

Chapter 4

Repression of MAL tumor suppressor activity by promoter methylation during cervical carcinogenesis

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Abstract

We recently identified *MAL* (T-lymphocyte maturation associated protein) as the most down-regulated gene in cervical oncogenesis. Here, we examined the mechanism underlying *MAL* silencing, its functional role in cervical carcinogenesis and the relevance of detecting *MAL* alterations for risk assessment of hrHPV-positive women.

MAL mRNA expression and promoter methylation were analysed in primary keratinocytes, hrHPV-immortalized keratinocytes, cervical cancer cell lines, biopsies and scrapings by quantitative (methylation-specific) PCR. SiHa cells were transfected with *MAL* cDNA and assayed for proliferation, migration and anchorage-independent growth.

MAL mRNA was (nearly) undetectable in all HPV-immortalized and cervical cancer cells, but could be up-regulated upon methylation inhibition. *MAL* promoter methylation at two promoter regions (M1 and M2) was detected in all HPV-immortalized cells and cancer cells. Ectopic expression of *MAL* in SiHa cells suppressed proliferation, migration and anchorage-independent growth. None (0/22) of normal cervical biopsies, 9% (6/66) of CIN1 lesions, 53% (34/64) of CIN3 lesions, 90% (85/94) of cervical squamous cell carcinomas (SCCs) and 93% (26/28) of cervical adenocarcinomas (AdCAs) demonstrated *MAL* promoter methylation at both promoter regions. Moreover, detection of *MAL* promoter methylation in cervical scrapings was predictive for underlying high-grade lesions. Both in biopsies and scrapings, *MAL* promoter methylation was significantly correlated with reduced mRNA expression.

MAL gene silencing by promoter methylation is a frequent and biologically essential event in HPV-induced cervical carcinogenesis. Hence, *MAL* promoter methylation and/or mRNA expression analysis on cervical scrapings may provide a valuable diagnostic tool to improve the detection of CIN3, SCC and AdCA.

Introduction

The development of cancer of the uterine cervix is a multistep process, which in virtually all cases results from a persistent infection with high-risk human papillomavirus (hrHPV) (1, 2). Cervical squamous cell carcinomas, representing 80% of cervical cancers, evolve from non-invasive premalignant lesions referred to as cervical intraepithelial neoplasias (CINs), graded from CIN1 to CIN3 (3, 4). The low-grade lesions (CIN1) are related to a productive infection with hrHPV, characterized by viral replication and production in differentiated cell layers. High-grade lesions (CIN2/3) and invasive cancer are associated with a so-called transforming infection in which viral oncogene (ie *E6* and *E7*) expression is evident in the dividing cell layers (5). Due to interference of the viral oncoproteins E6 and E7 with the p53 and pRb tumour suppressor pathways, chromosomal instability is induced in these dividing cells. Succeeding specific genetic and epigenetic alterations in host cell genes may incite malignant transformation of the hrHPV-infected cells (2, 6). Accordingly, knowledge on these (epi)genetic alterations may yield novel molecular biomarkers able to distinguish hrHPV-positive women with high-grade disease from those with clinically irrelevant infections.

To obtain insight in the host cell alterations that play a decisive role in cervical carcinogenesis, we previously generated both chromosomal and transcriptional profiles of cervical carcinomas (7, 8). Amongst the genes showing differential expression between invasive cancers and normal epithelial controls, the *MAL* gene was found to be most significantly down-regulated. Strongly reduced *MAL* mRNA expression was validated by real-time RT-PCR on an independent set of cervical cancers and normal cervical controls (8).

MAL (T-lymphocyte maturation associated protein) is a 17 kD membrane protein which constitutes an essential component of glycolipid-enriched membrane micro-domains or rafts involved in apical transport of membrane and secretory proteins (9, 10). *MAL* is located at chromosome 2q13 (11), a region which is commonly retained in cervical carcinomas (7). Here we endeavoured to unravel the mechanism underlying *MAL* silencing and analysed its potential functional role in cervical carcinogenesis and possible clinical utility.

Materials and Methods

Cell cultures and cell lines

Culturing of primary human foreskin keratinocytes (EK), HPV16-immortalized keratinocyte cell lines FK16A and FK16B, HPV18-immortalized keratinocyte cell lines FK18A and FK18B and cervical carcinoma cell lines SiHa, HeLa and CaSki, including incubations of SiHa and FK18B with 5 μ M 5-aza-2'-deoxycytidine (DAC; Sigma Chemical Co, St. Louis, MO, USA), was performed as described previously (6, 12). Genomic DNA and total RNA were isolated from cell lines with the use of the Puregene DNA isolation kit (Biozym, Landgraaf, The Netherlands) and RNAbee reagent (Tel-Test, Friendswood, TX, USA), respectively.

