Methylation-mediated silencing and tumour suppressive function of hsa-miR-124 in cervical cancer

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Abstract

Background: A substantial number of microRNAs (miRNAs) is subject to epigenetic silencing in cancer. Although epigenetic silencing of tumour suppressor genes is an important feature of cervical cancer, little is known about epigenetic silencing of miRNAs. Since DNA methylation-based silencing of hsa-miR-124 occurs in various human cancers, we studied the frequency and functional effects of hsa-miR-124 methylation in cervical carcinogenesis.

Results: Quantitative MSP analysis of all 3 loci encoding the mature hsa-miR-124 (hsa-miR-124-1/-2/-3) showed methylation in cervical cancer cell lines SiHa, CaSki and HeLa as well as in late passages of human papillomavirus (HPV) type 16 or 18 immortalised keratinocytes. Treatment of SiHa cells with a demethylating agent reduced hsa-miR-124 methylation levels and induced hsa-miR-124 expression. In HPV-immortalised keratinocytes increased methylation levels were related to reduced hsa-miR-124 expression and higher mRNA expression of IGFBP7, a potential hsa-miR-124 target gene. Ectopic hsa-miR-124 expression in SiHa and CaSki cells decreased proliferation rates and migratory capacity. Combined hsa-miR-124-1 and/or hsa-miR-124-2 methylation analysis of 139 cervical tissue specimens showed an increasing methylation frequency from 0% in normal tissues up to 93% in cervical carcinomas. Increased methylation levels of hsa-miR-124-1 and hsa-miR-124-2 were significantly correlated with reduced hsa-miR-124 expression in cervical tissue specimens. Combined hsa-miR-124-1 and/or hsa-miR-124-2 methylation analysis of 43 cervical scrapes of high-risk HPV positive women was predictive of underlying high-grade lesions.

Conclusions: DNA methylation-based silencing of hsa-miR-124 is functionally involved in cervical carcinogenesis and may provide a valuable marker for improved detection of cervical cancer and its high-grade precursor lesions.

Background

Cervical cancer is the second most common cancer in women worldwide and is caused by a persistent infection with high-risk types of the human papillomavirus (hrHPV) [1-3]. The development of cervical squamous cell carcinomas (SCCs, representing about 80% of cases) occurs via well-recognisable premalignant precursor lesions (cervical intraepithelial neoplasia (CIN), graded 1-3), whereas less is known about the different precursor stages preceding cervical adenocarcinomas (AdCAs, accounting for 10-20% of cases).

Even taking the promising results of recently introduced prophylactic HPV vaccines into account, cervical screening will remain necessary in the foreseeable future [4-7]. Recent studies have shown that the sensitivity of hrHPV testing is superior to that of cytology as a screening tool [8-10]. However, hrHPV testing also results in the identification of a considerable number of hrHPV-positive women without (pre)cancerous lesions, necessitating the development of proper triage tests for hrHPV-positive women. Assays detecting (epi)genetic changes that besides hrHPV are crucial for malignant progression will likely contribute to the discrimination of hrHPV-positive women with cervical (pre)cancer.

DNA methylation-mediated silencing of an increasing number of protein-coding tumour suppressor genes is...
known to be involved in cervical cancer. Therefore, the present study aimed to investigate whether epigenetic changes relevant in hrHPV-mediated cervical carcinogenesis may affect the expression microRNAs (miRNAs) as well [11-16]. miRNAs, ~23 nucleotide long, non-coding RNAs, regulate expression of protein-coding genes at the posttranscriptional level by sequence specific base pairing in the 3’ untranslated region (UTR) of the target mRNAs. Recent proteomic studies have shown that a single miRNA can regulate expression of hundreds of targets [17,18]. The potential importance of miRNAs in cervical carcinogenesis in general, is underlined by a number of studies. miRNA loci are significantly associated with fragile sites, which are known insertion sites of HPV in cervical cancers. In addition, even though no HPV-encoded miRNAs have been identified so far, HPV-encoded genes were shown to influence the miRNA expression of its host cell [19,20]. Altered miRNA expression was found in cervical cancer cell lines and/or cervical carcinomas compared to normal controls and a number of these differentially expressed miRNAs were shown to influence proliferation rates of cervical cancer cell lines SiHa or HeLa [20-24].

