discussed.

Cervical cancer and HPV

According to their epidemiological association with cervical cancer and consolidated by biological studies, 12 types of HPV (HPV-16, HPV-18, HPV-31, HPV-33, HPV-35, HPV-39, HPV-45, HPV-51, HPV-52, HPV-56, HPV-58 and HPV-59) have now been consistently classified as hrHPV (also known as IARC class I). HPV-68 has been classified as probable high-risk (also known as IARC class 2A), and another seven types have been classified as possible high-risk (HPV-26, HPV-53, HPV-66, HPV-67, HPV-70, HPV-73 and HPV-82; also known as IARC class 2B)[5].

Following an hrHPV infection, cervical cancer develops through a series of subsequent steps: hrHPV persistence, hrHPV-mediated epithelial transformation, development of precancerous lesions (cervical intraepithelial neoplasia graded 1 to 3 (CIN1–3)) and, finally, progression to invasive cervical cancer (FIG. 1). Cervical cancer development, in particular the step from precancer to invasive cancer, takes a long time in most patients. High-grade precancerous CIN2 and CIN3 lesions can develop within 3–5 years following an hrHPV infection[6], whereas further progression to invasive cancer can take up to 20–30 years[7,8]. This long period offers many opportunities for intervention and has probably contributed to

Key points

- Cervical intraepithelial neoplasia (CIN) lesions can be divided into productive (CIN1 and CIN2) and transforming (CIN2 and CIN3) lesions. Morphologically, productive CIN2 cannot be distinguished from transforming CIN2.
- Transforming CIN reflects a heterogeneous disease. Early and advanced transforming CIN lesions, displaying a low and high short-term progression risk for cancer, respectively, can be distinguished on the basis of molecular host cell alterations.
- When applied to cervical scrapings, specific methylation markers, such as cell adhesion molecule 1 (*CADM1*), myelin and lymphocyte (*MAL*) and *mir-124-2*, detect advanced transforming CIN and cancer with a high sensitivity.
- CIN2/CIN3 lesions detected by specific methylation markers are in need of immediate treatment, given their high short-term progression risk for cancer.
- Cytology detects morphological cellular abnormalities associated with CIN2, CIN3 and cancer with a moderate sensitivity, but may miss cancer and advanced transforming CIN with a high short-term progression risk for cancer.
- Human papilloma virus (HPV) testing will replace cytology as the primary screening tool for cervical cancer.
- Clinically validated panels of methylation markers, such as *CADM1*, *MAL* and *mir-124-2*, can be used as triage markers for HPV-positive women. Methylation marker panels with a high sensitivity for cancer have the potential to function as a primary screening tool.
- DNA methylation marker panels may also be used for the management of women with CIN lesions to prevent overtreatment of CIN2/CIN3 lesions.
- The compatibility of methylation markers with HPV testing and self-sampling has the potential for full molecular cervical screening in the near future.

the success of frequent Papanicolaou (Pap) screening to reduce the incidence and mortality of cervical cancer in the Western world[9].

Histomorphologically, most cervical cancers are squamous cell carcinomas (SCCs; accounting for 80% of cervical cancers). Adenocarcinomas (accounting for 10–20%) represent the second most common histotype, followed by a small proportion of adenosquamous carcinomas and other rare histotypes, including neuroendocrine carcinomas.

Productive versus transforming infections

A productive infection begins when viral particles gain access to the epithelial basement membrane, most probably via micro-abrasions, and subsequently enter the basal cells of squamous epithelium. In infected basal cells, the viral genome is replicated in conjunction with cellular DNA during S phase and maintained as stable episomes. In these cells, expression of the viral proteins occurs at very low levels, which probably facilitates escape from immune surveillance (reviewed in REF. 10). Following cell division, one of the daughter cells undergoes a differentiation process and exits the cell cycle. Subsequently, viral differentiation-dependent promoters become upregulated, which results in an increased expression of viral genes, including the viral early genes *E6* and *E7* (REFS 10–12). Expression of the *E6* and *E7* genes drives the differentiated cells into S phase, thereby creating environmental conditions that support vegetative viral genome replication. In the upper layer of the squamous epithelium, the last stage of the viral life cycle involves the generation of new viral particles that are released from shedding terminally differentiated cells (reviewed in REF. 13). Productive infections in the cervix may give rise to mild to moderate cellular abnormalities and, histologically, such conditions are manifested as CIN1 or CIN2 (CIN1/CIN2). In order to distinguish this condition from true cancer precursor lesions, such lesions are referred to here as productive CIN lesions. Usually, these lesions spontaneously regress within 1–2 years, a process that is accompanied by viral clearance resulting from cell-mediated immune responses to E2, E6 and E7. Immune evasion accompanied by viral and lesion persistence may result from various mechanisms, such as virus-mediated suppression of innate immunity, suppression of T cell effector function, increase in the number of regulatory T cells in the tumour microenvironment and frequent loss of human leukocyte antigen (HLA) expression resulting from genetic events (reviewed in REF. 14). Viral persistence facilitated by loss of immune control is crucial for HPV-mediated carcinogenesis, as HPV infections are essential for not only the initiation but also the maintenance of the transformed phenotype (reviewed in REF. 15).

Morphologically, CIN3 and a subset of CIN2 lesions typify transforming CIN lesions, in which the normal viral life cycle is aborted and the viral early genes *E6* and *E7* are overexpressed in proliferating cells. However, CIN2 lesions resulting from a productive infection cannot be morphologically distinguished from CIN2 lesions resulting from a transforming infection. In the context of dividing cells, the *E6*- and *E7*-encoded proteins function as oncoproteins and the respective genes are therefore referred to as viral oncogenes. A direct result from E6 and E7 deregulation in a transforming infection is the altered expression of cell cycle and DNA repair regulators. The exact mechanism that contributes to this rather unnatural E6 and E7 expression pattern has not been understood, but altered intraviral control of *E6* and *E7* expression by genetic alterations (for example, viral DNA integration) and epigenetic alterations (for example, methylation of viral promoter regions) of the viral genome have been suggested[16–18]. Alternatively, a different host cell environment that is non-permissive for viral replication could favour non-canonical regulation of E6 and E7 expression. A candidate cell type that could be highly susceptible to HPV transformation is the squamo-columnar junction (SCJ) cell. Herfs *et al.*[19] recently reported that this discrete population of single-layered, cuboidal epithelial cells of embryonic origin, which are localized between ectocervical squamous epithelium and endocervical glandular epithelium, represents the likely cellular precursor of most cervical cancers and their precursor lesions. By contrast, productive infections might arise exclusively from infection of basal cells of the squamous epithelium lining the ectocervix or adjacent transformation zone[20].

SCJ cells show a unique gene expression profile for several genes, including keratin 7 (*KRT7*), anterior gradient 2 (*AGR2*), matrix metalloproteinase 7 (*MMP7*) and guanine deaminase (*GDA*)[19]. The proteins that are encoded by these genes can be used as an SCJ-specific protein biomarker panel. The expression of these proteins is not induced by HPV E6 or E7 *in vitro* in squamous epithelial cells, and their expression is lost if the SCJ is removed by cone biopsy or loop electrical excision. Therefore, it seems that the SCJ-specific expression profile in CIN lesions and

Episomes
Extrachromosomal DNA elements that can replicate independently from host chromosomal DNA.