Clinical tissue specimens

This study followed the ethical guidelines of the Institutional Review Board of the VU University Medical Center. We obtained formalin-fixed, paraffin-embedded (FFPE) specimens of normal cervix ($n = 22$) of women treated for a non-malignant disorder, without a history of abnormal Pap scrapings or any form of cancer. Furthermore, we included FFPE specimens of CIN1 ($n = 66$), CIN3 ($n = 64$), cervical squamous cell carcinomas (SCCs; $n = 94$) and cervical adenocarcinomas (AdCAs; $n = 28$). For RNA expression analysis, frozen biopsies of normal cervix ($n = 12$), CIN3 lesions ($n = 15$) and SCC ($n = 11$) were included. All specimens were collected during the course of routine clinical practice from women who underwent biopsy or surgery and were stored at the tissue bank of the Department of Pathology at the VU University Medical Center (Amsterdam).

Cervical scrapings were collected from women participating in the population-based cervical screening trial POBASCAM2 (13, 14), an extension of the POBASCAM trial (15, 16). We included 17 cytomorphologically normal scrapings of hrHPV-positive women without CIN lesions during a 5-year follow-up and 21 scrapings classified as moderate dyskaryosis or worse of hrHPV-positive women that developed CIN3 within 18 months of follow-up.

Isolation of nucleic acids and HPV typing

Following proteinase K incubation, DNA of clinical specimens was isolated using the High Pure PCR Template Preparation kit (Roche Diagnostics,

Almere, The Netherlands) according to standard procedures (15), or as for 30 SCCs by phenol/chloroform extraction (7).

For mRNA expression analysis, frozen biopsies of normal cervix and CIN3 samples were first enriched for (dysplastic) epithelial cells by means of laser capture microdissection using a Leica ASLMD microscope (Leica, Heidelberg, Germany) (8). Total RNA was isolated from microdissected normal cervix, CIN3, SCC (>70% tumour cells) and cervical scrapings using TRIzol Reagent according to the manufacturer's instructions (8). Clinical specimens were HPV-typed using the general primer GP5+/6+ polymerase chain reaction (PCR) enzyme immunoassay (17), followed by reverse line blot analysis (18).

Quantitative reverse transcription-PCR for MAL expression

Real-time reverse transcription-PCR (RT-PCR) for MAL was performed as described previously (8). For cell lines, cervical tissues and scrapings, we obtained cDNA by specific reverse primers. In each PCR, a standard curve was included, using cDNA obtained from Universal Human Reference RNA (Stratagene, CA, USA). All PCR experiments were performed in duplicate and mean values were used for calculations. To determine the percentage of samples with reduced MAL expression, we calculated the 95% confidence interval of the normal specimens and used this as a cut-off value.

DNA modification and quantitative methylation specific PCR (qMSP) analysis

We analysed MAL promoter methylation by qMSP on sodium bisulphite-treated genomic DNA from cell lines, cervical tissue specimens and scrapings using an ABI 7500 Real-Time PCR system (Applied Biosystems, USA). For bisulphite treatment, we used the EZ DNA Methylation KitTM (Zymo Research, Orange, CA, USA). Quantitative MSPs (qMSPs) targeting two CpG-rich regions within the MAL promoter (ie from -680 to -573 and -92 to -7 relative to the first ATG; referred to as M1 and M2, respectively) were performed as described previously (19), using primer sets described in Table 1. The housekeeping gene β -actin (ACTB) was used as an internal reference (20). A standard curve of bisulphite-treated DNA of SiHa was included in each qMSP. Each sample was analysed in duplicate. Discrepant samples (<10% of cases) were analysed in quadruplicate and scored positive if at least two reactions were above the cut-off. qMSP values were adjusted for DNA input by expressing results as ratios between two absolute measurements (20-22). To determine the percentage of methylation-positive samples, we calculated the

99% confidence interval of 16 normal controls and used this as a cut-off value. For tissue biopsies and cervical scrapings, separate cut-off levels were determined.

Table 1. Primer and probe sequences for quantitative RT-PCR, qMSP and cloning

Assay	Gene	Forward 5'-3'primer	FAM 5'-3'-TAMRA probe	Reverse 5'-3'primer	bp
qRT-PCR	MAL	GCAAGACGGC TTCACCTACAC		GCAGAGTGGCTA TGTAGGAGAACA	74
qMSP	MAL M1	GCGTAGTATTAA GTAGAGAGGTTTCG	ACTAAACCGACG CTAATTCGACGCT		107
	MAL M2	TTAGGTTATTG GGTTTCGCG	TCCGCGCAAAC CTCTCGCTAAC		86
	β actin	TGGTGATGGAGG AGGTTTAGTAAGT	ACCACCACCCAACA CACATAACAAACACA	AACCAATAAAACC TACTCCTCCCTTAA	133
Cloning	MAL	ACGCCAGCAGC CCGTCATGGCC		CGGCCAGTTAA CACCATCTGGGT	526

Cloning and transfection of MAL complementary DNA

Full-length MAL cDNA was PCR-amplified from primary keratinocyte mRNA using the cloning primers listed in Table 1 and Phusion High-Fidelity DNA polymerase (Finnzymes, Espoo, Finland). The PCR product was cloned into pCR-BluntII-TOPO (Invitrogen Life Science) from which an *EcoRI* (Roche Diagnostics, Almere, The Netherlands) fragment containing the MAL cDNA was cloned into pcDNA3.1neo (Invitrogen Life Science). Upon subsequent digestion with *EcoRV* and *HindIII* (Roche Diagnostics), the MAL cDNA was ligated into pcDNA3.1hygro(+) (Invitrogen Life Science). The MAL cDNA sequence was verified by sequence analysis (BaseClear BV, Leiden, The Netherlands).