Interestingly, similar to protein-coding tumour suppressor genes, expression of a substantial number of miRNAs was shown to be under epigenetic regulation [25-31]. A well-known epigenetically silenced miRNA in human carcinogenesis is hsa-miR-124. DNA methylation of hsa-miR-124 was first shown by Lujambio et al in colon, breast and lung cancer, as well as in leukaemia and lymphoma [29]. Subsequent studies confirmed frequent hsa-miR-124 methylation in leukaemia affecting clinical outcome and additionally showed frequent hsa-miR-124 methylation in gastric cancer and hepatocellular carcinoma [25,26,30,32]. At present, no studies have been performed to investigate the role of epigenetic silencing of miRNAs in cervical cancer.

In this study we evaluated the potential role of DNA methylation-based silencing of hsa-miR-124 during cervical carcinogenesis. The mature hsa-miR-124 sequence is processed from 3 separate premature sequences, located at chromosomes 8p23.1 (miR-124-1), 8q12.3 (miR-124-2) and 20q13.33 (miR-124-3), all of which contain CpG islands in their promoter regions. We investigated the methylation status of all 3 genomic loci encoding the mature hsa-miR-124 in cervical cancer cell lines, a longitudinal in vitro model system of hrHPV-induced carcinogenesis [33], cervical tissue specimens (n = 139), and hrHPV-positive cervical scrapes (n = 43) of women with and without a CIN3 diagnosis in follow-up. In addition, effects of (ectopic) hsa-miR-124 expression on cellular proliferation, migration and mRNA expression of IGFBP7, a potential target gene, were studied.

Materials and methods
Cell lines and cell culture
Establishment and culture of the HPV16 (FK16A/FK16B) and HPV18 (FK18A/FK18B) immortalised cell lines have been described previously [33]. Primary human keratinocytes, referred to as EK cells, were isolated from foreskin and cultured as described previously [33]. The human cervical carcinoma cell lines SiHa, CaSki and HeLa were obtained from the American Type Culture Collection (Manassas, VA, USA). SiHa cells were treated with 5000 nM 5-aza-2’-deoxycytidine (DAC, Sigma Chemical Co., St. Louis, MO, USA) dissolved in PBS to analyse the effect of global methylation inhibition on hsa-miR-124 expression.

Clinical tissue specimens
We used frozen specimens of normal cervix (n = 5), CIN2/3 (n = 7), SCC (n = 9) and AdCA (n = 5) as well as formalin-fixed, paraffin-embedded (FFPE) biopsy specimens of normal cervix (n = 18), CIN1 (n = 36), CIN3 (n = 41), SCC (n = 29) and AdCA (n = 15). All specimens were collected during the course of routine clinical practice and stored at the Department of Pathology at the VU University Medical Center (Amsterdam, the Netherlands). Normal specimens were obtained from non-cancer patients undergoing hysterectomy. The mean age of all women included in this study was 40.3 years (range 18-79). Per histological subgroup the women had the following mean ages: 49.1 years (range 34-70) in the normal group; 35.9 (range 22-52) years in the CIN1 group; 35.9 years (range 27-74) in the CIN3 group; 53.0 years (range 35-61) in the SCC group; 45.6 years (range 28-79) in the AdCA group. The mean age in any of the groups of women with cervical (pre)malignant disease was not significantly higher than that of the women with normal histology.

Cervical scrapings were obtained from the population-based cervical screening trial POBASCAM, registered as an International Standard Randomized Controlled Trial under number ISRCTN20781131 [8,34]. For this study, we selected 22 cervical scrapes from hrHPV-positive women who had normal cytology without evidence of CIN disease up to the next screening round (i.e. 5 years) and 21 scrapings classified as severe dyskaryosis or worse from hrHPV-positive women who had a CIN3 diagnosis within 18 months of follow-up. The mean age of the women with normal cytology without CIN disease was 33.2 years (range 18-53), and that of women with abnormal cytology with CIN3 was 35.14 years (range 25-55). This study followed the ethical guidelines of the Institutional Review Board of the VU University Medical Center.
**Extraction of nucleic acids and HPV testing**

For methylation analysis, DNA was extracted from FFPE specimens by proteinase K digestion and purified using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Almere, The Netherlands) following the manufacturer’s recommendations. Genomic DNA from cell lines and frozen specimens was extracted by proteinase K digestion followed by standard phenol-chloroform extraction as described previously [35].

For hsa-miR-124 expression analysis, frozen specimens of normal cervix, CIN2/3 lesions, SCCs, and AdCAs were first enriched for epithelial cells by means of laser capture microdissection using a Leica ASLMD microscope (Leica, Heidelberg, Germany) as described before [36]. Subsequently, total RNA was isolated from these samples and cell lines using TRizol reagent (Life Technologies, Breda, The Netherlands), according to the manufacturer’s instructions.