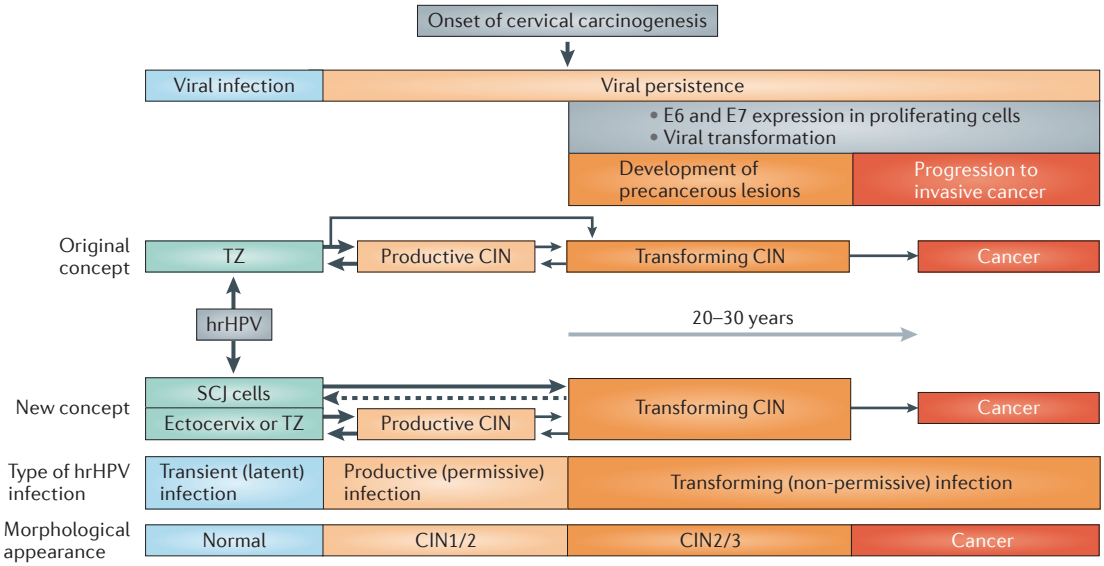

Figure 1 | **HPV-mediated cervical carcinogenesis.** The various outcomes of exposure of cervical epithelial cells to high-risk human papilloma virus (hrHPV) are represented as a transient infection (no pathology), a productive infection (productive cervical intraepithelial neoplasia (CIN); mainly representing CIN1 and a subset of CIN2) and a transforming infection (transforming CIN; mainly representing the remaining subset of CIN2 and CIN3). Morphologically, CIN2 that is associated with a productive HPV infection cannot be distinguished from CIN2 that is associated with a transforming HPV infection. Similarly, CIN1 lesions that may occasionally represent transforming infections are, morphologically, not distinguishable from productive counterparts. From the onset of a transforming CIN, it can take another 20–30 years before invasive cancer will develop. Transforming CIN represents a heterogeneous disease with varying duration of existence, which may either regress or progress to cancer. The risk of progression to cancer is dependent on molecular host cell alterations. A new concept suggests that most of the transforming CIN and cervical cancers arise from exposure of embryonic squamo-columnar junction (SCJ) cells to hrHPV[19], which suggests a high susceptibility of these cells to HPV transformation. The SCJ cells and corresponding lesions are characterized by a specific protein expression pattern (expression of keratin 7 (KRT7), anterior gradient 2 (AGR2), matrix metalloproteinase 7 (MMP7) and guanine deaminase (GDA)), and precursor lesions that arise from these SCJ cells are unlikely to be preceded by a productive CIN. Productive CIN lesions are suggested to arise from infection of cells in the ectocervix or transformation zone (TZ).

cervical cancers is not acquired during the transformation process and instead reflects the embryonal origin of the cells. Interestingly, of all cervical cancers analysed (both SCC and adenocarcinoma), most CIN2/CIN3 lesions and one-third of CIN1 lesions were positive for an SCJ expression profile[19,21]. The presumed high transformation susceptibility of these SCJ cells compared with squamous cells of the ectocervix and transformation zone is supported by the fact that HPV-associated high-grade precancerous lesions are up to 20-times more common in the cervix (which contains an SCJ) than in other genital sites that lack an SCJ, such as the vagina and the vulva[22].

The net result of deregulated expression of E6 and E7 in proliferating cells is chromosomal instability[23], which probably provides the driving force for accumulation of alterations in cancer genes of the host cell and consequently progression towards cancer.

In the following sections, the primary and secondary consequences of deregulated E6 and E7 expression on host cell genes and gene products will be discussed in the context of cervical cancer development.

Primary effects of E6 and E7 deregulation

It is now widely accepted that combined hyperactivity of E6 and E7 in proliferating cells represents the trigger for HPV-induced malignant transformation. Initially, the binding of tumour suppressor gene products, RB by E7 and p53 by E6, were thought to be the primary events responsible for malignant transformation. The targeting of RB by E7 leads to uncontrolled cell proliferation, which primarily results from increased E2F activity as evident through the upregulation of E2F-responsive gene products, such as proliferating cell nuclear antigen (PCNA), Ki-67, minichromosome maintenance proteins (MCMs), cyclin E and p21 (reviewed in REFS 24,25). Formation of a complex between the ubiquitin ligase E6AP (also known as UBE3A) and E6 results in the ubiquitin-mediated degradation of p53, thereby interfering with the normal p53-mediated apoptosis and cell cycle control mechanisms induced by genotoxic stress[24,25].

Currently, it has become evident that complex formation of E6 and E7 with other cellular proteins also contributes to the virus-mediated transformation process. Some of the interactions result in chromatin remodelling (reviewed in REF. 26). Both E6 and E7 can modulate the DNA methylation machinery, thereby influencing cellular and viral gene expression. HPV-16 E6 can induce upregulation of the DNA methyltransferase DNMT1 via suppression of p53 (REF. 27), whereas HPV-16 E7 can directly bind to and activate DNMT1 (REF. 28). In support of these *in vitro* findings, both

DNA methylation
The addition of a methyl group at a cytosine in a CpG dinucleotide pair. DNA methylation of CpG-rich areas in gene promoters can result in gene silencing.

(Micro)array comparative genomic hybridization ((Micro)arrayCGH). A platform on which DNA copy-number aberrations can be assessed at a genome-wide level in a single experiment.

DNMT1 and DNMT3B were shown to be upregulated in CIN3 lesions and cervical carcinomas[29–31]. A further modulating effect on epigenetic reprogramming can be accomplished by E7 via induction of the histone lysine demethylases KDM6A and/or KDM6B. This leads to histone demethylation of genes that were silenced by polycomb repressive complex (PRC)-mediated histone H3 lysine 27 (K27) trimethylation[32,33]. One of these genes encodes the cyclin-dependent kinase inhibitor p16 (also known as INK4A)[33]. Although this induction does not affect proliferation because of the downstream targeting of RB by E7, the overexpression of p16 is nowadays widely considered to be a hallmark of hrHPV activity[34] (FIG. 2).

In addition, HPV-16 E6 and E7 are also known to alter the expression of microRNAs (miRNAs) (BOX 1) through direct and indirect effects. HPV-16 E6 can downregulate miR-218, miR-23b and miR-34a expression[35–37], and miR-23b and miR-34a expression is linked to E6-induced p53 degradation. Reduced miR-203 and increased expression of the miR-15a/16-1 cluster is attributed to E2F release upon RB inactivation by hrHPV E7 (REF. 38). Conversely, miRNAs may also regulate viral gene expression[39], and the first indications of the existence of HPV-encoded miRNAs have been reported[40]. However, these findings await further confirmation.

Secondary effects of E6 and E7 deregulation

Although E6 and E7 are necessary for the initiation and maintenance of the transformed phenotype, the long duration of progression from precancer to invasive cancer indicates that several additional oncogenic events are pivotal for malignant progression. A well-known consequence of deregulated E6 and E7 expression is chromosome instability (reviewed in REFS 23,24). This genomic instability probably contributes to the accumulation of aberrations in host cell genes over time (FIG. 2). Such acquired aberrations can be both genetic and epigenetic, and some of them result in functional abrogation of human tumour suppressor genes or activation of oncogenes. Host cell aberrations observed in cervical cancers or precancers include deletions, copy number alterations, DNA mutations and epigenetic alterations, such as DNA methylation affecting both protein coding genes and non-coding genes such as miRNAs. An overview is available in the form of a recently established database of genes that have been found to be altered in cervical cancer[41]. Various aberrations in cervical cancers and CIN lesions are described below.

Chromosomal aberrations. A meta-analysis of 12 (micro) array comparative genomic hybridization ((micro)array-CGH) studies covering a total of 293 samples showed that the most frequent DNA copy-number alterations

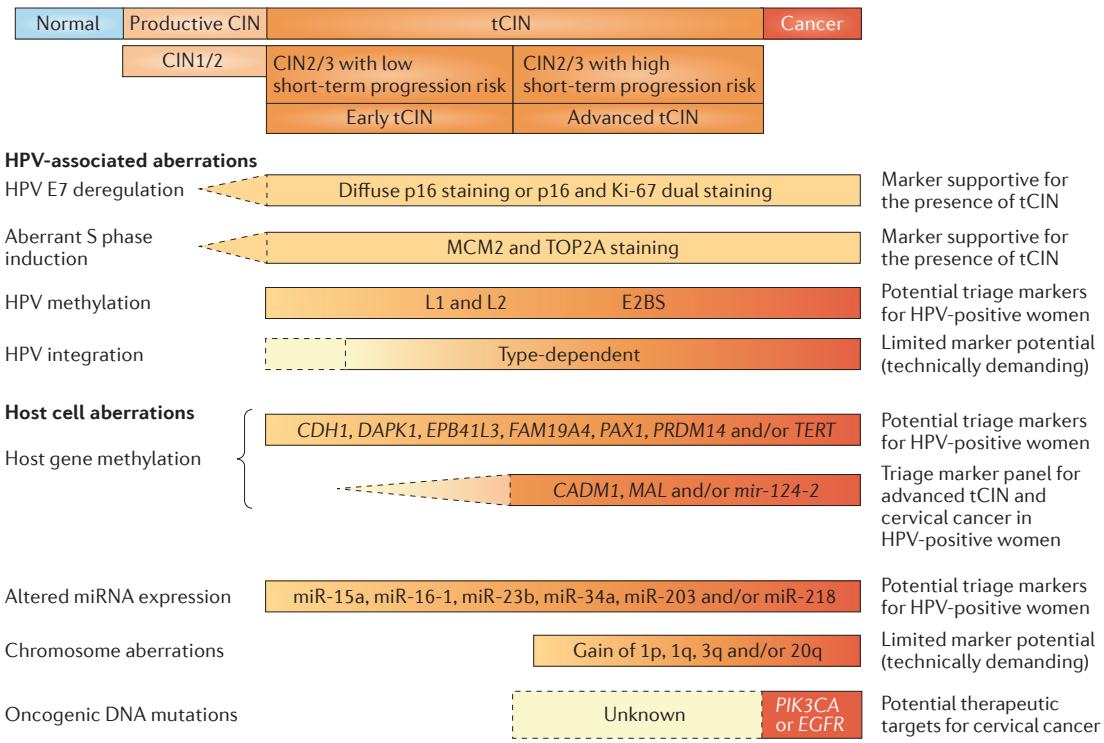

Figure 2 | **Cellular changes required for the progression of transforming cervical intraepithelial neoplasia (tCIN) to cancer.** The human papilloma virus (HPV)-related and host cell aberrations that are associated with disease progression are indicated below the concept of HPV-induced cervical carcinogenesis. Colour intensities indicate their level or frequency of detection (with red being high level or highly frequent) and dashed lines indicate their infrequent or unknown detection. The potential applications of the viral and host cell aberrations as markers for screening, diagnosis and treatment strategies are listed on the right. *CADM1*, cell adhesion molecule 1; *CDH1*, cadherin 1; *DAPK1*, death-associated protein kinase 1; E2BS, E2 binding site; *EGFR*, epidermal growth factor receptor; *MAL*, myelin and lymphocyte; MCM2, minichromosome maintenance protein 2; miRNAs, microRNAs; *PAX1*, paired box 1; *PRDM14*, PR domain containing 14; *TERT*, telomerase reverse transcriptase; TOP2A, topoisomerase 2A.

