Focal aberrations indicate EYA2 and hsa-miR-375 as oncogene and tumor suppressor in cervical carcinogenesis

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Cervical cancer results from persistent infection with high-risk human papillomavirus (hrHPV). Common genetic aberrations in cervical (pre)cancers encompass large genomic regions with numerous genes, hampering identification of driver genes. This study aimed to identify genes functionally involved in HPV-mediated transformation by analysis of focal aberrations (<3 Mb) in high-grade cervical intraepithelial neoplasia (hgCIN). Focal chromosomal aberrations were determined in high-resolution array comparative genomic hybridization data of 60 hgCIN. Genes located within focal aberrations were validated using 2 external gene expression datasets or qRT-PCR. Functional roles of candidate genes EYA2 (20q13) and hsa-miR-375 (2q35) were studied by siRNA-mediated knock-down and overexpression, respectively, in hrHPV-containing cell lines. We identified 74 focal aberrations encoding 305 genes. Concurrent altered expression in hgCIN and/or cervical carcinomas compared with normal cervical samples was shown for ATP13A3, HES1, OPA1, HRASLS, EYA2, ZMYND8, APOBEC2, and NCR2. Gene silencing of EYA2 significantly reduced viability, migratory capacity, and anchorage-independent growth of HPV16-transformed keratinocytes. For hsa-miR-375, a direct correlation between a (focal) loss and significantly reduced expression was found. Downregulation of hsa-miR-375 expression was confirmed in an independent series of cervical tissues. Ectopic expression of hsa-miR-375 in 2 cervical carcinoma cell lines reduced cellular viability. Our data provide a proof of concept that chromosomal aberrations are actively contributing to HPV-induced carcinogenesis and identify EYA2 and hsa-miR-375 as oncogene and tumor suppressor gene, respectively.
INTRODUCTION

Persistent infection with high-risk human papillomavirus (hrHPV) has been causally related to cervical cancer development. Squamous cell carcinomas (SCCs) are preceded by precursor lesions, called cervical intraepithelial neoplasia (CIN). Based on histology, CIN can be classified as low-grade (CIN1) or high-grade (CIN2/3). Low-grade CIN are mainly associated with productive viral infections. High-grade CIN (hgCIN) usually harbor transforming HPV infections and are characterized by deregulated expression of viral oncoproteins E6 and E7 in proliferating cells and diffuse CDKN2A overexpression. Overexpression of the viral oncoproteins leads to accumulation of (epi)genetic changes in the host-cell genome and may drive progression to cervical cancer. Array comparative genomic hybridization (arrayCGH) on hgCIN has revealed common chromosomal aberrations, e.g., gain of 3q. Although these aberrations are in part lesion, tumor, or HPV-type related, it has not been unequivocally demonstrated if they actively contribute to HPV-induced carcinogenesis.

Each tumor genome harbors a mixture of genetic aberrations with genes directly responsible for its development (drivers) and random events (passengers). Distinguishing driver from passenger aberrations is crucial for understanding cervical cancer development and will aid in the identification of functional biomarkers or even molecular targets for therapeutic intervention. Identification of driver genes has been a major challenge in early (array)CGH studies due to the large size of detected aberrations and the numerous genes located therein, making functional validation of potential candidates virtually impossible. Additionally, cancer genomes generally display a high degree of chromosomal instability, including many passenger aberrations. The use of premalignant lesions aids in the identification of driver genes due to a lower degree of genetic chaos.

Increased resolution of arrayCGH platforms and their applicability to formalin-fixed paraffin-embedded (FFPE) tissues have yielded new insights into small chromosomal aberrations of only several Mb previously undetected or erroneously classified as outliers. Small somatic aberrations, so-called focal aberrations (commonly regarded as <3 Mb), harbor only a small number of genes and thereby aid the identification of drivers in a cancer genome. Recent studies showed focal aberrations to be enriched for cancer genes and genes frequently mutated in cancer. Analysis of focal aberrations and genes therein by combined bio-informatical analysis, and experimental biology has led to the discovery of novel tumor suppressor genes and oncoproteins in various nonviral induced epithelial tumors such as lung, colon, and breast cancer, including CDH20, PRKAA2, GPR124, ARFRP1, and hsa-miR-101. The role and order of chromosomal aberrations in the genetic history of these epithelial tumors is unclear. This is different for cervical cancer where persistent infection with hrHPV may provoke tumor
development by inducing genetic instability, chronologically succeeded by selected (epi)genetic changes providing a growth advantage, which yet become evident in hgCIN. In this study, we aimed to identify potential driver genes of HPV-induced cervical carcinogenesis by analysis of focal aberrations in 60 hgCIN.