SiHa was transfected with *BglIII*- (Roche Diagnostics) linearized pcDNA3.1hygro(+)/MAL and pcDNA3.1hygro(+)/(-) (ie empty vector) using lipofectamine 2000 transfection reagent (Invitrogen Life Science). SiHa_MAL cells or SiHa (-) cells were selected by continuous culturing of the transfected SiHa cells in the presence of 300 μ g/mL hygromycin B (Roche Diagnostics) and bulk cultures were used for functional analysis.

Cell proliferation and anchorage independence assays

Cell proliferation was measured using a colorimetric (MTT-tetrazolium) assay (ICN Biomedicals Inc, OH, USA). In brief, 5000 cells were seeded in triplicate in 96-wells plates and assayed for MTT conversion at day 0 and day 5. The proliferation rate was determined by subtracting the OD of day 0 from the measurement at day 5. Anchorage-independent growth was analysed as described previously (6). All cell lines were seeded in duplicate and after three weeks of culture, colonies containing at least 50 cells were counted.

Migration assay

Cells were grown to confluence in 24-well plates and a single uniform scratch was made in all wells using a sterile tip, resulting in a cell-free gap. Photographs were taken immediately after the scratch and after 24 h.

Statistical analysis

Mean quantities of methylation or MAL expression between the different cervical specimens and the association between MAL expression and methylation status were compared using the Mann–Whitney *U*-test (SPSS 12.0, Chicago, IL, USA). A two-sided *p* value of ≤ 0.05 was considered significant.

Results

MAL mRNA expression in HPV-immortalized keratinocytes and cervical carcinoma cell lines

We measured *MAL* mRNA levels in primary keratinocytes, three hrHPV-containing cervical cancer cell lines (SiHa, HeLa and CaSki) and four hrHPV-immortalized keratinocyte cell lines (FK16A, FK16B, FK18A and FK18B) at early and late passages. The latter, representing intermediate states in the transformation process reminiscent of premalignant lesions (6, 23), have acquired an immortal phenotype but are not tumourigenic in nude mice. Relative to primary keratinocytes (set at 100%), all cervical cancer cells and early (passage 29–35) and late (passage 80–106) passages of hrHPV-immortalized cell lines and cervical cancer cell lines showed very low or undetectable *MAL* mRNA expression levels, with highest expression in early passage FK16A cells (12%; Figure 1A).

MAL promoter methylation

Since losses of the *MAL* locus are uncommon in cervical carcinomas, we examined whether *MAL* silencing resulted from DNA methylation. We treated FK18B and SiHa cells with the DNA methylation inhibitor 5-aza-2'-deoxycytidine (DAC) and determined the *MAL* mRNA levels relative to primary keratinocytes. After 5 days of DAC treatment, the *MAL* mRNA levels in FK18B cells increased to 97% and those in SiHa cells to 68% (Figure 1B). These data suggest that *MAL* gene silencing could be due to methylation of the *MAL* promoter or methylation of direct or indirect regulators of *MAL* transcription.