HPV typing of clinical specimens was performed using the general primer GP5+/6+ PCR, followed by reverse line blot, as described previously [37,38] (Additional file 1).

**DNA modification and quantitative methylation-specific PCR (qMSP) analysis**

The DNA methylation status of the CpG-island containing promoter regions associated with the three genomic loci encoding hsa-miR-124 (hsa-miR-124-1, hsa-miR-124-2 and hsa-miR-124-3) was determined by qMSP analysis on sodium bisulfite-treated genomic DNA from cell lines, tissue specimens and scrapings. In brief, genomic DNA was modified using the EZ DNA Methylation kit (Zymo Research, Orange, CA, USA), which induces chemical conversion of unmethylated cytosines into uracils, whereas methylated cytosines are protected from this conversion. Specific primers were designed to amplify the methylated DNA sequence of all 3 promoter regions. Amplicons (hsa-miR-124-1: -191 to -97; hsa-miR-124-2: -301 to -163; hsa-miR-124-3: -106 to -11 relative to the transcription start site, respectively) were detected and quantified using TaqMan probes (Table 1). In addition, the modified, unmethylated sequence of the housekeeping gene β-actin (ACTB) was amplified as a reference [39]. qMSP reactions were carried out in a 12 μl reaction volume containing 50 ng of bisulfite-treated DNA, 417 nM of each primer, 208 nM probe and 1× QuantiTect Probe PCR Kit master mix (Qiagen, Westburg, Leusden, The Netherlands) using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands).

All PCR experiments were performed in duplicate (delta Ct ≤1.5 between replicates) and mean values were used for calculations. Methylation values of the 3 target regions were normalised to the reference gene ACTB using the comparative Ct method (2^-ΔCT) [40]. All methylation negative samples in our study had a Ct for ACTB below 32, indicating sufficient DNA quality and thereby excluding false negative results. To ensure the detection of distinguishable increases in methylation level in (pre)malignant cervical lesions over normal cervical controls, we used the 99% confidence interval of the methylation levels obtained in normal cervical controls as cut-off value. Samples above this threshold were considered positive for methylation. For tissue specimens and scrapings separate cut-off values were determined using the appropriate normal controls.

**Retroviral transduction**

Retroviral hsa-miR-124 or empty vector (ctrl) constructs previously described by Voorhoeve et al [41] were transfected into the Phoenix A retrovirus producer cell line and supernatants containing the replication-deficient hsa-miR-124-expressing retrovirus or empty vector retrovirus were harvested 48 hours post-transfection. For the transduction experiments, SiHa and CaSki cells were incubated for 16 hours at 37°C with filtered viral supernatants supplemented with polybrene (15 μg/ml). SiHa/CaSki_miR-124 cells or SiHa/CaSki_ctrl cells were selected by continuous culturing of the transfected cells in the presence of blasticidin (3 μg/ml).

**Quantitative Reverse Transcription-PCR (qRT-PCR)**

Expression of hsa-miR-124 was measured using TaqMan microRNA assays following the manufacturer’s instructions (00046 and 001182; Applied Biosystems) on the ABI 7500 Fast Real-Time PCR System (Applied Biosystems). The small nucleolar RNA transcript RNU43 was included as internal reference for hsa-miR-124 expression (001095; Applied Biosystems). Hsa-miR-124 expression values were normalised to the reference again by using the comparative Ct method as described above.

Intron-flanking primers for IGFBP7, a potential target gene of hsa-miR-124, were selected using Primer Express 3.0 (Applied Biosystems) (Table 1). Total RNA was reverse transcribed using the specific reverse primer and the resulting cDNA was used for real-time PCR. cDNA corresponding to 25 ng of total RNA was amplified in a total reaction volume of 25 μl containing 12.5 μl 2x Sybr Green master mix (Perkin Elmer/Applied Biosystems) and 0.5 μM primers. The housekeeping gene U1 small nuclear ribonucleoprotein specific A protein (snRNP U1A) was included as internal reference (Table 1). Expression values of IGFBP7 were normalized to this reference by using the comparative Ct method as described above.
Cellular proliferation and migration assays

Cell proliferation was measured using a colorimetric (MTT-tetrazolium) assay (ICN Biomedicals Inc, OH, USA). In this assay the amount of dye conversion, as measured by the optical density at a wavelength of 540 nm, is directly related to the number of viable cells in each well. In brief, 5000 cells (CaSki) or 10000 cells (SiHa) were seeded in triplicate in 96-well plates and assayed for MTT conversion at day 0, day 1, day 2 and day 5 for CaSki and day 0, day 2, day 3 and day 6 for SiHa. The proliferation rate was determined by subtracting the measurement of day 0 from all other time points.