MATERIALS AND METHODS

Tissue Samples and Cell Lines
Previously, 60 HPV-positive, FFPE hgCIN (of which 56 CIN3 and 4 CIN2) were analyzed by high-resolution comparative genomic hybridization microarray (arrayCGH) (Bierkens et al., 2012a, b). HPV types of CIN3 were: HPV16 n = 25, HPV18 n = 4, HPV31 n = 14, HPV33 n = 2, HPV45 n = 3, HPV51 n = 2, HPV52 n = 3, HPV58 n = 2, and HPV69 n = 1; of CIN2: HPV16 n = 1, HPV31 n = 2, and HPV45 n = 1. Samples were compared with pooled DNA from FFPE cervical biopsies of 5 women diagnosed with leiomyoma, but with no cervical disease. These biopsies were negative for the presence of either low- or high-risk HPV.

All lesions analyzed showed strong diffuse staining for CDKN2A, indicative of a transforming HPV-infection, and were obtained from the population based screening study Amsterdam (POBASCAM). An additional set of HPV-positive frozen biopsies of 6 normal cervical squamous epithelial specimens, 13 CDKN2A-positive hgCIN, and 9 SCC was used to validate altered expression of hsa-miR-375. These specimens were collected during routine clinical practice at the Department of Obstetrics and Gynecology and stored at the Department of Pathology of the VU University Medical Center (Amsterdam, The Netherlands).

The HPV16-immortalized keratinocyte cell line FK16A was established and cultured as described previously. Human cervical carcinoma cell lines SiHa and CaSki were obtained from the American Type Culture Collection (Manassas, VA) and cultured as described previously.

All cell lines were around the time of functional experiments tested for the presence of the correct HPV type, the presence of known copy number, and DNA methylation alterations for authentication of the cell lines.

ArrayCGH Procedures and Data Analysis of Potential Focal Aberrations
Microdissection and DNA extraction of the hgCIN were performed as described previously. Whole genome amplification using the Bioscore kit (Enzo Bioscore™ Screening and Amplification, Enzo Life Sciences, Farmingdale, NY) was performed as described by Buffart et al. Labeling of DNA with Cy3 or Cy5 was performed with the Enzo Genomic DNA labeling kit according to the manufacturer’s instructions (Enzo Life Sciences) using 500 ng of amplified
Hybridizations were performed on slides containing 2× 105K arrays. Each array contained 99,000 synthetic 60-mer oligonucleotides, with an overall median probe spacing of 21.7 kb (Agilent Technologies, Palo Alto, CA). Across array CGH as well as across slide comparison was used, in which a sample was either hybridized to a reference, or to another sample as described by Buffart et al. Dye channels were digitally swapped to compare each sample with a reference. Data extraction and calling of gains and losses were performed as described previously.

Downstream analysis for focal aberrations was performed and plots were made using the statistical programming language R version 2.11.1. Log2-ratios of signal intensities between hgCIN and reference for every probe were median-normalized and postsegmentation mode normalization was performed. To discriminate between somatic focal aberrations and germ-line copy number variants (CNVs), all aberrations smaller than 3 Mb were identified using the processed copy number data and compared with a database of known CNVs in the healthy population using the database of genomic variants.

Only gains and losses detected at least twice (recurrent) in the dataset were considered. These recurrent focal aberrations were further filtered for aberrations that were only affected in a single direction (gain or loss). For each recurrent focal aberration, the smallest genomic overlap of the focal aberrations and the frequency in the dataset was determined (the high frequency region (HFR)). Genes and miRNAs were retrieved using biomaRt (R/Bioconductor) and Ensembl (hg18/NCBI 36, ensemble 54). Potential driver genes were assumed to be located completely within the HFR in case of both gains and losses and were selected based on the total frequency of occurrence in the data series.