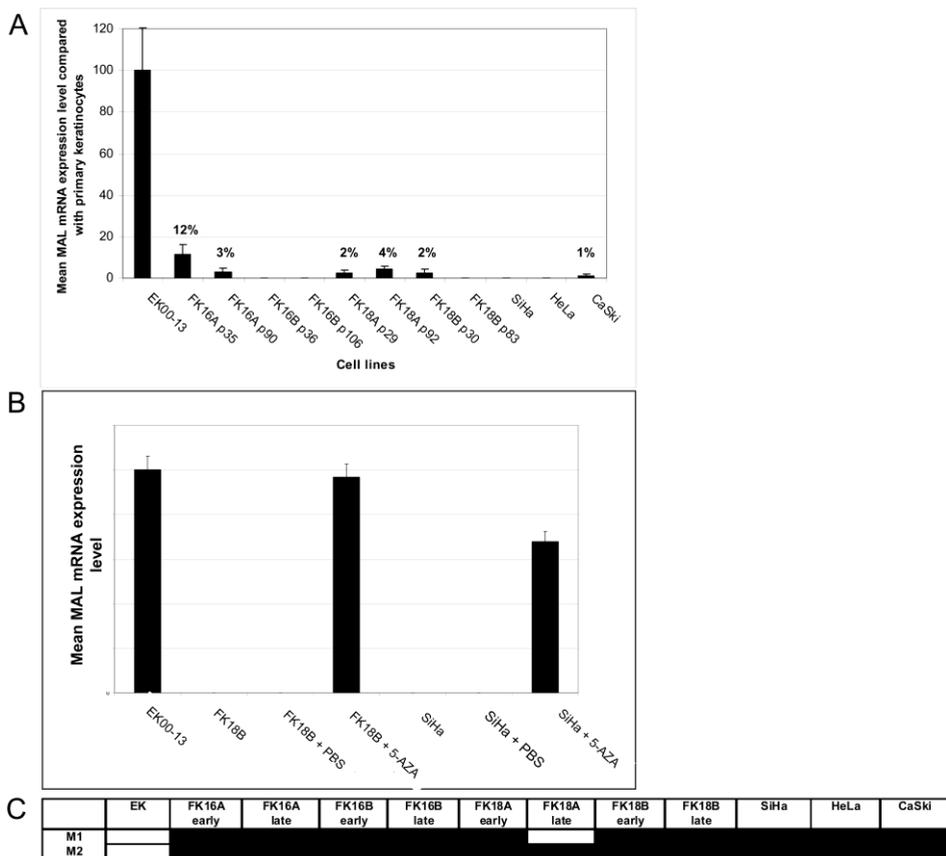


Figure 1. (A) *MAL* mRNA expression in primary keratinocytes (EK), early and late passages of HPV-immortalized keratinocytes (FK16A, FK16B, FK18A and FK18B) and cervical carcinoma cell lines (SiHa, HeLa and CaSki). The mean *MAL* mRNA level of EK was set to 100%. (B) *MAL* mRNA expression in FK18B and SiHa cells with and without DAC treatment. The mean *MAL* mRNA level of untreated EK was set to 100%. (C) *MAL* promoter methylation at the M1 and M2

regions in primary keratinocytes (EK), early and late passages of HPV-immortalized keratinocytes (FK16A, FK16B, FK18A and FK18B) and cervical carcinoma cell lines (SiHa, HeLa and CaSki). DNA methylation is depicted in black; white boxes indicate unmethylated DNA.

Subsequently, we examined the methylation status of two regions within the MAL promoter by qMSP. We observed no methylation in primary human keratinocytes, whereas early and late passages of FK16A, FK16B and FK18B cells, early passage FK18A cells and SiHa, HeLa and CaSki cells were positive for methylated DNA at both the MAL M1 and the MAL M2 promoter region. Late passage FK18A cells showed methylation of MAL M2 only (Figure 1C). qMSP analysis on FK18B and SiHa cells treated with DAC showed that increased MAL mRNA expression was associated with reduced methylation at both promoter regions (data not shown).

Effects of MAL expression on cellular proliferation, migration and anchorage-independent growth

To determine the potential functional role of MAL in cervical carcinogenesis, we stably transfected SiHa cells with a MAL expression vector (SiHa_MAL) or an empty control vector (SiHa (-)). Ectopic MAL expression was confirmed by RT-PCR (Figure 2A). Bulk cultures of both transfectants were examined for their proliferation rate, migration capacity and ability to grow in soft agarose. As shown in Figure 2B, SiHa_MAL transfectants showed a 43% reduction in proliferation rate as compared with SiHa (-) cells, indicating that ectopic expression of MAL has an anti-proliferative effect *in vitro*.

The cellular migration capacity of both SiHa transfectants was assessed by a scratch assay. We found that migration was strongly inhibited in SiHa_MAL transfectants compared with the empty vector control cells (Figure 2C). SiHa_MAL cells also displayed a 53% reduction in anchorage-independent growth compared with SiHa transfectants bearing the empty vector (Figure 2D).