in cervical SCC include gain at 3q (rate 0.55), loss at 3p (rate 0.36) and loss at 11q (rate 0.33)[42]. Gain at 3q was particularly frequent in HPV-16-positive SCC (rate 0.84). Gain at 17q (rate 0.36) was most frequent in adenocarcinoma (4 studies, with a total of 58 samples). Gain at 1p was the most frequent aberration in high-grade CIN (rate 0.34). This was followed in decreasing order of frequency (from 0.27 to 0.08) by gain at 3q and loss at 4q, 2q, 4p, 11p and 3p. From these regions, candidate driver genes can be extracted by analysis of recurrent focal aberrations and/or expression profiling supplemented with functional analysis. This approach has led to the identification of eyes absent homologue 2 (*EYA2*) and *mir-375* as novel oncogenes and tumour suppressor genes, respectively, in cervical cancer[43]. In support of these findings, *EYA2* has recently been identified as a target of viral integration and a tumour-suppressive function of miR-375 has also been corroborated in other studies[44,45]. These data provide a proof-of-concept that specific chromosomal aberrations can contribute to HPV-induced carcinogenesis.

DNA mutations. To date, relatively few reports on mutations in oncogenes or tumour suppressor genes have been described for cervical cancer or its precursor lesions. Because the gene products of *TP53* and *RB1* are inactivated by E6 and E7, they are only rarely mutated in cervical cancer (5% and 3%, respectively; COSMIC catalogue of somatic mutations)[46]. Other somatic mutations that are found in cervical cancers mainly involve members of signalling pathways. The highest mutation rates are reported for *PIK3CA* in both SCC and adenocarcinoma, as corroborated in two recent papers (that is, mutation rates in SCC: 37.5% (REF. 47) and 14% (REF. 48); and mutation rates in adenocarcinoma: 25% (REF. 47) and 16% (REF. 48)). Wright *et al.*[47] also identified *KRAS* mutations in adenocarcinoma only (17.5%) and epidermal growth factor receptor (*EGFR*) mutations in SCC only (7.5%). In addition, Ojesina *et al.*[48] showed recurrent mutations in E1A binding protein p300 (*EP300*; 16%), F-box and WD repeat domain containing 7 (*FBXW7*; 15%), *HLAB* (9%), *MAPK1* (8%), *PTEN* (6%), serine/threonine kinase 11 (*STK11*; encoding LKB1; 4%) and nuclear factor, erythroid 2-like 2 (*NFE2L2*; 4%) in SCC, as well as E74-like factor 3 (*ELF3*; 13%) and core-binding factor, β-subunit (*CBFB*; 8%) in adenocarcinoma. So far, CIN lesions have neither been studied nor analysed at a substantial sample size.

Aberrant DNA methylation. Epigenetic mediators include histone modifications, nucleosome occupancy and positioning, protein and non-coding RNA interactions, as well as direct DNA modifications (reviewed in REF. 49). In cervical lesions, DNA methylation has gained the most attention. DNA methylation involves the covalent binding of a methyl group (CH_3) at the carbon-5 position of cytosine located 5′ of a guanine to generate a 5-methylcytosine. In general, increased methylation of CpG-rich human gene promoters represses gene transcription and often involves (candidate) tumour suppressor genes. However, methylation of viral DNA is thought to both negatively and positively regulate viral gene transcription.

A rapidly growing number of studies have analysed the occurrence and role of viral DNA methylation in the development of cervical cancer. Although an altered HPV methylation pattern during disease progression is a common finding — being most pronounced in the L1 and L2 viral late regions — data are inconsistent (reviewed in REFS 50,51). Besides technical differences and differences in the CpG sites analysed, the nature of the samples may account for the discrepant findings. It is currently unclear whether viral methylation is of any biological importance to malignant transformation in terms of providing the infected cell with a growth advantage. It has been suggested that viral DNA methylation represents a generic phenomenon of *de novo* methylation of foreign DNA, serving as a host defence mechanism to silence viral replication and transcription[52,53]. DNA methylation of the viral upstream regulatory region (URR) has been associated with latent infection, which is proposed to facilitate and preserve a long-latency infection[54]. Methylation of the four E2 binding sites (E2BSs; each containing one or two CpG dinucleotides) in the viral URR reduces E2 binding[55], thereby contributing to deregulated E6 and E7 expression, which is the driving force of a transforming HPV infection. A gradual increase in E2BS methylation is thought to result in a further increase in E6 and E7 expression during disease progression. In line with this concept, methylation of the E2BS has been reported to increase with disease progression, with methylation at E2BS2 in the HPV-16 enhancer region being the most consistent finding across the various methylation studies[50,51].

Aberrant methylation patterns have been described for a diverse number of (candidate) tumour suppressor genes in CIN lesions and cervical cancers (reviewed in REFS 56,57). The methylation patterns are, in part, histotype-dependent, with cell adhesion molecule 1 (*CADM1*), cadherin 1 (*CDH1*), death-associated protein kinase 1 (*DAPK1*), *EPB41L3*, *FAM19A4*, myelin and lymphocyte (*MAL*), paired box 1 (*PAX1*),

Box 1 | microRNAs and their differential expression in transforming CIN

MicroRNAs (miRNAs) are non-coding regulatory RNAs of 18–25 nucleotides in length that can bind to the 3′ untranslated regions (3′ UTRs) of target mRNAs, thereby inhibiting protein translation, inducing mRNA degradation, or both. As such, altered expression of miRNAs may affect tumour suppressor or oncogene protein expression. To date, more than 2,500 human mature miRNAs have been annotated in the miRNA database (miRBase 20, release date June 2013). miRNA expression profiles are highly tissue- and/or differentiation-specific and are often altered in cancers, which may, at least in part, result from DNA copy-number alterations, as well as epigenetic alterations[74,76,122].

For a summary of differentially expressed miRNAs in transforming cervical intraepithelial neoplasia (CIN) lesions compared to normal cervical biopsies, and of which altered expression persists or increases in cervical carcinomas, see Supplementary information S1 (table). At present, there is relatively little overlap in the altered miRNAs detected in the various studies, and further research using independent platforms is warranted to extract the most powerful miRNA signature predicting cervical cancer risk. Nonetheless, preliminary data indicate that miRNA expression analysis of a subset of differentially expressed miRNAs in cervical scrapings enables the detection of underlying transforming CIN (S. Wilting, personal communication).

PR domain containing 14 (*PRDM14*) and telomerase reverse transcriptase (*TERT*) belonging to the most frequently methylated genes in both SCC and adenocarcinoma. Of these genes in transforming CIN lesions, the weighted mean methylation frequencies were highest for *CADM1*, followed by *CDH1*, *DAPK1* and *TERT*[56]. A number of recent genome-wide methylation profiling studies have identified a substantial number of additional genes that are methylated in CIN lesions and cervical cancers, and these findings warrant further validation studies[58–63]. For a small subset of genes, including *CADM1,* dickkopf WNT signalling pathway inhibitor 3 (*DKK3*), *MAL,* secreted frizzled-related protein 2 (*SFRP2*) and *C13ORF18* (also known as *KIAA0226L*), tumour suppressive activity in cervical cancer cells has been shown[64–69]. The biological relevance of most other methylation events described in cervical lesions remains elusive.

miRNAs. Several genome-wide studies on miRNA expression in cervical carcinomas have resulted in the identification of a relatively low number of miRNAs that are consistently altered across studies. These include miR-126, miR-143 and miR-145 downregulation and miR-15b, miR-16, miR-146a and miR-155 upregulation (reviewed in REFS 39,70,71). Further independent validation studies are required for a larger number of miRNAs that might have altered expression. Another future challenge includes the identification of the target genes that are affected by the altered miRNAs and the determination of their functional relevance in HPV-induced transformation. Only a small proportion of miRNAs (miR-9, miR-203, miR-375, miR-143, miR-145, miR-146a and miR-199a) have been shown to have a mechanistic role in cervical cancer cells or HPV-immortalized cells[43,72–75]. Four studies that included transforming CIN lesions in their analysis showed that altered expression of several miRNAs represents a rather early event in HPV-induced carcinogenesis that is detectable in CIN lesions[76–79] (BOX 1 and see Supplementary information S1 (table)). Most miRNA alterations, however, are not directly induced following an HPV-infection and are secondary alterations[76] that might in part be a consequence of a copy-number gain at chromosome 5p encoding the miRNA processor Drosha[80,81]. Downregulation of miRNAs could be accomplished by methylation of the CpG-rich regulatory sequences. Indeed, downregulation of *mir-124-1*, *mir-124-2*, *mir-124-3*, *mir-149*, *mir-203*, *mir-375*, *mir-641* and *mir-1287* in cervical cancers has been linked to increased promoter methylation of respective genes[73,74,82,83].