Data access

Expression Analysis by Quantitative Reverse-Transcription PCR (qRT-PCR)
Expression of EYA2 was measured on the ABI7500 Fast Real-Time PCR System (Applied Biosystems) using primers and probe described in. The house keeping gene snRNP U1A was included for normalization purposes, as described by Henken et al. Expression values were normalized to the reference using the comparative Ct method (2−ΔΔCt) (Schmittgen and Livak, 2008). qRT-PCRs were performed using Universal PCR master mix (Applied Biosystems).

Expression of hsa-miR-375 was measured using TaqMan microRNA assays following the manufacturer’s instructions (Assay ID: 000564; Applied Biosystems, Nieuwerkerk a/d IJssel,
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The Netherlands) on the ABI7500 Fast Real-Time PCR System. The small nucleolar RNA transcript RNU43 (Assay ID 001095; Applied Biosystems) was included as internal reference for hsa-miR-375 expression. Expression values were normalized to the reference using the comparative Ct method (2−ΔΔCt)27.

Validation of Candidate Driver Genes in Independent Expression Array Studies

Two independent single-channel, expression microarray datasets (Affymetrix Human Genome U133A Array, HG-U133A) were used to validate the candidate genes in the most frequently occurring focal gains and losses. Normalized expression data were downloaded from the Gene Expression Omnibus (GEO) database: GSE7803 and GSE975028,29. From GSE7803, 38 samples were used, including 10 normal cervical samples, 7 hgCIN, and 21 SCC. From GSE9750, 57 samples were used, including 24 normal cervical samples and 33 SCC. Probes were annotated using Ensembl (ensembl.org) based on hg18/NCBI36 (ensembl 54).

siRNA and cDNA Transfection

Transfections were performed using Dharmafect2 (Dharmacon, Lafayette, LA) and Lipofectamine 2000 (Invitrogen) for siRNA and cDNA, respectively, according to the manufacturer’s recommendations.

FK16A cells, passage 255, were used for experiments using siRNA targeting either EYA2 “ON-TARGETplus SMARTpool EYA2” (Cat#L-017233-00-0005, Dharmacon, Lafayette, USA) or nontargeting siRNA pool “ON-TARGETplus Nontargeting Pool” (Cat#D-001810-10-05, Dharmacon) according to the manufacturer’s instructions. As positive control for transfection efficiency, PLK1 specific siRNAs “ON-TARGETplus SMARTpool PLK1” (Cat#L-003290-00-0005, Dharmacon) was used.

SiHa and CaSki cells were transfected with either hsa-miR-375 construct pcDNA4/H1/miR-375 or empty vector pcDNA4/H1 (a generous gift from Dr. R. J. Perera, Mazar et al., 2011).

After transfection, silencing (EYA2) and ectopic overexpression (hsa-miR-375), respectively, were checked by qRT-PCR as described above.

Cell Viability Analysis

Cell viability was measured using a colorimetric (MTT-tetrazolium) assay (ION Biomedicals, Cleveland, OH) according to the manufacturer’s instructions. Cells were transfected with the respective siRNA and cDNA constructs and equal cell numbers for all conditions were seeded in triplicate in 96-well plates. MTT conversion was measured at days 0 and 2. Cellular viability was calculated for all tested conditions by subtracting the measurement of day 0 from day 2. All experiments were performed in duplicate, starting from independent transfections.
Migration and Anchorage-Independent Growth Analysis

Migration and anchorage-independent growth was determined as described previously\textsuperscript{26}. In brief, for migration analysis, siRNA transfected cells were plated at high-confluency and uniformly scratched to create a cell-free gap. Plates were photographed again after 30 hr.

To examine anchorage-independent growth, 5,000 siRNA transfected cells were plated in semi-solid agarose. Colonies were photographed and counted after 3 weeks of incubation.

Statistical Analysis

To test whether focal aberrations were significantly enriched for cancer related genes as published in the cancer census list\textsuperscript{30}, enrichment analysis was performed. Ten thousand sets of random aberrations were generated of the same size as the focal aberrations identified in our study, and genes therein, were matched to genes of the cancer census list. Overlap of genes for each random set with genes of the cancer census list was calculated and expressed as a P-value\textsuperscript{17}.