Cellular migration in vitro was determined using a so-called wound-healing assay. Cells were grown to confluency in 24-well plates and a single linear scratch was made in duplicate for all conditions using a sterile tip, resulting in a cell-free zone. Photographs of the scratch were taken immediately post-scratching and following 24 hours of incubation at 37°C. After 48 hours cells were fixed with methanol and stained with crystal violet solution.

Statistical analysis

Proliferation rates between hsa-miR-124-expressing cells and control cells were compared using the Student’s t test. The frequency of methylation between normal and low-grade (CIN1) lesions on one hand and high-grade (CIN3) lesions and SCCs on the other hand were compared using χ²-testing. The difference in hsa-miR-124 expression between normal cervical epithelium and CIN2/3 lesions and carcinomas was compared using the Wilcoxon rank test. Linear (Pearson) correlation was determined between hsa-miR-124 methylation levels and hsa-miR-124 expression.

Results

Methylation of hsa-miR-124 during hrHPV-mediated transformation in vitro

To determine whether hsa-miR-124 may be silenced due to promoter hypermethylation in cervical cancer, we assessed DNA methylation at the 3 promoter regions of hsa-miR-124 (hsa-miR-124-1 located at 8p23.1; hsa-miR-124-2 located at 8q12.3; and hsa-miR-124-3 located at 20q13.3) in cervical cancer cell lines using qMSP analysis. Methylation of all 3 promoter regions of hsa-miR-124 was observed in SiHa cells as well as in another cervical cancer cell line, CaSki. A third cervical cancer cell line, HeLa, showed lower levels of methylation for all 3 regions and especially methylation levels of hsa-miR-124-1 were extremely low compared to SiHa and CaSki. Primary keratinocytes isolated from 3 independent donors, on the other hand, were completely unmethylated at all 3 regions (Figure 1A-C). Treatment of the cervical cancer cell line SiHa with the demethylating agent DAC resulted in > 50% decrease of methylation levels at all 3 regions and increased expression of hsa-miR-124 compared to untreated and mock-treated SiHa cells (PBS) (Figure 1D and 1E).

To investigate at which stage during hrHPV-mediated transformation hsa-miR-124 becomes methylated we subsequently performed qMSP analysis for all 3 regions on early and late passages of HPV16 (FK16A and FK16B)
and HPV18 (FK18A and FK18B) immortalised keratinocyte cell lines. Whereas early passages (range: p23-p43) of all 4 cell lines showed little to no methylation, increased methylation of hsa-miR-124-1 and hsa-miR-124-2 was observed in late passages (range: p70-p96) (Figure 2A and 2B). For hsa-miR-124-1 an increase in methylation levels was observed in late passages of FK16B and FK18A cells. However, the levels of methylation were still very low compared to those observed in SiHa and CaSki cells (Figure 1A). For hsa-miR-124-2, late passages of FK18A and FK18B cells showed increased methylation, of which the methylation level in late passage FK18B cells was comparable to that observed in SiHa cells (Figure 1B). Methylation of the hsa-miR-124-3 region was not detected in any of the HPV-immortalised keratinocytes (data not shown). The increase in hsa-miR-124-1 methylation in late passage FK16B cells, though being quite low compared to SiHa and CaSki cells, was associated with reduced hsa-miR-124 expression compared to its corresponding earlier passage (Figure 2C). Similarly, in late passage FK18B cells, showing methylation levels for hsa-miR-124-2 comparable to SiHa cells, lower hsa-miR-124 expression was found compared to its earlier passage. Together, these results further support a (direct) correlation between

Figure 1 Hsa-miR-124 methylation in primary keratinocytes (EK cells) and cervical cancer cell lines SiHa, CaSki and HeLa. A. hsa-miR-124-1 methylation. B. hsa-miR-124-2 methylation. C. hsa-miR-124-3 methylation. Whereas in primary keratinocytes no methylation was detectable, all cervical cancer cell lines were positive for methylation of hsa-miR-124-1, hsa-miR-124-2 and hsa-miR-124-3. D. In SiHa cells treated with 5000 nM DAC, methylation levels of hsa-miR-124-1 (black), hsa-miR-124-2 (white) and hsa-miR-124-3 (grey) were reduced by more than 50%. Methylation levels of all regions in untreated cells were set to 100%. E. Whereas in untreated and mock (PBS) treated SiHa cells no hsa-miR-124 expression was detectable, SiHa cells treated with 5000 nM DAC showed clear hsa-miR-124 expression.
methylation and expression of hsa-miR-124. Methylation-mediated silencing of hsa-miR-124 appears to occur during HPV-induced carcinogenesis at the post-immortalisation stage and is not directly related to the presence of hrHPV.