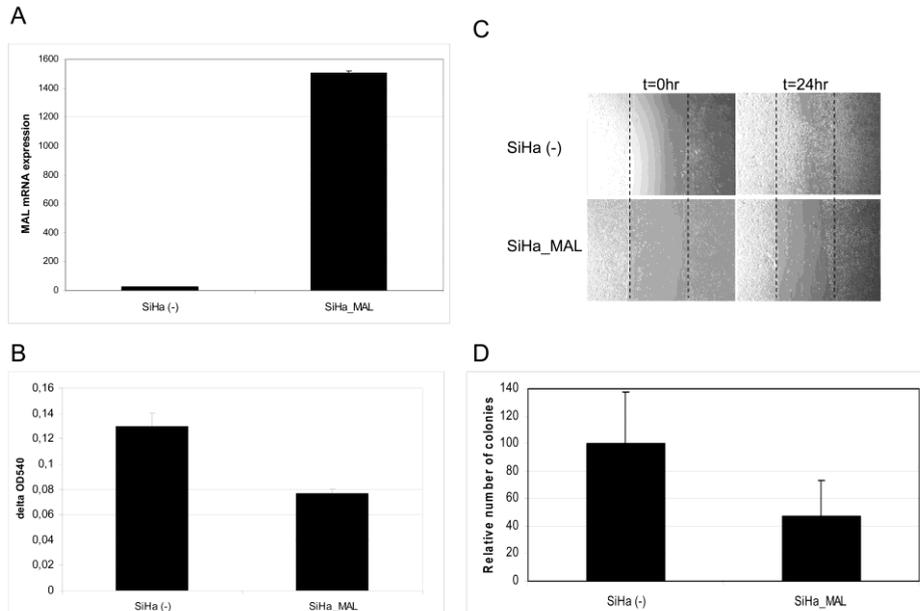


Figure 2. (A) MAL mRNA expression in SiHa transfected with an empty vector (SiHa (-)) and SiHa transfected with a MAL expression vector (SiHa_MAL). (B) Cellular proliferation in SiHa (-) and SiHa_MAL. The Y-axis indicates the ΔOD_{540} following 5 days of culturing as measured using a MTT assay. (C) Representative pictures of cellular migration as determined by a scratch assay. SiHa (-) and SiHa_MAL are shown at the starting point ($t = 0$ h) and after 24 hours of culturing ($t = 24$ h). Lines indicate the width of the cell-free gap introduced upon scratching. (D) Anchorage-independent growth of SiHa (-) and SiHa_MAL. The number of colonies in SiHa (-) was set to 100%.

Methylation status of the MAL promoter during cervical carcinogenesis

To determine whether and when the MAL promoter becomes hypermethylated during cervical carcinogenesis *in vivo*, we analysed the methylation status of the MAL promoter in DNA isolated from cervical tissue specimens. High-risk HPV DNA was detected in 0% (0/22) of normal cervical epithelium specimens, 35% (23/66) of CIN1 lesions, 86% (55/64) of CIN3 lesions, 100% (94/94) of cervical SCCs and 93% (26/28) of AdCAs.

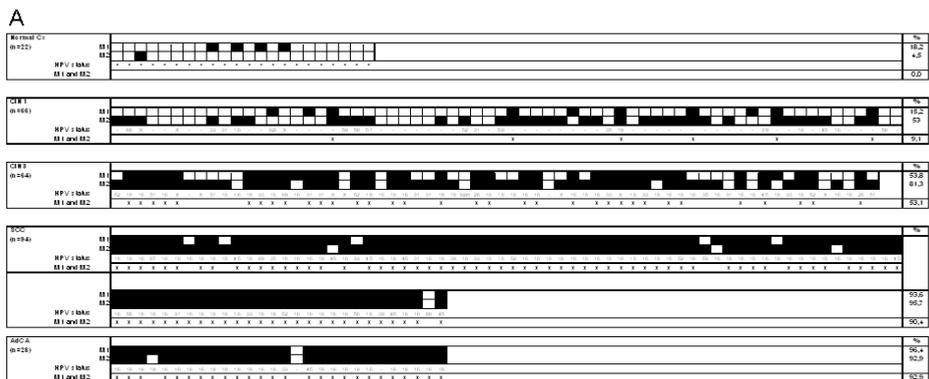
qMSP analysis demonstrated MAL M1 methylation in 18% (4/22) and MAL M2 methylation in 5% (1/22) of normal cervical biopsy samples. In CIN1 lesions, MAL M1 methylation was evident in 15% (10/66) of cases and MAL M2 methylation in 53% (35/66) of lesions. In CIN3 lesions, the frequencies of MAL M1 and M2 methylation were 58% (37/64) and 81% (52/64), respectively. Ninety-four per cent (88/94) of SCCs showed MAL M1

methylation and 96% (90/94) MAL M2 methylation. In AdCAs, MAL M1 methylation was detected in 96% (27/28) and MAL M2 methylation in 93% (26/28) of tumours (Figure 3A). Methylation of both regions was detected in 0% (0/22) of normal cervical biopsies, while 9,1% (6/66) of CIN1 lesions, 53% (34/64) of CIN3 lesions, 90% (85/94) of SCCs and 93% (26/28) of AdCAs showed methylation of M1 and M2 (Figure 3A). As is shown in Figure 3B, the level of MAL M1 and MAL M2 methylation also increases with severity of cervical disease.

MAL mRNA expression in relation to MAL promoter methylation in cervical lesions

MAL mRNA expression was determined by quantitative RT-PCR on 12 normal cervical biopsies, 15 microdissected CIN3 lesions and 11 SCCs. Only one out of 12 (8%) normal biopsies showed reduced *MAL* mRNA expression compared with 87% (13/15) of CIN3 lesions and 100% (11/11) of cervical SCCs. The differences in *MAL* mRNA expression levels between normal epithelium and CIN3 lesions as well as normal epithelium versus SCC and CIN3 versus SCC were statistically significant (Figure 3C; $p < 0.0005$, $p < 0.0005$ and $p = 0.001$, respectively).

Subsequent qMSP analysis of these samples (Supporting information, Supplementary Figure 1) showed that *MAL* mRNA expression was significantly lower in samples with promoter methylation at M2 or M1 plus M2 (Figure 3D; $p = 0.001$ and $p < 0.0005$, respectively). The group of specimens with only MAL promoter methylation at the M1 region was too small ($n=1$) to draw any conclusions.