Gene expression between histological groups was assessed using the Mann-Whitney U-test for both qRT-PCR and microarray data. Association between the number of gains and losses, exclusively gained or lost, and CNVs or somatic focal aberrations was determined using a χ\textsuperscript{2}-test. P-values smaller than 0.05 were considered statistically significant.

RESULTS

Focal Aberrations in hgCIN

A total of 235 recurrent focal aberrations (<3 Mb) were detected in 60 hgCIN. The frequency with which these chromosomal gains and losses were detected is shown in Figure 1. Overlap with known germ-line CNVs in the healthy population marked 138 of these aberrations as CNVs (Conrad et al., 2010). Differences were observed between gains and losses overlapping known CNVs and remaining potential somatic aberrations. For example, 84% of CNVs versus 97% of somatic aberrations contained a gene. There was a significant difference in the number of aberrations exclusively affected as either a gain or a loss between CNVs and somatic aberrations, i.e., 42% and 76%, respectively (χ\textsuperscript{2}-test, P < 0.01). Therefore, all focal aberrations showing both gains and losses in the dataset were considered potential CNVs and excluded from further analysis. This reduced the list of somatic focal aberrations to 74 genomic regions, indicated in black in Figure 1 and listed in Supporting Information Table I. The most frequent aberrations and genes therein are summarized in Table 1. For each recurrent focal aberration, the HFR was identified. A total of 305 genes were located in these HFRs (range: 0–28 genes per focal aberration). Focal aberrations were significantly enriched for known
Figure 1: Frequency plot of all copy number aberrations and focal aberrations. Percentages of gains (positive Y-axis) and losses (negative Y-axis) for each dinucleotide are shown for the 60 hgCIN. Focal aberrations are indicated in black.

Table 1: Summary of the most frequent focal aberrations (exclusively affected as a gain or a loss) detected and associated full-length genes therein.

<table>
<thead>
<tr>
<th>Cytoband</th>
<th>Start</th>
<th>End</th>
<th>Mb</th>
<th>Gain Loss</th>
<th>Gain Loss</th>
<th>Genes within HFR</th>
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<td>45671015</td>
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<td>0</td>
<td>14/0</td>
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<td></td>
<td>3q29</td>
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<td>195671889</td>
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<td>2/0</td>
<td>27/0</td>
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<td>178392811</td>
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<td>2/0</td>
<td>17/0</td>
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<td>20q11.22</td>
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<td>31700661</td>
<td>0.15</td>
<td>2/0</td>
<td>14/0</td>
</tr>
<tr>
<td>Loss</td>
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<td>219664836</td>
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<td>0/5</td>
<td>0/10</td>
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<td>73417685</td>
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<td>0/2</td>
<td>0/4</td>
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</table>

* The number of samples found to have a focal aberration
* The total number of samples in which this genomic location was altered
* All genes that are located full-gene within the high frequency region (HFR) of the focal aberration.
cancer related genes (enrichment analysis: $P = 0.02$) based on the Cancer Gene Census database (http://www.sanger.ac.uk/genetics/CGP/Census), including *ABL1*, *ARHGEF12*, *CDX2*, *ELN*, *EML4*, *FEV*, *FLT3*, *FNBP1*, *HOXD11*, and *JAK2* (Supporting Information Table I).

**Expression of Protein-Coding Genes within Focal Aberrations**

Expression of genes located within the 2 most frequent focal gains (20q13 and 3q29) and losses (2q35 and 6p21.1) (Table 1) was examined in 2 independent cervical cancer mRNA expression microarray datasets (GSE7803 and GSE9750)\(^{28,29}\). The mRNA expression of genes within the 4 focal aberrations, showing altered expression concurrent with the chromosomal aberration in either one or both datasets, was plotted per histological grade (Figure 2).