Tumour suppressive activities of hsa-miR-124 in cervical cancer cell lines

The fact that methylation of hsa-miR-124 is consistently found in cervical cancer cell lines and appears to be associated with gene silencing, suggests that hsa-miR-124 may possess tumour suppressive traits in cervical cancer. To test this hypothesis, we stably transduced SiHa and CaSki cells with a retroviral hsa-miR-124-containing vector (SiHa_miR-124 and CaSki_miR-124, respectively) and an empty vector (SiHa_ctrl and CaSki_ctrl, respectively). Ectopic expression of hsa-miR-124 was confirmed by qRT-PCR (Figure 3A). To determine the effects of ectopic hsa-miR-124 expression on proliferation we measured cell viability using the MTT assay. The proliferation rate was significantly lower in SiHa_miR-124 compared to SiHa_ctrl cells and parental SiHa cells (Figure 3B; p < 0.01). In CaSki cells effects were less pronounced, but proliferation rates were still significantly lower in CaSki_miR-124 compared to both CaSki_ctrl and CaSki parental cells (Figure 3C; p < 0.05). The somewhat reduced effect observed in CaSki_miR-124 cells compared to SiHa_miR-124 cells is likely correlated to the levels of hsa-miR-124 overexpression (Figure 3A).

To measure effects of hsa-miR-124 expression on the migratory capacity of SiHa cells we performed a wound-healing assay. Duplicate experiments consistently showed decreased migratory capacity of SiHa_miR-124 compared to SiHa_ctrl cells and parental SiHa cells at 24 and 48 hours (Figure 3D).

In conclusion, these results suggest that ectopic expression of hsa-miR-124 in cervical cancer cells has tumour suppressive effects.

**IGFBP7** is a potential target of hsa-miR-124 in a subset of cervical cancers

To identify potential hsa-miR-124 target genes in cervical cancer we used data from a recent study by Baek et al., in which the impact of hsa-miR-124 expression in HeLa cells on mRNA and protein output was determined [17]. Interestingly, they found that for targets undergoing robust (>1.5 fold) repression, the major mechanism of repression was mRNA destabilisation. Therefore we compared their proteomics data on HeLa cells ectopically expressing hsa-miR-124 to our own dataset of genome-wide mRNA expression in cervical SCCs, all of which showed hsa-miR-124 methylation by qMSP analysis (data not shown) [36]. This comparison highlighted 1 gene containing an hsa-miR-124 target site, namely IGFBP7, that showed 3.7 fold protein downregulation in HeLa_miR124 cells and a significant (false discovery rate (FDR) = 0.036) mRNA upregulation in cervical SCCs displaying hsa-miR-124 methylation.

Quantitative RT-PCR was performed for IGFBP7 in early and late passages of FK16B and FK18B cells as well as SiHa and CaSki cells with and without ectopic hsa-miR-124 expression, to determine the effects of hsa-miR-124 on the mRNA level of IGFBP7. Interestingly, late passages of FK16B and FK18B cells, which were shown to
Figure 3 Ectopic expression of hsa-miR-124 in SiHa and CaSki cells. A. Whereas parental cell lines and empty vector control cells (SiHa_ctrl and CaSki_ctrl) showed no detectable expression of hsa-miR-124, cells transduced with hsa-miR-124 (SiHa_miR-124 and CaSki_miR-124) expressed hsa-miR-124. Ectopic hsa-miR-124 expression resulted in decreased proliferation rates of B. SiHa_miR-124 (red) and C. CaSki_miR-124 (red) compared to parental (black) and empty vector control cells (grey). In D, results of wound-healing assays in SiHa_miR-124, SiHa_ctrl and SiHa cells are shown, indicating decreased migratory capacity in cells expressing hsa-miR-124.
have increased levels of hsa-miR-124-1 and hsa-miR-124-2 methylation and decreased levels of hsa-miR-124 expression (Figure 2), showed increased levels of IGFBP7 expression compared to their corresponding early passages (Figure 4A). In CaSki cells ectopic hsa-miR-124 expression resulted in reduction of IGFBP7 expression of more than 50% compared to the empty vector control cells and parental cells, however, no effect was observed in SiHa_miR-124 cells. As a control, SLC25A36, a gene without any hsa-miR-124 target sites, was included in this analysis and showed similar expression levels in cells with and without ectopic hsa-miR-124 expression (Figure 4B).