Molecular profile reflects CIN duration

As indicated above, CIN3 lesions and a subset of CIN2 lesions constitute transforming CIN lesions. Although CIN3 is morphologically regarded as the immediate, most advanced cervical cancer precursor, it in fact represents a rather heterogeneous disease[84–86]. This heterogeneity probably reflects the variable duration that the lesion has existed in the patient relative to the long timeline of 20–30 years that is necessary for the progression to invasive carcinoma in most patients[8]. In addition, natural history studies have revealed that, if not treated, only a subset of CIN3 lesions would progress to invasive cancer[7,87]. Therefore, the short-term risk of progression of transforming CIN to cancer is highly variable. Cross-sectional studies have revealed variable frequencies of genetic and epigenetic alterations in CIN lesions and cervical scrapings thereof (see Supplementary information S2 (table) and reviewed in REFS 42,56,57). As some of the observed molecular aberrations overlap with those found in cervical cancers, it seems obvious that these molecular changes represent more advanced transforming CIN lesions with a longer duration of existence. This is supported by recent findings showing that a longer duration of preceding HPV infection — considered a surrogate for the duration of existence of a transforming CIN lesion — is associated with an increase in the number of chromosome aberrations[88]. Transforming CIN lesions found in women with long-term preceding hrHPV infections (≥5 years) had a significantly higher average percentage of chromosome aberrations (that is,16.5% of microarray-CGH probes deviated from normal state) than women with a preceding HPV infection of less than 5 years (2.8% deviating microarrayCGH probes). By comparison, CIN3 lesions adjacent to cervical SCC — considered to represent the most advanced transforming CIN lesions — had, on average, 28.8% deviating microarray-CGH probes. The genomic profiles of most CIN3 with a long-term preceding hrHPV infection were similar to those of invasive carcinomas and tumour-adjacent CIN3 lesions. More recently, it was also found that methylation levels of two host cell genes, *CADM1* and *MAL,* in cervical scrapings were increased in CIN3 lesions of women with long-term preceding hrHPV infections and reached the highest values in women with cervical cancer[89]. These data are fully in line with the concept that an increase in specific genetic and epigenetic alterations reflects a longer duration of existence of the underlying lesion.

Biomarkers for cervical cancer screening

Owing to its high sensitivity for detecting CIN2, CIN3 and cervical cancer (referred to here as CIN2+ lesions), testing for hrHPV DNA is likely to become the predominant method for cervical screening in the western world in the near future[90,91]. The main drawback of this screening tool is a 2–4% lower specificity for CIN2+ than cytology, as the hrHPV test also detects transient HPV infections, which results in overdiagnosis and overtreatment. To compensate for this limitation, different triage algorithms have been suggested in order to keep the follow-up procedures, and associated costs, within acceptable limits. Cytology, with and without HPV-16 and HPV-18 genotyping, is a currently widely-accepted triage tool for HPV-positive women[92–94]. Alternative algorithms to triage HPV-positive women for colposcopy are based on morphological or molecular biomarkers. For biomarker validation in cervical screening, a five-phase framework has been proposed[95], based on recommendations

Self-samples
Self (at home)-collected cervicovaginal specimens, collected using a lavage or brush-based sampler. The self-collected cells can be used for cervical cancer screening by human papilloma virus (HPV) detection and triage by methylation marker analysis.

Methylation marker panel
A panel of genes, most often involving gene promoter sequences, in which methylation of CpG sites represents a biomarker for a specific condition, such as a cancerous or precancerous lesion of the cervix.

made by Pepe *et al.*[96,97] on biomarker development for the early detection of cancer. The designated phases are: preclinical exploratory studies (phase 1); clinical assay development for clinical disease and assessment in non-invasive samples (phase 2); retrospective longitudinal repository studies (phase 3); prospective screening studies (phase 4); and prospective intervention studies (phase 5). Phase 5 preferentially concerns a population-based randomized controlled trial in which a new biomarker test is applied and evaluated against the reference.

At present, most biomarkers are in phase 1 or 2, and only a few have reached later phases (discussed below).

Morphological biomarkers for triage of HPV-positive women. Cross-sectional and longitudinal studies have shown that p16 or dual p16 and Ki-67 immunostaining on cytological preparations gives a promising triage strategy for HPV-positive women[98,99]. Other candidates explored by immunostaining include the overexpression of topoisomerase 2A (TOP2A) and MCM2, which reflects aberrant S phase induction and correlates with severity of cervical disease (reviewed in REFS 100,101).

These immunohistochemical candidate triage tests, however, are microscopy-dependent and require the use of a well-fixed specimen with preserved morphology and a skilled (cyto)pathologist. Self-sampling of cervicovaginal material has recently proved to be a promising new sampling technique to test for hrHPV. However, these specimens have shown decreased numbers of cervical cells, often with poor morphology, in a background of excess vaginal cells, and this has resulted in a low sensitivity of cytology for transforming CIN. A systematic review and a meta-analysis showed that hrHPV testing on self-samples can be similarly accurate as on physician-taken cervical scrapings when a validated combination of sampling device and HPV test is used[102,103], whereas cytology has been shown to be inferior on self-samples[104]. Accordingly, triage of women with an hrHPV-positive self-sample by cytology-based tests would require an extra visit to the physician for making a cervical smear for cytological examination. Therefore, molecular, non-morphology-based triage tools, which are also directly applicable to self-samples, are of great interest for future cervical screening programmes.

Molecular biomarkers for triaging HPV-positive women. To date, those molecular biomarkers that are based on DNA methylation have gained the most attention, because altered DNA methylation in cervical cancer has been well established and DNA methylation can be easily detected in both histological and cytological cervical specimens. Other cellular gene alterations, such as DNA mutations and DNA copy-number aberrations, are currently less attractive as molecular triage markers. DNA mutations in transforming CIN are not sufficiently well defined to be used as a triage marker. Moreover, studies on cancers indicate that mutations in proto-oncogenes or tumour suppressor genes are insufficiently prevalent to enable the identification of all cancers[47,48]. Although better defined, the detection of DNA copy-number aberrations in cervical scrapings is expected to suffer from relatively limited sensitivity for advanced disease by current assays, owing to a dilution of cells from the lesion, and it therefore awaits further technical developments and clinical evaluation. Conversely, several sensitive methods are available to analyse DNA methylation in cervical scrapings and cervicovaginal self-samples (BOX 2).

For current data on methylated host-cell gene promoters investigated in cervical scrapings, see Supplementary information S2 (table). Studies on HPV DNA methylation have recently been reviewed elsewhere[50,51], and combinations based on viral and host cell gene promoter methylation are currently being explored[105].

So far, only a limited number of the host cell methylation markers have been extensively tested for their use as triage markers of HPV-positive women. These studies indicate that a methylation marker panel is needed to reach high sensitivities for transforming CIN. These panels include various combinations of the markers SRY-box 1 (*SOX1*), *PAX1*, LIM homeobox transcription factor 1α (*LMX1A*) and NK6 homeobox 1 (*NKX6-1*)[106], the four-marker panel junctional adhesion molecule 3 (*JAM3*), *EPB41L3, TERT, C13ORF18* (REF. 107), and the bi-marker panel *CADM1* and *MAL*[108]. With respect to the five-phase framework of biomarker validation[95], most markers or marker panels tested on cervical scrapings have so far only reached early phases. One biomarker panel (that is, *CADM1* and *MAL*) has been validated in a population-based screening setting,

Box 2 | **Methods for DNA methylation detection applicable to cervical scrapings and self-samples**

Sensitive methods to detect aberrant DNA methylation in cervical scrapings or self-samples include (quantitative) methylation-specific PCR ((q)MSP), MethyLight, methylation-specific high-resolution melting (MS-HRM) analysis and pyrosequencing. Each of these techniques is based on sodium bisulphite treatment of DNA, which results in the conversion of unmethylated cytosines into uracils, while leaving methylated cytosines unaffected. qMSP, MethyLight and MS-HRM have similar analytical sensitivities and can detect as little as 0.1–1.0% of methylated DNA in a background of unmethylated DNA[123–125]. The sensitivity for bisulphite pyrosequencing is approximately 5% (REF. 126). Although the sensitivity of bisulphite sequencing analysis can be increased when converted to a massive parallel sequencing-by-synthesis approach[127], its high-throughput application on large sample series awaits further developments.

A major advantage of the quantitative real-time PCR technologies is the option to analyse multiple methylation targets and an internal control in a multiplex reaction using a single aliquot of sample material, thereby saving material, time and costs, and improving quality control, as recently developed for cell adhesion molecule 1 (*CADM1*), myelin and lymphocyte (*MAL*) and *mir-124-2*, and the reference gene β-actin[124].

thereby reaching phases 3 and 4 of the biomarker validation framework. On HPV-positive cervical scrapings, this panel was, depending on the threshold setting, equally discriminatory for CIN3+ as cytology at similar specificity[109].