Focal gain at 20q13 was detected 4 times and the genomic region was gained in 10 other samples as part of a larger aberration. It harbored 2 genes: *ZMYND8* and *EYA2*. In the first dataset, both genes showed increased expression in hgCIN and SCC versus normal. Increased expression was only significant for *ZMYND8* in SCC versus normal in the second dataset ($P = 0.03$). Increase of *EYA2* expression nearly reached significance for hgCIN versus normal in the first dataset ($P = 0.06$) and for SCC versus normal in the second dataset ($P = 0.06$). Focal gain at 3q29 was detected 2 times and the genomic region was gained in 25 other samples as part of a larger aberration. Expression data in the external datasets was available for *ATP13A3*, *GP5*, *LRRC15*, *CPN2*, *HES1*, *OPA1*, and *HRASLS*, of which *ATP13A3*, *HES1*, *OPA1*, and *HRASLS* showed altered expression in SCC and/or hgCIN consistent with the copy number data. Expression of *ATP13A3* was significantly higher in SCC versus normal in both datasets (both $P < 0.001$) and in SCC versus hgCIN ($P < 0.001$). Expression of *HES1* was significantly higher in hgCIN versus normal ($P = 0.003$), but there was no differential expression between SCC and normal in either dataset. *OPA1* expression was significantly increased in SCC versus normal in both datasets ($P < 0.001$ and $P = 0.02$, respectively), yet expression in hgCIN and normal was similar. *HRASLS* was significantly higher expressed in SCC versus normal ($P = 0.02$ and $P = 0.04$) as well as SCC versus hgCIN ($P = 0.04$). For the other genes at 3q29, *ATP13A4* and *ATP13A5*, no expression data were available.

Focal losses at both 2q35 and 6p21.1 were detected 5 times and the genomic region was lost in 5 other samples as part of a larger aberration. Expression of *FEV* (2q35) was slightly decreased in hgCIN and SCC compared with normal. No (reliable) data were available for the genes, *CRYBA2* and *hsa-miR-375*. Expression of *APOBEC2* (6p21.1) was slightly decreased in hgCIN versus normal and was (significantly) lower in SCC compared with normal in both datasets ($P = 0.07$ and $P = 0.02$, respectively). Expression of *NCR2* (6p21.1) was significantly reduced in hgCIN versus normal ($P = 0.04$), but not in SCC versus normal in both datasets. The remaining genes located at 6p21.1 either showed no (concurrent) change in expression...
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Figure 2: Expression of candidate driver genes. Boxplots showing mRNA expression of genes located within the 2 most common focal gains: 20q13 (ZMYND8, EYA2) and 3q29 (ATP13A3, HES1, OPA1, HRASLS) and the 2 most common focal losses: 2q35 (FEV) and 6p21 (APOBEC2, NRC2) as obtained from 2 external datasets: left GSE7803; right GSE9750.

For dataset GSE7803 expression data was available for normal cervix (n=10), hgCIN (CIN2/3, n=7) and squamous cell carcinoma (SCC, n=21). For dataset GSE9750 expression data was available for normal cervix (n=24) and SCC (n=33).

*p<0.05, **p<0.01.
Focal Abberations in hgCIN

(TREM1, TREML2, NFYA, and BZRPL1) or were absent in the datasets (MDFI, FOXP4, TREML4, TREML3, TREML2P, TREM2, TREML1, C6orf130, and UNCSCL).

Increased Expression of EYA2 Associated with Focal Gain at 20q13 is Functionally Relevant

To investigate the potentially functional relevance of 20q13 focal gain in cervical carcinogenesis, EYA2 expression was silenced in late passage (p255) HPV16-immortalized keratinocytes (FK16A) using siRNA. These cells are characterized by both copy number gain of 20q (Wilting et al., 2006) and increased EYA2 expression compared with primary foreskin keratinocytes (Supporting Information Figure I). Silencing of EYA2 expression was confirmed by qRT-PCR (Figure 3A). Cell viability was significantly reduced in cells transfected with EYA2 siRNA compared with nontargeting siRNA transfected cells (Figure 3B). Cells transfected with EYA2 siRNA showed reduced migratory capacity and significantly reduced anchorage-independent growth compared with nontargeting siRNA transfected cells (Figures 3C–3E).