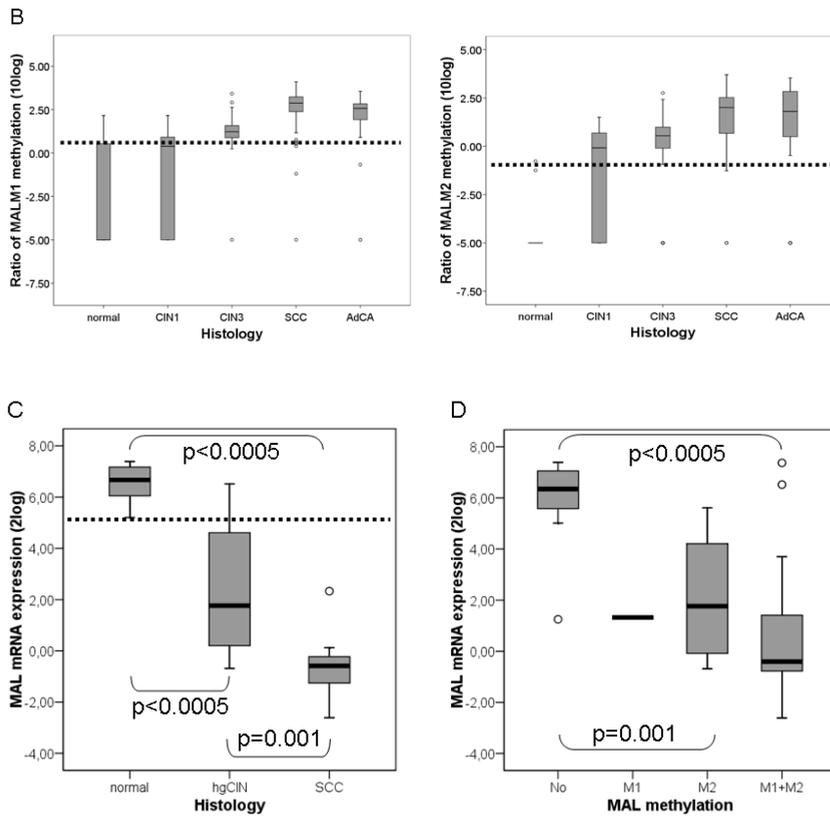


Figure 3. (A) Summary of MAL qMSP results on cervical tissue specimens. DNA methylation is depicted in black; white boxes indicate unmethylated DNA; HPV status of specimens is depicted as specific HPV type present or negative (-). (B) Box plots of levels of MAL M1 and M2 methylation (expressed as 10log-transformed values) in normal cervix, CIN1, CIN3, SCC and AdCA. (C) Box plot of 2log-transformed *MAL* mRNA expression levels in cervical tissue specimens. (D) Box plot of the correlation between the 2log-transformed *MAL* mRNA expression levels and the MAL promoter methylation status in cervical tissue specimens. Dotted lines indicate the cut-off values used. The upper and lower boundaries of the boxes represent the 75th and 25th percentiles, respectively. The black line within the box represents the median; the whiskers represent the minimum and maximum values that lie within 1.5 inter-quartile range from the end of the box. Values outside this range are represented by circles.

MAL promoter methylation in cervical scrapings

Finally, we performed a pilot study to assess whether the detection of MAL promoter methylation in cervical scrapings can predict underlying high-grade cervical disease. To this end, we compared 17 cytologically normal scrapings of hrHPV-positive women who did not develop CIN lesions during 5 years of follow-up (controls) with 21 cytologically abnormal (ie

moderate dyskaryosis or worse) scrapings of hrHPV-positive women who developed CIN3 within 18 months after scraping collection (cases).