For HPV-positive self-samples, methylation analysis of various marker combinations, such as the four-marker panel *JAM3, EPB41L3, TERT* and *C13ORF18* and the *MAL–mir-124-2* panel, seemed to be a feasible triage tool[107,110]. The use of methylation analyses would obviate the need for HPV-positive women to make an extra visit to a physician for a subsequent cervical sample for morphology-based triage testing. The *MAL–mir-124-2* panel recently passed the later phases of biomarker validation, first by a test-definition on self-samples collected in a prospective screening study[110], and subsequently by a prospective, randomized clinical trial with intervention among non-attendees of the regular cervical screening programme[111]. In the prospective, randomized clinical trial, DNA methylation analysis using the *MAL–mir-124-2* panel on HPV-positive self-samples (intervention arm) was compared with an additional physician-collected cervical scraping (control arm) for CIN2+ detection. The results indicate that direct DNA methylation-based molecular triage was at least as sensitive as cytology triage for the detection of CIN2+[111]. Unlike cytology, methylation analysis on self-samples scored all women with cervical carcinoma positive. The results also showed a better compliance and shorter diagnostic track, but this was at the cost of a higher colposcopy referral rate.

The question can be raised whether methylation markers can be used in clinical practice, as these markers do not detect all CIN3 lesions and tend to detect less CIN2 lesions than cytology at the same specificity[109]. However, these markers can still be considered to be eligible for triage when they at least detect all invasive cancers and advanced transforming CIN with a high short-term progression risk for cancer and when test-negative women have a sufficiently low risk of cervical cancer that they can be dismissed from direct colposcopy referral.

In this context, the following observations are important. Increasing methylation levels of genes such as *CADM1* and *MAL* have been shown to parallel the increasing severity and duration of CIN disease. High methylation levels of *CADM1* and *MAL* were detected in cervical scrapings of women with advanced transforming CIN lesions, and methylation levels in scrapings of women with cervical cancer were exceptionally high[89]. Consistent with these findings, several studies showed that all (100%) cervical scrapings of women with underlying cervical cancer were positive for DNA methylation using *PAX1* ($n = 14$), tissue inhibitor of metalloproteinases 3 (*TIMP3*; $n = 11$) or a tri-marker panel consisting of *CADM1*, *MAL* and *mir-124-2* ($n = 79$) (L. De Strooper and M. van Zummeren, personal communication)[112,113]. From these findings, it can be concluded that methylation analysis has a high detection sensitivity for cancer and advanced lesions with a high short-term progression risk for cancer, thereby missing less advanced lesions with a low short-term probability of progression to cancer. Cytology, however, detects with a moderate sensitivity all morphological cellular abnormalities associated with most CIN2 lesions, CIN3 lesions and cancer (FIG. 3), but it misses a proportion of advanced transforming CIN lesions and cancers[114,115].

This concept implies that HPV-positive women with a positive methylation test should be sent for colposcopy because of the presence of cancer or advanced transforming CIN lesions with a high short-term progression risk for cancer. It follows that methylation-negative women are not in need of immediate colposcopy because of a very low short-term progression risk for cancer. Instead, these women could be offered a repeat test after 12–18 months. For pregnant women, this approach seems to be particularly important, as only the treatment of methylation-positive lesions is indicated, thereby limiting the risk of preterm delivery that might result from the treatment[116–118].

The above-mentioned methylation studies also point to the possibility of using methylation analysis as a primary screening tool in cervical screening. When these findings — in particular, the high sensitivity for cancer — can be confirmed by others, primary methylation testing may provide a screen and treatment approach in developing countries. This is particularly attractive because, in such countries, quality-controlled cytology is absent and the implementation of follow-up algorithms for HPV-positive women is very complicated.

Molecular markers in management of CIN lesions

In most European countries, women who are treated for CIN2 and CIN3 are monitored by cervical cytology at 6, 12 and 24 months after treatment. After three consecutive negative test results, women return to the screening programme (interval 3–5 years) or are recalled within 5 years. The risk of recurrent CIN2+ disease recently proved to be similar when combined cytology and hrHPV testing at 6 and 24 months only was used[119,120].

An interesting perspective is the surveillance of women who are treated for CIN2+ disease using a combination of hrHPV testing and methylation marker analysis. Residual advanced CIN2/CIN3 lesions that result from incomplete excision of the original CIN lesion are expected to have higher methylation levels compared with *de novo* or incident recurrent CIN2+ lesions because of their longer duration of existence. This would imply that methylation marker testing could be helpful in differentiating between cervical cancer and advanced CIN2/CIN3 lesions that result from residual disease, and *de novo* or incident CIN2/CIN3 disease, although this is awaiting clinical confirmation. The clinical value of post-treatment monitoring by combined HPV and methylation marker testing is currently being evaluated (M. Uijterwaal, personal communication). If successful, it is anticipated that, in the future, women who are treated for CIN2/CIN3 could self-collect a cervicovaginal specimen for post-treatment surveillance by combined HPV and methylation marker testing.

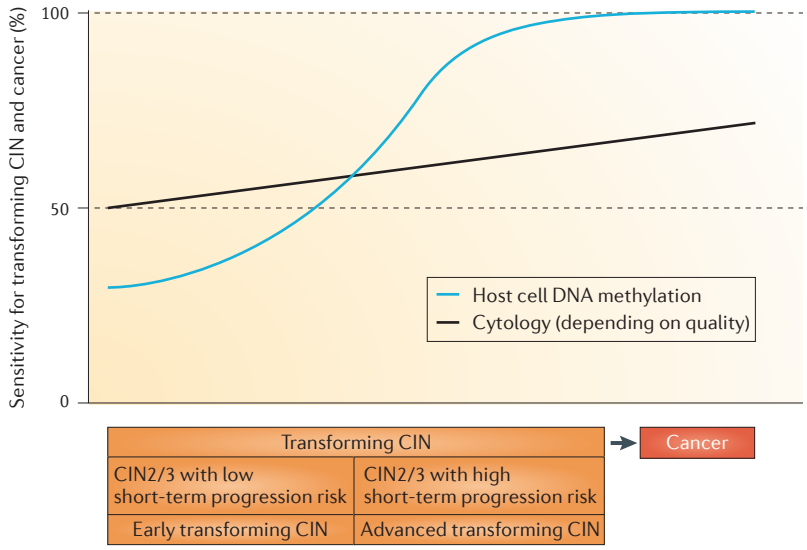

Figure 3 | **Triage tools in cervical scrapings of human papilloma virus (HPV)-positive women.** A schematic representation of the sensitivity (y-axis) of different triage methods for women with HPV-test-positive cervical scrapings along the timeline of transforming cervical intraepithelial neoplasia (CIN) towards invasive cervical cancer (x-axis).

Post-vaccination and therapeutic options

Prophylactic vaccination against HPV-16 and HPV-18 has been introduced in many countries. In post-vaccination screening cohorts, the probability of a high-grade lesion after a positive screening result, either by cytology or an HPV test, should be lower. In this context, the use of a methylation marker assay might help to identify women with progressive CIN lesions with a high short-term cancer risk in need of treatment and help to prevent overtreatment.

Current advances in genome-wide analyses that show the molecular alterations driving cervical carcinogenesis will also provide the opportunity for targeted drug development, such as small molecules that target altered cancer-associated gene products, and personalized treatment regimens. The reversible nature of the epigenetic alterations in transforming CIN and cervical cancers offers alternative options for pharmaceutical intervention. Demethylating agents, such as 5-azacytidine and 5-aza-2′-deoxycytidine (also known as decitabine), have been approved by the US Food and Drug Administration (FDA) for the treatment of haematological malignancies and are in Phase I clinical trials for the treatment of solid tumours. Their application is limited by a high toxicity and poor chemical stability. DNMT inhibitors, such as zebularine and small non-nucleoside analogues, are being developed but await clinical testing[121].

Future perspectives

The distinction between productive CIN1/CIN2 lesions and transforming CIN2/CIN3 lesions has consequences for the clinical management of women with these lesions. At present, a productive CIN2 lesion and a transforming CIN2 lesion cannot be distinguished morphologically, and this results in overtreatment of these lesions. Determining the molecular alterations that are associated with the transition from viral infection to cervical cancer can be used for a molecular classification of cervical lesions, over and above the current morphological (histological) classification — that is, CIN1, CIN2 and CIN3. Using molecular means, the histological changes currently reported as CIN1 or CIN2 lesions that coincide with viral production (productive CIN), which have a very low cancer progression rate, can be distinguished from CIN2 or CIN3 representing viral transformation (transforming CIN). Transforming CIN can, in turn, be subdivided by the level of genetic and epigenetic alterations, such as DNA copy-number aberrations and DNA methylation, into early and advanced transforming CIN. Women with early transforming CIN, which is characterized by low levels of molecular aberrations, have a low short-term progression risk for cancer and could be managed by close surveillance. Women with advanced transforming CIN, which is characterized by increased levels of molecular aberrations, have a high short-term progression risk for cancer and are in need of immediate treatment. Accordingly, the detection of increased DNA methylation gives an indication for treatment of CIN2/CIN3 lesions. This molecular distinction allows for the better management of women who are diagnosed with CIN lesions, and it may be particularly beneficial to women of reproductive age, as treatment of CIN lesions coincides with some degree of morbidity of the cervix and can give rise to preterm delivery[116–118].

HPV testing will probably become the primary screening tool for cervical cancer. Owing to the slightly lower specificity compared with cytology-based screening, triage of hrHPV-positive women is required to keep follow-up procedures and associated costs within acceptable limits. The increase in DNA methylation of tumour suppressor genes associated with the development of advanced transforming CIN and cervical cancer provides valuable objective molecular triage markers. Such markers will probably replace current triage algorithms based on cytology, with or without HPV-16 or HPV-18 genotyping. DNA methylation can be easily detected in cervical scrapings and self-samples, and methylation analysis has a high sensitivity for cervical cancer and advanced transforming CIN lesions in both sample types. The compatibility of methylation markers with HPV testing and self-sampling allows for full molecular cervical screening in the near future. In addition, the methylation markers could be used as molecular tools to monitor women for CIN2+ lesions post-treatment.