Reduced Expression of hsa-miR-375 Associated with Focal Loss at 2q35 is Functionally Relevant

The most frequent focal loss at 2q35 was detected in 5 samples and as a larger aberration in 5 extra samples (Table 1). The HFR contained CRYBA2, FEV, and hsa-miR-375 (Figure 4). Expression of FEV was not significantly decreased in the external datasets (Figure 2). Although no (reliable) expression data were available for CRYBA2 or hsa-miR-375, we further investigated hsa-miR-375 because the stability of miRNAs allowed us to determine hsa-miR-375 expression in a subset of the FFPE hgCIN used in the arrayCGH experiments. Hsa-miR-375 expression could be measured in 4 out of 5 samples with focal loss, 5 samples with a larger loss, and in 28 samples with no aberration at that locus. Hsa-miR-375 expression was significantly lower in hgCIN with (focal) loss compared with lesions with no aberration (P = 0.04, Mann-Whitney U test; Figure 5A). Reduced hsa-miR-375 expression was verified in an independent series of samples consisting of frozen specimens of 6 normal cervices, 13 hgCIN, and 9 SCC (Figure 5B). Expression of hsa-miR-375 decreased in a step-wise pattern from normal cervical epithelium to hgCIN to SCC (SCC compared with normal: P = 0.003). To investigate the effect of hsa-miR-375 on cell viability, SiHa and CaSki cells were transiently transfected with either an empty vector or a hsa-miR-375 expression vector. Ectopic expression of hsa-miR-375 was confirmed by qRT-PCR (Figure 6A). Cell viability was significantly reduced in SiHa cells transfected with hsa-miR-375 compared with the empty vector control (t-test, P < 0.05; Figure 6B). Cell viability was also reduced in CaSki cells upon ectopic hsa-miR-375 expression, yet this did not reach significance (Figure 6B).
Figure 3: The effect of siRNA mediated silencing of EYA2 in FK16A cells. A) Relative EYA2 mRNA expression levels in FK16A cells transfected with non-targeting siRNAs (left) and with a pool of EYA2-specific siRNAs (right), showing significantly reduced EYA2 mRNA expression in EYA2 siRNA transfectants. B) Silencing of EYA2 significantly decreased cell viability. C) Results of the scratch assay indicate decreased migratory capacity in cells transfected with EYA2 siRNAs. Photographs in left panel are taken immediately after scratching (t=0 hours). Middle and right panel shows same cells after 30 hours of incubation, the right panel being stained with crystal violet. D) Results of anchorage-independent growth assay indicate decreased colony formation ability of cells transfected with EYA2 siRNAs. E) Quantification of anchorage-independent growth assay.
Figure 4: Focal loss at chromosome 2q35. Schematic representation of the focal deletion at chromosome 2q35 as detected in 5 of 60 hgCIN (A-E). X-axis represent the log2-ratio of the probes, Y-axis the genomic location. The grey line indicates the segmented values as obtained using circular binary segmentation (CBS). Bars at the top represent the exact location of the genes. Deleted genomic regions are indicated with grey fields. All samples, except C, show a focal deletion in an otherwise unaffected region; C shows a focal deletion within a larger loss at 2q35.
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Figure 5: Hsa-miR-375 expression in cervical tissue samples. A) HgCIN with a (focal) loss of 2q35 show significantly decreased hsa-miR-375 expression compared to lesions with a normal copy number. B) Relative hsa-miR-375 expression in an independent series of cervical specimens (6 normal cervices, 13 hgCIN and 9 SCC). Hsa-miR-375 expression is significantly decreased in SCC compared to normal cervical samples as well as hgCIN. *p<0.05, NS is not significant.

Figure 6: Ectopic expression of hsa-miR-375 in SiHa and CaSki cells. A) SiHa and CaSki cells transiently transfected with hsa-miR-375 show significantly increased hsa-miR-375 expression compared to cells transfected with empty vector. B) Ectopic of hsa-miR-375 resulted in decreased cellular viability. *p<0.05, NS is not significant.
DISCUSSION

In early stages of cervical cancer development, we identified 74 recurrent focal aberrations encoding 305 genes using high-resolution arrayCGH data of 60 hgCIN. These focal aberrations were significantly enriched for cancer census genes. Using 2 independent mRNA expression datasets, a number of genes located within the focal aberrations were identified as candidate oncogenes and tumor suppressors, including ZMYND8 and EYA2 (20q13). Additionally, hsa-miR-375 (2q35) was acknowledged as a candidate tumor suppressor gene by expression analysis of 2 series of cervical specimens. Functional validation studies on EYA2 and hsa-miR-375 provided proof of concept that chromosomal aberrations are actively contributing to HPV-induced carcinogenesis.