These results indicate that IGFBP7 may be a target of hsa-miR-124 in part of the cervical carcinomas.

**hsa-miR-124 methylation and silencing is frequent in cervical (pre)malignant lesions**

Since methylation of hsa-miR-124 becomes detectable in late passages of HPV-immortalised keratinocytes mimicking premalignant cervical disease [33], these regions may provide markers for the detection of cervical cancer and its high-grade precursor lesions. We therefore determined the methylation levels of all promoter regions in 18 normal cervical specimens, 36 CIN1 lesions, 41 CIN3 lesions, 29 SCCs and 15 AdCAs (Figure 5A-C). Using a cut off value based on the 99% confidence interval of the mean value measured in the normal specimens, only 1 normal sample (5.6%) scored positive for hsa-miR-124-3 methylation, whereas no normal samples scored positive for hsa-miR-124-1 and hsa-miR-124-2 methylation. For hsa-miR-124-1 and hsa-miR-124-2, respectively, the percentages of methylation positivity increased from 27.8% and 5.6% in CIN1, to 46.3% and 19.5% in CIN3, to 86.2% and 82.8% in SCCs. In addition, high percentages of methylation positivity for hsa-miR-124-1 and hsa-miR-124-2 were found in AdCAs as well (93.3% and 80% respectively). In concordance with the fact that in vitro hsa-miR-124-3 was only methylated in cervical cancer cell lines and not in HPV-immortalised precursor cell lines, the frequency of hsa-miR-124-3 methylation was low in CIN1 (11.1%) and CIN3 (9.8%) lesions, but increased to 72.4% and 73.3% in SCCs and AdCAs, respectively (Table 2).

A combined scoring system for hsa-miR-124-1 and/or hsa-miR-124-2 methylation resulted in 0% positivity in normal cervix, 30.6% in CIN1 lesions, 58.5% in CIN3 lesions and 93.1% in SCCs (Table 2). The difference in methylation frequency between normal samples and low-grade lesions (CIN1) on one hand and high-grade lesions (CIN3) and SCCs on the other hand was highly significant (p < 0.001). Addition of hsa-miR-124-3 methylation resulted in the detection of one extra AdCA as well as one extra normal specimen.

To determine whether hsa-miR-124 methylation also resulted in silencing of hsa-miR-124 expression in cervical lesions, we measured the expression of hsa-miR-124 in a panel of frozen specimens of normal cervical squamous epithelium (n = 5), CIN2/3 (n = 7), cervical SCCs (n = 9) and AdCAs (n = 5). To eliminate the possibility of confounding results due to stromal expression, all samples were microdissected. The average expression of hsa-miR-124 in CIN2/3 lesions and cervical carcinomas compared to normal cervical epithelium was 4.4 fold decreased (p = 0.001). In addition, we determined the correlation between hsa-miR-124 expression and methylation levels for the 3 regions in CIN2/3 lesions and carcinomas (Figure 6). Methylation levels of hsa-miR-124-1 and hsa-miR-124-2 were significantly negatively correlated with hsa-miR-124 expression levels (R = -0.451, p =
Figure 5 Hsa-miR-124 methylation levels in cervical specimens. Dotplots showing the levels of methylation for A. hsa-miR-124-1, B. hsa-miR-124-2 and C. hsa-miR-124-3 in normal cervical specimens, CIN1 lesions, CIN3 lesions, SCCs and AdCAs. Methylation levels in cervical scrapes of women with normal cytology without underlying CIN and women with abnormal cytology with underlying CIN3 lesions are shown in D. for hsa-miR-124-1, E. for hsa-miR-124-2 and F. for hsa-miR-124-3.
### Table 2: Frequencies of *hsa-miR-124* methylation detected by qMSP analysis