In conclusion, recent insight into genetic and epigenetic alterations associated with cervical cancer development has offered opportunities for the molecular distinction of cervical cancer precursor lesions, thereby paving the way for new biomarkers that are useful for screening, diagnosis and management of cervical cancer precursor lesions.

1. Arbyn, M. *et al.* Worldwide burden of cervical cancer in 2008. *Ann. Oncol.* **22**, 2675–2686 (2011).
2. Ferlay, J. *et al.* Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int. J. Cancer* **127**, 2893–2917 (2010).
3. Walboomers, J. M. *et al.* Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *J. Pathol.* **189**, 12–19 (1999).
4. Baseman, J. G. & Koutsky, L. A. The epidemiology of human papillomavirus infections. *J. Clin. Virol.* **32** (Suppl. 1), S16–S24 (2005).
5. IARC. A review of human carcinogens — PartB: biological agents. **100B**, (2011).
6. Snijders, P. J., Steenbergen, R. D., Heideman, D. A. & Meijer, C. J. HPV-mediated cervical carcinogenesis: concepts and clinical implications. *J. Pathol.* **208**, 152–164 (2006).
7. McCredie, M. R. *et al.* Natural history of cervical neoplasia and risk of invasive cancer in women with cervical intraepithelial neoplasia 3: a retrospective cohort study. *Lancet Oncol.* **9**, 425–434 (2008).
8. Vink, M. A. *et al.* Clinical progression of high-grade cervical intraepithelial neoplasia: estimating the time to preclinical cervical cancer from doubly censored national registry data. *Am. J. Epidemiol.* **178**, 1161–1169 (2013).
References 7 and 8 describe the natural history of transforming CIN and cervical cancer and provide an estimate of the duration of progression to cervical cancer.
9. Peto, J., Gilham, C., Fletcher, O. & Matthews, F. E. The cervical cancer epidemic that screening has prevented in the UK. *Lancet* **364**, 249–256 (2004).
10. Doorbar, J. Molecular biology of human papillomavirus infection and cervical cancer. *Clin. Sci.* **110**, 525–541 (2006).
11. Stoler, M. H. *et al.* Human papillomavirus type 16 and 18 gene expression in cervical neoplasias. *Hum. Pathol.* **23**, 117–128 (1992).
12. Crum, C. P., Nagai, N., Levine, R. U. & Silverstein, S. In situ hybridization analysis of HPV 16 DNA sequences in early cervical neoplasia. *Am. J. Pathol.* **123**, 174–182 (1986).
13. Chow, L. T., Broker,T. R., & Steinberg, B. M. The natural history of human papillomavirus infections of the mucosal epithelia. *APMIS* **118**, 422–449 (2010).
14. Stern, P. L. *et al.* Therapy of human papillomavirus-related disease. *Vaccine* **30** (Suppl. 5), F71–F82 (2012).
15. zur Hausen, H. Papillomaviruses causing cancer: evasion from host-cell control in early events in carcinogenesis. *J. Natl Cancer Inst.* **92**, 690–698 (2000).
16. Schwarz, E. *et al.* Structure and transcription of human papillomavirus sequences in cervical-carcinoma cells. *Nature* **314**, 111–114 (1985).
17. Duensing, S. & Munger, K. The human papillomavirus type 16 E6 and E7 oncoproteins independently induce numerical and structural chromosome instability. *Cancer Res.* **62**, 7075–7082 (2002).
18. Bhattacharjee, B. & Sengupta, S. CpG methylation of HPV 16LCR at E2 binding site proximal to P97 is associated with cervical cancer in presence of intact E2. *Virology* **354**, 280–285 (2006).
19. Herfs, M. *et al.* A discrete population of squamocolumnar junction cells implicated in the pathogenesis of cervical cancer. *Proc. Natl Acad. Sci. USA* **109**, 10516–10521 (2012).
This paper reports on a new type of cell, referred to as SCJ cells, that may represent the cells of origin of cervical cancer.
20. Mcnairn, A. J. & Guasch, G. Epithelial transition zones: merging microenvironments, niches, and cellular transformation. *Eur. J. Dermatol.* **21** (Suppl. 2), 21–28 (2011).
21. Herfs, M. *et al.* Cervical squamocolumnar junction-specific markers define distinct, clinically relevant subsets of low-grade squamous intraepithelial lesions. *Am. J. Surg. Pathol.* **37**, 1311–1318 (2013).
22. de Martel, C. *et al.* Global burden of cancers attributable to infections in 2008: a review and synthetic analysis. *Lancet Oncol.* **13**, 607–615 (2012).
23. Korzeniewski, N., Spardy, N., Duensing, A. & Duensing, S. Genomic instability and cancer: lessons learned from human papillomaviruses. *Cancer Lett.* **305**, 113–122 (2011).
24. Moody, C. A. & Laimins, L. A. Human papillomavirus oncoproteins: pathways to transformation. *Nature Rev. Cancer* **10**, 550–560 (2010).
25. Klingelhutz, A. J. & Roman, A. Cellular transformation by human papillomaviruses: lessons learned by comparing high- and low-risk viruses. *Virology* **424**, 77–98 (2012).
26. McLaughlin-Drubin, M. E. & Munger, K. Biochemical and functional interactions of human papillomavirus proteins with polycomb group proteins. *Viruses.* **5**, 1231–1249 (2013).
27. Au Yeung, C. L. *et al.* HPV-16 E6 upregulation of DNMT1 through repression of tumor suppressor p53. *Oncol. Rep.* **24**, 1599–1604 (2010).
28. Burgers, W. A. *et al.* Viral oncoproteins target the DNA methyltransferases. *Oncogene* **26**, 1650–1655 (2007).
29. Wilting, S. M. *et al.* Increased gene copy numbers at chromosome 20q are frequent in both squamous cell carcinomas and adenocarcinomas of the cervix. *J. Pathol.* **209**, 220–230 (2006).
30. Sawada, M. *et al.* Increased expression of DNA methyltransferase 1 (DNMT1) protein in uterine cervix squamous cell carcinoma and its precursor lesion. *Cancer Lett.* **251**, 211–219 (2007).
31. Leonard, S. M. *et al.* Oncogenic human papillomavirus imposes an instructive pattern of DNA methylation changes which parallel the natural history of cervical HPV infection in young women. *Carcinogenesis* **33**, 1286–1293 (2012).
32. Hyland, P. L. *et al.* Evidence for alteration of EZH2, BMI1, and KDM6A and epigenetic reprogramming in human papillomavirus type 16 E6/E7-expressing keratinocytes. *J. Virol.* **85**, 10999–11006 (2011).
33. Laughlin-Drubin, M. E., Crum, C. P. & Munger, K. Human papillomavirus E7 oncoprotein induces KDM6A and KDM6B histone demethylase expression and causes epigenetic reprogramming. *Proc. Natl Acad. Sci. USA* **108**, 2130–2135 (2011).
This paper reports on the mechanisms underlying p16 overexpression in transforming CIN and cervical cancer.