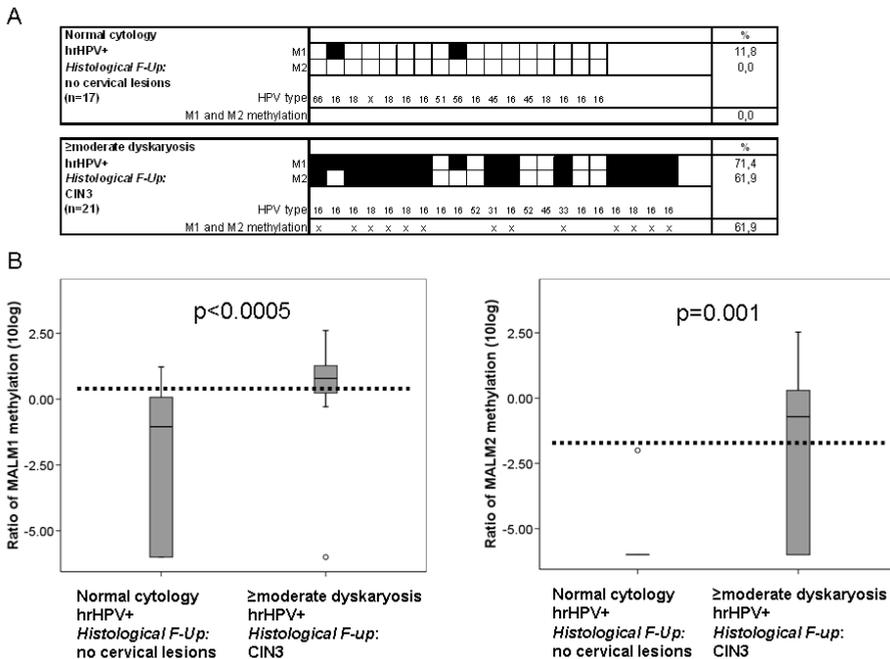


Figure 4. (A) Summary of MAL qMSP data on cervical scrapings. The upper section represents cytomorphologically normal scrapings of hrHPV-positive women without CIN lesions during 5-year follow-up. The lower section represents scrapings classified as moderate dyskaryosis or worse of hrHPV-positive women who developed CIN3 within 18 months of follow-up. DNA methylation is depicted in black; white boxes indicate unmethylated DNA. The HPV status of specimens is depicted as specific HPV type present, type X when no specific type could be determined by RLB (X), or negative (–). (B) Box plots of levels of MAL M1 and M2 methylation (expressed as 10log-transformed values) in cytomorphologically normal scrapings of hrHPV-positive women without CIN lesions during 5-year follow-up versus scrapings classified as moderate dyskaryosis or worse of hrHPV-positive women who developed CIN3 within 18 months of follow-up.

MAL M1 methylation was detected in 12% (2/17) of controls and 71% (15/21) of cases. Methylation of MAL M2 was found in 0% (0/17) and 62% (13/21), respectively, of controls and cases. Methylation at both regions was observed in 0% (0/17) of controls and 62% (13/21) of cases (Figure 4A). As is shown in Figure 4B, the level of MAL M1 and MAL M2 methylation was also increased in cases compared with controls ($p < 0.0005$ and $p = 0.001$, respectively). Similar to tissue specimens, scrapings also revealed a significant

correlation between MAL methylation and reduced MAL mRNA expression (Supporting information, Supplementary Figure 2; $p = 0.001$).

Discussion

By genome-wide expression analysis, we previously identified MAL as the most significantly down-regulated gene in both SCC and AdCA (8).

Here, we report that MAL gene silencing is a highly common event in HPV-immortalized cells and cervical cancer cells, which can be attributed to promoter methylation. The biological significance of MAL silencing in HPV-mediated transformation was supported by its tumour suppressor activities, as established in functional assays using bulk cultures of the cervical cancer cell line SiHa stably transfected with MAL. MAL overexpression in SiHa cells reduced the proliferation rate and repressed tumour cell characteristics such as migration and anchorage-independent growth.

Tumour suppressive traits have been assigned to MAL before in HPV-independent malignancies. Mimori *et al* showed that ectopic expression of MAL in an oesophageal cancer cell line reduced migration *in vitro* and tumourigenicity in nude mice, most likely resulting from enhanced Fas-induced apoptosis (24). However, in SiHa cells, known to be insensitive to death receptor-induced apoptosis (25), we could not show evidence for induction of either deathreceptor (Fas/TRAIL) or DNA damage-(cisplatin) induced apoptosis upon MAL overexpression (data not shown).

As for all the functional studies described here, which are performed on a bulk culture of SiHa cells stably transfected with MAL, additional functional assays on single cell clones and using other cervical cancer cell lines would provide further support for the biological role of MAL silencing in cervical carcinogenesis. Moreover, functional studies using RNA interference would be of interest to assess whether MAL silencing is sufficient to promote the transformation of hrHPV-containing primary keratinocytes.

MAL is a necessary element of glycolipid-enriched membrane (GEM) microdomains or lipid rafts. These rafts are implicated in polarized sorting of apical membrane proteins in epithelial cells and also in signal transduction from the cell membrane (9). MAL is an itinerant protein that constantly shuttles between the trans-Golgi network, the plasma membrane and endosomes (26). As such, loss of MAL expression in HPV-transformed cells may affect signaling

pathways and cell polarity, the latter being a commonly lost feature in human epithelial cancers.

Analysis of a large series of cervical biopsies revealed that both the frequency and level of MAL promoter methylation increase with the severity of disease. In tissue specimens, methylation of sole MAL M2 and MAL M2 in combination with MAL M1 were significantly correlated to reduced *MAL* mRNA expression. This confirms our *in vitro* findings identifying promoter methylation as the main mode of MAL silencing and underscores the biological role of MAL promoter methylation in cervical cancer development.

Although down-regulation of *MAL* mRNA in cervical carcinomas has been described before, no promoter methylation was detected using a *HpaII*-PCR-based methylation assay (27). This apparent discrepancy may be explained by the use of different assays, but is most likely also related to the analysis of different promoter regions. Previous studies indicate that *MAL* mRNA down-regulation was not correlated to *MAL* gene mutations or a genetic loss (7, 8, 27). This confirms our finding that promoter methylation is the driving force for *MAL* gene silencing. MAL promoter methylation and concomitant reduced *MAL* mRNA expression have also been described in gastric, colorectal and breast cancer (19, 27, 28). In addition, loss of *MAL* mRNA and protein expression is highly frequent in oesophageal dysplasia and neoplasia (29).