|                     | methylation positivity (%) | Combined (and/or) methylation positivity (%) |
|---------------------|----------------------------|---------------------------------------------|
|                     | hsa-miR-124-1 | hsa-miR-124-2 | hsa-miR-124-3 | hsa-miR-124-1/-2/-3 | hsa-miR-124-1/-2 | hsa-miR-124-1/-3 | hsa-miR-124-2/-3 |
| Normal (n = 18)     | 0.0           | 0.0           | 5.6           | 5.6                | 0.0             | 5.6            | 5.6            |
| CIN1 (n = 36)       | 27.8          | 5.6           | 11.1          | 30.6               | 30.6            | 27.8           | 13.9           |
| CIN3 (n = 41)       | 46.3          | 19.5          | 9.8           | 58.5               | 58.5            | 48.8           | 26.8           |
| SCC (n = 29)        | 86.2          | 82.8          | 72.4          | 93.1               | 93.1            | 89.7           | 86.2           |
| AdCA (n = 15)       | 93.3          | 80.0          | 73.3          | 100.0              | 93.3            | 100.0          | 93.3           |
| normal cytology without CIN (n = 22) | 4.5 | 0.0 | 4.5 | 9.1 | 4.5 | 9.1 | 4.5 |
| abnormal cytology with CIN3 (n = 21) | 47.6 | 47.6 | 23.8 | 71.4 | 71.4 | 57.1 | 47.6 |
To be considered as a candidate disease marker that potentially could be of value for the detection of high-grade CIN and carcinoma in cervical screening, methylation of \( hsa\text{-miR-124} \) should be detectable in cervical scrapes containing few abnormal cells in a background of normal cells. As a proof of principle we analysed the methylation levels of all 3 loci in 22 hrHPV-positive cytologically normal cervical scrapes of women without evidence of CIN disease in the subsequent 5 years and 21 hrHPV-positive cytologically abnormal scrapes of women with CIN3, diagnosed within 18 months of follow-up (Figure 5D-F). Using the same analysis method as described above, we found methylation of \( hsa\text{-miR-124}-1 \) and/or \( hsa\text{-miR-124}-2 \) in 4.5% (1/22) of the women without disease versus 71.4% (15/21) of women with CIN3. Methylation analysis of \( hsa\text{-miR-124}-3 \) had no additive value in this sample series (Table 2).

Collectively, these results show that methylation analysis of \( hsa\text{-miR-124}-1 \) and \( hsa\text{-miR-124}-2 \) provides an attractive candidate marker for the triage of hrHPV-positive women.

**Discussion**

In this study we showed that epigenetic silencing of \( hsa\text{-miR-124} \) is functionally involved in cervical carcinogenesis and may provide a valuable marker for risk stratification of hrHPV-positive women. Using qMSP analysis, we found methylation of \( hsa\text{-miR-124}-1 \) and/or \( hsa\text{-miR-124}-2 \) in none of the normal tissues, 58.5% of CIN3 lesions, 93.1% of SCCs and 93.3% of AdCAs. Increased methylation levels of \( hsa\text{-miR-124}-1 \) and \( hsa\text{-miR-124}-2 \) in cervical tissue specimens were significantly correlated with lower \( hsa\text{-miR-124} \) expression levels. Analysis of cervical scrapes showed that only 4.5% of hrHPV-positive scrapes without CIN disease in follow-up was positive compared to 71.4% of hrHPV-positive scrapes with CIN3 in follow-up. To the best of our knowledge this study provides the first evidence of DNA methylation-based silencing of a miRNA in cervical cancer.

Methylation of \( hsa\text{-miR-124} \) was found in cervical cancer cell lines SiHa, CaSki and HeLa as well as in late passages of HPV16/18 immortalised keratinocytes, reminiscent of high grade cervical precursor lesions, but not in normal primary keratinocytes. The fact that methylation of \( hsa\text{-miR-124} \) and concomitant reduced \( hsa\text{-miR-124} \)
miR-124 expression was found in late passages of HPV-immortalised keratinocytes but not in early passages, indicates that this event takes place post-immortalisation and is not directly related to the presence of hrHPV. Ectopic expression of hsa-miR-124 decreased the proliferation rate of both SiHa and CaSki cells and also inhibited the migratory capacity of SiHa cells. Effects on the migratory capacity of CaSki cells were difficult to ascertain due to the specific growth characteristics of this cell line. Consistent with our findings, both Agirre et al and Furuta et al observed inhibitory effects on cellular growth upon reintroduction of hsa-miR-124 expression in acute lymphoblastic leukaemia (ALL)-derived cells and hepatocellular carcinoma cell lines [26,32].