34. Wentzensen, N. & von Knebel, D. M. Biomarkers in cervical cancer screening. *Dis. Markers* **23**, 315–330 (2007).
35. Martinez, I. *et al.* Human papillomavirus type 16 reduces the expression of microRNA-218 in cervical carcinoma cells. *Oncogene* **27**, 2575–2582 (2008).
36. Wang, X. *et al.* Oncogenic HPV infection interrupts the expression of tumor-suppressive miR-34a through viral oncoprotein E6. *RNA* **15**, 637–647 (2009).
37. Au Yeung, C. L., Tsang, T. Y., Yau, P. L. & Kwok, T. T. Human papillomavirus type 16 E6 induces cervical cancer cell migration through the p53/microRNA-23b/urokinase-type plasminogen activator pathway. *Oncogene* **30**, 2401–2410 (2011).
38. Melar-New, M. & Laimins, L. A. Human papillomaviruses modulate expression of microRNA 203 upon epithelial differentiation to control levels of p63 proteins. *J. Virol.* **84**, 5212–5221 (2010).
39. Zheng, Z. M. & Wang, X. Regulation of cellular miRNA expression by human papillomaviruses. *Biochim. Biophys. Acta* **1809**, 668–677 (2011).
40. Qian, K. *et al.* Identification and validation of human papillomavirus encoded microRNAs. *PLoS ONE.* **8**, e70202 (2013).
41. Agarwal, S. M., Raghav, D., Singh, H. & Raghava, G. P. CCDB: a curated database of genes involved in cervix cancer. *Nucleic Acids Res.* **39**, D975–D979 (2011).
42. Thomas, L. K. *et al.* Chromosomal gains and losses in human papillomavirus-associated neoplasia of the lower genital tract - A systematic review and meta-analysis. *Eur. J. Cancer* **50**, 85–98 (2014).
43. Bierkens, M. *et al.* Focal aberrations indicate EYA2 and hsa-miR-375 as oncogene and tumor suppressor in cervical carcinogenesis. *Genes Chromosomes. Cancer* **52**, 56–68 (2013).
44. Wang, F. *et al.* miR-375 is down-regulated in squamous cervical cancer and inhibits cell migration and invasion via targeting transcription factor SP1. *Am. J. Pathol.* **179**, 2580–2588 (2011).
45. Akagi, K. *et al.* Genome-wide analysis of HPV integration in human cancers reveals recurrent, focal genomic instability. *Genome Res.* **24**, 185–199 (2014).
46. Forbes, S. A. *et al.* COSMIC: mining complete cancer genomes in the Catalogue of Somatic Mutations in Cancer. *Nucleic Acids Res.* **39**, D945–D950 (2011).
47. Wright, A. A. *et al.* Oncogenic mutations in cervical cancer: genomic differences between adenocarcinomas and squamous cell carcinomas of the cervix. *Cancer* **119**, 3776–3783 (2013).
48. Ojesina, A. I. *et al.* Landscape of genomic alterations in cervical carcinomas. *Nature* **506**, 371– 375 (2013).
This is the first landscape paper on cervical carcinomas describing genome-wide analysis of DNA mutations.
49. Shen, H. & Laird, P. W. Interplay between the cancer genome and epigenome. *Cell* **153**, 38–55 (2013).
50. Clarke, M. A. *et al.* Human papillomavirus DNA methylation as a potential biomarker for cervical cancer. *Cancer Epidemiol. Biomarkers Prev.* **21**, 2125–2137 (2012).
51. Johannsen, E. & Lambert, P. F. Epigenetics of human papillomaviruses. *Virology* **445**, 205–212 (2013).
References 50 and 51 provide a detailed overview of the current literature regarding HPV DNA methylation and its biological and clinical implications.
52. Doerfler, W. *et al.* The fate of foreign DNA in mammalian cells and organisms. *Dev. Biol.* **106**, 89–97 (2001).
53. Fernandez, A. F. *et al.* The dynamic DNA methylomes of double-stranded DNA viruses associated with human cancer. *Genome Res.* **19**, 438–451 (2009).
54. Vinokurova, S. & von Knebel, D. M. Differential methylation of the HPV 16 upstream regulatory region during epithelial differentiation and neoplastic transformation. *PLoS ONE.* **6**, e24451 (2011).
55. Thain, A., Jenkins, O., Clarke, A. R. & Gaston, K. CpG methylation directly inhibits binding of the human papillomavirus type 16 E2 protein to specific DNA sequences. *J. Virol.* **70**, 7233–7235 (1996).
56. Wentzensen, N., Sherman, M. E., Schiffman, M. & Wang, S. S. Utility of methylation markers in cervical cancer early detection: appraisal of the state-of-the-science. *Gynecol. Oncol.* **112**, 293–299 (2009).
57. Szalmas, A. & Konya, J. Epigenetic alterations in cervical carcinogenesis. *Semin. Cancer Biol.* **19**, 144–152 (2009).
58. Chen, Y. C. *et al.* Methylomics analysis identifies epigenetically silenced genes and implies an activation of β-catenin signaling in cervical cancer. *Int. J. Cancer* **135**, 117–127 (2013).
59. Lendvai, A. *et al.* Genome-wide methylation profiling identifies hypermethylated biomarkers in high-grade cervical intraepithelial neoplasia. *Epigenetics* **7**, 1268–1278 (2012).
60. Farkas, S. A., Milutin-Gasperov, N., Grce, M. & Nilsson, T. K. Genome-wide DNA methylation assay reveals novel candidate biomarker genes in cervical cancer. *Epigenetics.* **8**, 1213–1225 (2013).
61. Brebi, P. *et al.* Genome-wide methylation profiling reveals Zinc finger protein 516 and FK-506-binding protein 6 promoters frequently methylated in cervical neoplasia, associated with HPV status and ethnicity in a Chilean population. *Epigenetics.* **9**, 308–317 (2013).
62. Steenbergen, R. D. *et al.* Methylation-specific digital karyotyping of HPV16E6E7-expressing human keratinocytes identifies novel methylation events in cervical carcinogenesis. *J. Pathol.* **231**, 53–62 (2013).
63. Huang, R. L. *et al.* Methylomic analysis identifies frequent DNA methylation of zinc finger protein 582 (ZNF582) in cervical neoplasms. *PLoS ONE* **7**, e41060 (2012).
64. Chung, M. T. *et al.* SFRP1 and SFRP2 suppress the transformation and invasion abilities of cervical cancer cells through Wnt signal pathway. *Gynecol. Oncol.* **112**, 646–653 (2009).
65. Steenbergen, R. D. *et al.* TSLC1 gene silencing in cervical cancer cell lines and cervical neoplasia. *J. Natl Cancer Inst.* **96**, 294–305 (2004).
66. Overmeer, R. M. *et al.* Association between dense CADM1 promoter methylation and reduced protein expression in high-grade CIN and cervical SCC. *J. Pathol.* **215**, 388–397 (2008).
67. Overmeer, R. M. *et al.* Repression of MAL tumour suppressor activity by promoter methylation during cervical carcinogenesis. *J. Pathol.* **219**, 327–336 (2009).
68. Huisman, C. *et al.* Functional validation of putative tumor suppressor gene C13ORF18 in cervical cancer by artificial transcription factors. *Mol. Oncol.* **7**, 669–679 (2013).