This study is the first to identify EYA2 as a candidate oncogene in cervical carcinogenesis. Next to increased EYA2 expression in hgCIN and carcinomas compared with controls, its functional relevance in HPV-mediated transformation was shown. Silencing of EYA2 in HPV-immortalized cells reduced viability, migration, and anchorage-independent growth, substantiating its potential oncogenic role. EYA2 has been suggested to function as a transcriptional co-activator. Increased expression of EYA2 has been seen in prostate, breast, urinary tract, and lung cancer11,32. Overexpression of EYA2 has been implicated in increased cell proliferation, migration, invasion, transformation, and metastasis in mammary carcinoma cells25,33. Upregulation of EYA2 has also been shown to promote tumor growth of ovarian cancer cell lines and was shown to be partly related to genomic amplification of its locus in ovarian cancer specimens32.

The most frequent focal loss contained hsa-miR-375 and a direct correlation between (focal) loss and reduced expression in hgCIN was shown. Downregulation of hsa-miR-375 expression in a second series of cervical tissues was confirmed, indicating continual decrease with disease progression. To the best of our knowledge, we are the first to show a direct correlation between a focal aberration and altered gene expression in the same tissue specimens. Ectopic expression of hsa-miR-375 in SiHa and CaSki cells reduced viability. Cell migration could not be examined due to strong induction of cell death upon ectopic hsa-miR-375 expression. In an independent and parallel study, downregulation of this miRNA was reported to be functionally involved in cervical cancer progression34, and36 reported decreased expression of hsa-miR-375 in high-grade CIN and SCC. A tumor suppressive function of hsa-miR-375 is further supported by the observed reduced expression in gastric, head and neck, pancreatic, hepatocellular carcinomas, and melanomas as well as the induction of apoptosis and reduced cell viability upon re-expression in gastric, hepatocellular, head and neck, breast cancer, and melanoma cell lines36-48. Candidate target genes of hsa-miR-375 include SP1, AEG1, and MTDH34,48,49.
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Functional studies were restricted to EYA2 and hsa-miR-375, and further mechanistic studies are warranted for the remaining genes validated in the external datasets. Additional validation studies on independent series of high-grade CIN analyzed by high-resolution copy number platforms are required to underline the translational relevance of current findings. Unfortunately, such datasets are not yet available.

The current study was performed on precancerous lesions induced by a viral infection, rather than on advanced tumors with variable and often unknown initiating events previously studied for focal aberrations; thus, the genetic chaos is likely modest, enhancing the chance of identifying actual driver genes. In this regard, also increased HES1 expression (3q29), restricted to hgCIN, is of particular interest. The mRNA expression pattern is in line with recent findings showing an increase in NOTCH1 expression, an upstream regulator of HES1, in nontumorigenic HPV-transformed keratinocytes, yet reduced NOTCH1 expression in tumorigenic cervical carcinoma cell lines. This is suggestive of a transient induction of NOTCH signaling driving HPV-mediated malignant transformation. Hence, it can be speculated that increased NOTCH signaling, either or not supplemented with the focal gain at 3q29, results in elevated HES1 expression in hgCIN. Genes within the focal aberrations that could not be verified using the external datasets due to their absence await future expression analysis.

Despite the fact that the number of chromosomal aberrations in hgCIN is in part HPV-type dependent, this study revealed no correlations between specific focal aberrations and the HPV-type present.

To conclude, analysis of focal chromosomal aberrations in hgCIN identified new candidate driver genes in HPV-induced carcinogenesis of the cervix. In addition to demonstrating a direct association between focal loss at 2q35 and decreased expression of hsa-miR-375, functional proof is provided for focal chromosomal aberrations actively contributing to cervical carcinogenesis. EYA2 was identified as a novel candidate oncogene and hsa-miR-375 as a tumor suppressor gene in HPV-mediated transformation.

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REFERENCES

Focal Abberations in hgCIN