Interestingly, we showed MAL promoter methylation to be histotype-independent, which is in contrast to most of the methylation markers presently analysed in both cervical SCC and AdCA (30, 31). A well studied example is *CADM1*, showing promoter methylation in 83% of SCCs and only 23% of AdCAs (32). The high frequency of MAL methylation in AdCAs is of particular interest, since the incidence of AdCA and its glandular precursor lesions (ACIS) has remained the same or is even increasing, despite cervical screening programs (33-35). Accordingly, MAL methylation may emerge as a particularly valuable marker to improve the early detection of cervical AdCA and ACIS.

The diagnostic value of *MAL* gene silencing by promoter methylation as a diagnostic marker for high-grade CIN is further strengthened by our preliminary results on cervical scrapings obtained from a prospective cohort study (13-16). We showed that MAL M1 and M2 methylation and a concomitant reduction in *MAL* mRNA expression were significantly more common in hrHPV-positive women who developed CIN3 than in hrHPV-

positive women with normal cytology who did not develop CIN lesions during a 5-year follow-up.

In conclusion, we report that *MAL* silencing is functionally involved in HPV-mediated transformation and that *MAL* promoter methylation, which is predictive of decreased *MAL* mRNA expression in both tissues and cervical scrapings, is significantly associated with the development of CIN3, SCC and AdCA of the cervix. Our data strongly suggest that *MAL* gene silencing may be a useful diagnostic marker for (pre-)invasive cervical neoplasms of both histotypes.

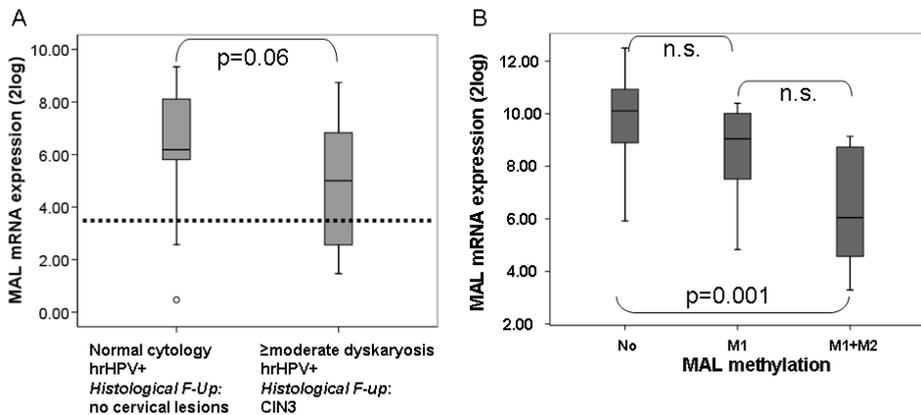
Acknowledgements

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Supplementary figures

Normal Cx (n=12)	M1	[Bar chart showing DNA methylation for M1 in Normal Cx]											%
	M2	[Bar chart showing DNA methylation for M2 in Normal Cx]											8,3
	HPV type	35 - - ND 16 - 39 16 ND - - 16											8,3
	M1 and M2 methylation	x											8,3
CIN3 (n=15)	M1	[Bar chart showing DNA methylation for M1 in CIN3]											%
	M2	[Bar chart showing DNA methylation for M2 in CIN3]											33,3
	HPV type	18 31 66 16 16 67 58 33 58 16 31 16 51 31											80,0
	M1 and M2 methylation	x x x x x x x x x x x x											26,7
SCC (n=11)	M1	[Bar chart showing DNA methylation for M1 in SCC]											%
	M2	[Bar chart showing DNA methylation for M2 in SCC]											90,1
	HPV type	16 16 35 16 33 45 16 45 31 16 33											100,0
	M1 and M2 methylation	x x x x x x x x x x x											90,1

Supplementary Figure 1. Summary of qMSP results on frozen cervical tissue specimens: normal biopsies ($n = 12$), CIN3 lesions ($n = 15$) and SCC ($n = 11$). DNA methylation is depicted in black, white boxes indicate unmethylated DNA; HPV status of specimens is depicted as specific HPV type present, negative (-) or not done (ND).



Supplementary Figure 2. (A) Box plot of 2log-transformed *MAL* mRNA expression levels in cervical scrapings. (B) Box plot of the correlation between the 2log-transformed *MAL* mRNA expression levels and *MAL* promoter methylation status in cervical scrapings. Dotted lines indicate cut-off values used. The upper and lower boundaries of the boxes represent the 75th and 25th percentiles, respectively. The black line within the box represents the median, the whiskers represent the minimum and maximum values that lie within 1.5 inter quartile range from the end of the box. Values outside this range are represented by circles.

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