69. Lee, E. J. *et al.* Dkk3, downregulated in cervical cancer, functions as a negative regulator of β-catenin. *Int. J. Cancer* **124**, 287–297 (2009).
70. Kaczkowski, B., Morevati, M., Rossing, M., Cilius, F. & Norrild, B. A decade of global mRNA and miRNA profiling of HPV-positive cell lines and clinical specimens. *Open. Virol. J.* **6**, 216–231 (2012).
71. Saavedra, K. P., Brebi, P. M. & Roa, J. C. Epigenetic alterations in preneoplastic and neoplastic lesions of the cervix. *Clin. Epigenet.* **4**, 13 (2012).
72. Lee, J. W. *et al.* Altered MicroRNA expression in cervical carcinomas. *Clin. Cancer Res.* **14**, 2535–2542 (2008).
73. Wilting, S. M. *et al.* Methylation-mediated silencing and tumour suppressive function of hsa-miR-124 in cervical cancer. *Mol. Cancer* **9**, 167 (2010).
74. Wilting, S. M. *et al.* Methylation-mediated transcriptional repression of microRNAs during cervical carcinogenesis. *Epigenetics.* **8**, 220–228 (2013).
75. Wang, X. *et al.* Aberrant expression of oncogenic and tumor-suppressive microRNAs in cervical cancer is required for cancer cell growth. *PLoS ONE.* **3**, e2557 (2008).
76. Wilting, S. M. *et al.* Altered microRNA expression associated with chromosomal changes contributes to cervical carcinogenesis. *Oncogene* **32**, 106–116 (2013).
77. Li, Y. *et al.* Progressive miRNA expression profiles in cervical carcinogenesis and identification of HPV-related target genes for miR-29. *J. Pathol.* **224**, 484–495 (2011).
78. Cheung, T. H. *et al.* Dysregulated microRNAs in the pathogenesis and progression of cervical neoplasm. *Cell Cycle* **11**, 2876–2884 (2012).
79. Pereira, P. M., Marques, J. P., Soares, A. R., Carreto, L. & Santos, M. A. MicroRNA expression variability in human cervical tissues. *PLoS ONE* **5**, e11780 (2010).
80. Scotto, L. *et al.* Identification of copy number gain and overexpressed genes on chromosome arm 20q by an integrative genomic approach in cervical cancer: potential role in progression. *Genes Chromosomes Cancer* **47**, 755–765 (2008).
81. Muralidhar, B. *et al.* Functional evidence that Drosha overexpression in cervical squamous cell carcinoma affects cell phenotype and microRNA profiles. *J. Pathol.* **224**, 496–507 (2011).
82. Yao, T. *et al.* Exploration of tumor-suppressive microRNAs silenced by DNA hypermethylation in cervical cancer. *Virol. J.* **10**, 175 (2013).
83. Botezatu, A. *et al.* Quantitative analysis of the relationship between microRNA124a, -34b and -203 gene methylation and cervical oncogenesis. *Mol. Med. Rep.* **4**, 121–128 (2011).
84. Yang, H. P. *et al.* Clinical and pathological heterogeneity of cervical intraepithelial neoplasia grade 3. *PLoS ONE* **7**, e29051 (2012).
85. Wentzensen, N. *et al.* Heterogeneity of high-grade cervical intraepithelial neoplasia related to HPV16: implications for natural history and management. *Int. J. Cancer* **132**, 148–154 (2013).
References 84 and 85 provide insight into the clinical, colposcopical and pathological heterogeneity of transforming CIN.
86. Castle, P. E., Gravitt, P. E., Wentzensen, N. & Schiffman, M. A descriptive analysis of prevalent versus incident cervical intraepithelial neoplasia grade 3 following minor cytologic abnormalities. *Am. J. Clin. Pathol.* **138**, 241–246 (2012).
87. Ostor, A. G. Natural history of cervical intraepithelial neoplasia: a critical review. *Int. J. Gynecol. Pathol.* **12**, 186–192 (1993).
88. Bierkens, M. *et al.* Chromosomal profiles of high-grade cervical intraepithelial neoplasia relate to duration of preceding high-risk human papillomavirus infection. *Int. J. Cancer* **131**, E579–E585 (2011).
89. Bierkens, M. *et al.* CADM1 and MAL promoter methylation levels in hrHPV-positive cervical scrapes increase proportional to degree and duration of underlying cervical disease. *Int. J. Cancer* **133**, 1293–1299 (2013).
References 88 and 89 are the first studies indicating that the number of chromosome aberrations and levels of DNA methylation are associated not only with severity of CIN disease but also with the duration of transforming CIN.
90. Arbyn, M. *et al.* Evidence regarding human papillomavirus testing in secondary prevention of cervical cancer. *Vaccine* **30** (Suppl. 5), F88–F99 (2012).
91. Ronco, G. *et al.* Efficacy of HPV-based screening for prevention of invasive cervical cancer: follow-up of four European randomised controlled trials. *Lancet* **383**, 524–532 (2014).
This is a pooled analysis showing that the HPV test gives a 70% greater protection against cervical cancer compared with the cytology test.
92. Katki, H. A. *et al.* Benchmarking CIN 3+ risk as the basis for incorporating HPV and Pap cotesting into cervical screening and management guidelines. *J. Low Genit. Tract. Dis.* **17**, S28–S35 (2013).
93. Dijkstra, M. G. *et al.* Primary hrHPV DNA testing in cervical cancer screening: how to manage screen-positive women? A POBASCAM trial substudy. *Cancer Epidemiol. Biomarkers Prev.* **23**, 55–63 (2013).
94. Rijkaart, D. C. *et al.* HPV DNA testing in population-based cervical screening (VUSA-Screen study): results and implications. *Br. J. Cancer* **106**, 975–981 (2012).
95. Arbyn, M., Ronco, G., Cuzick, J., Wentzensen, N. & Castle, P. E. How to evaluate emerging technologies in cervical cancer screening? *Int. J. Cancer* **125**, 2489–2496 (2009).
96. Pepe, M. S. *et al.* Phases of biomarker development for early detection of cancer. *J. Natl Cancer Inst.* **93**, 1054–1061 (2001).
97. Pepe, M. S., Feng, Z., Janes, H., Bossuyt, P. M. & Potter, J. D. Pivotal evaluation of the accuracy of a biomarker used for classification or prediction: standards for study design. *J. Natl Cancer Inst.* **100**, 1432–1438 (2008).
98. Carozzi, F. *et al.* Risk of high-grade cervical intraepithelial neoplasia during follow-up in HPV-positive women according to baseline p16-INK4A results: a prospective analysis of a nested substudy of the NTCC randomised controlled trial. *Lancet Oncol.* **14**, 168–176 (2013).
99. Petry, K. U. *et al.* Triaging Pap cytology negative, HPV positive cervical cancer screening results with p16/Ki-67 Dual-stained cytology. *Gynecol. Oncol.* **121**, 505–509 (2011).
100. Conesa-Zamora, P. Role of cell cycle biomarkers in human papillomavirus related uterine lesions. *Curr. Pharm. Des.* **19**, 1412–1424 (2013).
101. Reuschenbach, M. & von Knebel, D. M. Diagnostic tests for the detection of human papillomavirus-associated cervical lesions. *Curr. Pharm. Des.* **19**, 1358–1370 (2013).
102. Snijders, P. J. *et al.* High-risk HPV testing on self-sampled versus clinician-collected specimens: a review on the clinical accuracy and impact on population attendance in cervical cancer screening. *Int. J. Cancer* **132**, 2223–2236 (2013).
103. Arbyn, M. *et al.* Accuracy of human papillomavirus testing on self-collected versus clinician-collected samples: a meta-analysis. *Lancet Oncol.* **15**, 172–183 (2014).
104. Brink, A. A. *et al.* High concordance of results of testing for human papillomavirus in cervicovaginal samples collected by two methods, with comparison of a novel self-sampling device to a conventional endocervical brush. *J. Clin. Microbiol.* **44**, 2518–2523 (2006).
105. Kalantari, M. *et al.* Methylation of human papillomavirus 16, 18, 31, and 45 L2 and L1 genes and the cellular DAPK gene: Considerations for use as biomarkers of the progression of cervical neoplasia. *Virology* **448**, 314–321 (2014).
106. Lai, H. C. *et al.* Quantitative DNA methylation analysis detects cervical intraepithelial neoplasms type 3 and worse. *Cancer* **116**, 4266–4274 (2010).
107. Eijsink, J. J. *et al.* Detection of cervical neoplasia by DNA methylation analysis in cervico-vaginal lavages, a feasibility study. *Gynecol. Oncol.* **120**, 280–283 (2011).
108. Overmeer, R. M. *et al.* Combined CADM1 and MAL promoter methylation analysis to detect (pre-) malignant cervical lesions in high-risk HPV-positive women. *Int. J. Cancer* **129**, 2218–2225 (2011).
109. Hesselink, A. T. *et al.* Combined promoter methylation analysis of CADM1 and MAL: An objective triage tool for high-risk human papillomavirus DNA-positive women. *Clin. Cancer Res.* **17**, 2459–2465 (2011).
110. Hesselink, A. T. *et al.* Methylation marker analysis of self-sampled cervico-vaginal lavage specimens to triage high-risk HPV-positive women for colposcopy. *Int. J. Cancer* http://dx.doi.org/10.1002/ijc.28723 (2014).
111. Verhoef, V. M. *et al.* A randomised controlled trial on triage of HPV positive women on self-collected cervico-vaginal specimens: direct methylation marker testing versus indirect cytology. *Lancet Oncol.* **15**, 315–322 (2014).
This is the first randomized controlled clinical trial on implementation of methylation markers in cervical screening using self-samples.
112. Widschwendter, A. *et al.* Analysis of aberrant DNA methylation and human papillomavirus DNA in cervicovaginal specimens to detect invasive cervical cancer and its precursors. *Clin. Cancer Res.* **10**, 3396–3400 (2004).
113. Huang, T. H. *et al.* Quantitative analysis of methylation status of the PAX1 gene for detection of cervical cancer. *Int. J. Gynecol. Cancer* **20**, 513–519 (2010).
114. Cuzick, J. *et al.* Overview of the European and North American studies on HPV testing in primary cervical cancer screening. *Int. J. Cancer* **119**, 1095–1101 (2006).
115. Kitchener, H. C., Castle, P. E. & Cox, J. T. Chapter 7: Achievements and limitations of cervical cytology screening. *Vaccine* **24** (Suppl. 3), 63–70 (2006).
116. Bruinsma, F. J. & Quinn, M. A. The risk of preterm birth following treatment for precancerous changes in the cervix: a systematic review and meta-analysis. *BJOG.* **118**, 1031–1041 (2011).
117. Arbyn, M. *et al.* Perinatal mortality and other severe adverse pregnancy outcomes associated with treatment of cervical intraepithelial neoplasia: meta-analysis. *BMJ* **337**, a1284 (2008).
118. Kyrgiou, M. *et al.* Obstetric outcomes after conservative treatment for intraepithelial or early invasive cervical lesions: systematic review and meta-analysis. *Lancet* **367**, 489–498 (2006).
119. Kocken, M. *et al.* Risk of recurrent high-grade cervical intraepithelial neoplasia after successful treatment: a long-term multi-cohort study. *Lancet Oncol.* **12**, 441–450 (2011).
120. Kocken, M. *et al.* High-risk human papillomavirus testing versus cytology in predicting post-treatment disease in women treated for high-grade cervical disease: a systematic review and meta-analysis. *Gynecol. Oncol.* **125**, 500–507 (2012).
121. Gnyszka, A., Jastrzebski, Z. & Flis, S. DNA methyltransferase inhibitors and their emerging role in epigenetic therapy of cancer. *Anticancer Res.* **33**, 2989–2996 (2013).
122. Lopez-Serra, P. & Esteller, M. DNA methylation-associated silencing of tumor-suppressor microRNAs in cancer. *Oncogene* **31**, 1609–1622 (2012).
123. Wojdacz, T. K. & Dobrovic, A. Methylation-sensitive high resolution melting (MS-HRM): a new approach for sensitive and high-throughput assessment of methylation. *Nucleic Acids Res.* **35**, e41 (2007).
124. Snellenberg, S. *et al.* Development of a multiplex methylation-specific PCR as candidate triage test for women with an HPV-positive cervical scrape. *BMC. Cancer* **12**, 551 (2012).
125. Eads, C. A. *et al.* MethyLight: a high-throughput assay to measure DNA methylation. *Nucleic Acids Res.* **28**, E32 (2000).
126. Mikeska, T., Felsberg, J., Hewitt, C. A. & Dobrovic, A. Analysing DNA methylation using bisulphite pyrosequencing. *Methods Mol. Biol.* **791**, 33–53 (2011).
127. Taylor, K. H. *et al.* Ultradeep bisulfite sequencing analysis of DNA methylation patterns in multiple gene promoters by 454 sequencing. *Cancer Res.* **67**, 8511–8518 (2007).

Acknowledgements
The research in the laboratory of R.D.M.S., P.J.F.S., D.A.M.H. and C.J.L.M.M. is supported by grants from the Dutch Cancer Society (KWF2007-3771, KWF2009-4413, KWF2009-4522 and KWF2010-4668), Zorgonderzoek Nederland-Medische Wetenschappen (ZON-MW), the European community Program Health-FP7 Program (PreHdIct FP7-Health 2009-24206- and CoheaHr-Health-F3-2013-603019) and the European Research Council (ERC advanced 2012-AdG, proposal 322986 Mass-care).

Competing interests statement
The authors declare competing interests: see Web version for details.

DATABASES
COSMIC catalogue of somatic mutations: http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/
miRBase 20: www.mirbase.org

SUPPLEMENTARY INFORMATION
See online article: S1 (table) | S2 (table)

ALL LINKS ARE ACTIVE IN THE ONLINE PDF