One of the previously identified targets mediating the tumour suppressive function of hsa-miR-124 is CDK6, via CDK6 mediated phosphorylation and subsequent inactivation of the tumour suppressor pRb [29,32]. However, in cervical cancer the virally encoded oncoprotein E7 is thought to bind and inactivate pRb, suggesting hsa-miR-124 may (partly) function via other targets in this type of cancer. Using data from a recent study by Baek et al, we identified IGFBP7 as a promising target gene in cervical cancer [17]. IGFBP7 mRNA levels were increased in late passages of FK16B and FK18B cells compared to their corresponding early passages, which also showed increased levels of hsa-miR-124-1 and hsa-miR-124-2 methylation and decreased levels of hsa-miR-124 expression. In addition, IGFBP7 showed decreased mRNA expression in CaSki cells ectopically expressing hsa-miR-124 compared to empty vector control cells and parental cells. No effect was seen in SiHa_miR-124 cells, however. These results indicate that IGFBP7 may be a potential target of hsa-miR-124 in part of the cervical cancers, but other targets may be relevant for the tumour suppressive function of hsa-miR-124 in cervical cancer as well. IGFBP7 is part of the insulin-like growth factor (IGF) axis, which has been implicated in cervical cancer before. It was shown that the IGF-axis may influence the persistence of hrHPV infections and that abnormally balanced co-expression of IGFBP family members is associated with gynaecological malignancy [42,43]. IGFBP7 is the only member of the IGFBP family that binds insulin instead of IGF and is relatively unknown compared to its family members. On one hand IGFBP7 has recently been described as a tumour suppressor gene in colorectal cancer and was shown to induce senescence in cells harbouring oncogenic BRAF [44-46], whereas on the other hand IGFBP7 was shown to have oncogenic properties in gliomas [47]. Further functional studies are needed to investigate whether the tumour suppressive function of hsa-miR-124 in cervical cancer may in part be mediated via IGFBP7.

Hsa-miR-124 was originally described as a brain-specific miRNA, involved in neuronal differentiation. Lujiangio et al were the first to show methylation-mediated silencing of hsa-miR-124 in different human cancer types, reaching the highest frequency in colorectal cancer (75%) [29]. In acute lymphoblastic leukaemia (ALL) methylation of hsa-miR-124 was shown to negatively affect clinical outcome [30,32]. In ALL hsa-miR-124-1 showed the highest frequency of methylation as was also found for cervical cancer in our study. Interestingly, in gastric cancer and hepatocellular carcinoma, hsa-miR-124-3 showed the highest frequency of methylation [25,26], whereas in ALL and cervical cancer hsa-miR-124-3 showed the lowest frequencies of methylation positivity. This may indicate that hsa-miR-124-3 methylation is tissue or tumour type dependent. The fact that hsa-miR-124-3 methylation was infrequent in cervical precursor lesions and HPV-immortalised keratinocyte cell lines supports the notion that at least in cervical carcinogenesis hsa-miR-124-3 methylation is a rather late event. Overall, the methylation positivity rates for hsa-miR-124 found in our study rank among the highest currently reported, although the use of different assays and methods, including non-quantitative MSP and combined bisulfite restriction analysis, makes a direct comparison difficult. Importantly, hsa-miR-124 methylation in cervical cancer was histotype-independent and could already be detected in CIN3 lesions and scrapes of women with underlying CIN3, underlining its potential value for cervical cancer screening.

Conclusions

This study shows that methylation of hsa-miR-124 is a frequent and functionally relevant event in cervical carcinogenesis. The high positivity rates in CIN3 lesions and carcinomas as well as in scrapes with underlying CIN3 lesions combined with the fact that hsa-miR-124 methylation is not directly related to the presence of hrHPV, indicate that hsa-miR-124 methylation may provide a valuable marker for the triage of hrHPV-positive women. Future studies in large population-based cohorts will determine whether testing for hsa-miR-124 methylation either or not in combination with other promising methylation markers such as CADM1 and MAL [13,14] can improve future cervical screening strategies based on primary hrHPV testing.

Additional material

Additional file 1 HPV typing results of clinical specimens included in this study. In this additional table, for all clinical specimens included in this study, HPV typing results by general primer GP5+/6+ PCR and reverse line blot are given.
Competing interests
The authors declare that they have no competing interests.

Authors' contributions
SMW and RAaV8 performed all experiments and data analysis and drafted the manuscript. RDMS and PIFS participated in the design of the study and the drafting of the manuscript. FEH, RA and CIS greatly contributed to the functional experiments with retroviral constructs. CILM, BD and GAM contributed to the conception of the study and critically revised the manuscript. All authors read and approved the final manuscript.

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