

p16^{INK4a} and p14^{ARF} mRNA expression in Pap smears is age-related

Helmut von Keyserling, Wolfgang Kühn, Achim Schneider, Thomas Bergmann and Andreas M Kaufmann

Klinik für Gynäkologie, Charité-Universitätsmedizin Berlin, Campus Mitte and Benjamin Franklin, Berlin, Germany

Expression of high-risk HPV oncogenes results in a strong overexpression of cellular protein p16^{INK4a}. Immunohistochemical staining for p16^{INK4a} is widely used as diagnostic marker. However, p16^{INK4a} upregulation was also described as a biomarker of age. Here we analyzed p16^{INK4a} expression in cervical smears to investigate if patient age may influence p16^{INK4a}-based cervical cancer diagnosis. p14^{ARF} was analyzed as a related supportive biomarker. Cervical scrapes were taken and stored in RNAlater. Total RNA was extracted, and cDNA was analyzed for expression of p16^{INK4a} and p14^{ARF} relative to β -actin, by real-time reverse transcriptase PCR SYBR-Green I assays. Patient-derived smears referred as HSIL ($n = 45$) had 6.27-fold higher p16^{INK4a} mRNA expression than smears of cytologically normal and HPV-negative persons ($n = 48$). Expression of p14^{ARF} was 4.87-fold higher. When women with normal diagnoses were stratified for age, a significantly enhanced p16^{INK4a} (2.88-fold) and p14^{ARF} (1.9-fold) expression was observed as a consequence of ageing. A significant age-dependent upregulation was also observed in older HSIL patients (2.54-fold). Our study revealed significantly enhanced expression of p16^{INK4a}/p14^{ARF} mRNA in cervical scrapes referred to as HSIL compared with normal women. An age-dependent bias has to be considered when quantifying these tumor suppressor genes, with respect to cervical cancer development.

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Cervical cancer is the second leading cause of cancer deaths for women worldwide.¹ Today, cervical cancer screening programs mostly rely on cytology. The Pap-test introduced more than 60 years ago by Papanicolaou² remarkably reduced the mortality by cervical cancer.³ Pap screening has high specificity for disease, but suffers from suboptimal sensitivity and observer subjectivity that is partially compensated by frequent testing.⁴ Therefore, supportive molecular alternative methods are highly desirable.

Human papillomavirus is a necessary cause of invasive cervical cancer.⁵

Therefore, HPV testing is being discussed as a primary screening method, for triaging of equivocal results, and follow-up after therapeutic intervention.⁶ High-risk HPV infections are not sufficient for the development of cervical cancer. Most individuals remain asymptomatic and clear HPV infec-

tions spontaneously within approximately 8–10 months.⁷ The immortalization of primary human keratinocytes can be sufficiently maintained by the expression of the high-risk HPV E6 and E7 oncogenes. These oncogenes are required for initiation and all subsequent stages of carcinogenic progression.⁸ The focus on HPV oncogene mRNA expression could be a promising strategy to identify transformation events, but requires multiplex approaches to deal with HPV type-specific transcripts. The proteins p16^{INK4a} (*CDKN2A*) and p14^{ARF} (*CDKN2B*) are generally upregulated in transformed cells as a consequence of high-risk HPV E6 and E7 expression.

The *CDKN2A/B* gene locus encodes two proteins, p14^{ARF} and p16^{INK4A}, generated by alternative mRNA splicing.⁹ The p14^{ARF} consists of the unique first exon, 1-beta, 15 kb upstream of the exon 1-alpha of p16^{INK4A}. Both contain the common exons 2 and 3. p14^{ARF} and p16^{INK4A} are involved in cell cycle regulation, but despite their functional relationship, distinct promoters independently control the mRNA transcription of p14^{ARF} and p16^{INK4A}, and different reading frame usage results in unique amino acid sequences.¹⁰

Correspondence: Dr AM Kaufmann, PhD, Klinik für Gynäkologie, Charité-Universitätsmedizin Berlin, Campus Benjamin Franklin and Campus Mitte, Hindenburgdamm 30, Berlin 12200, Germany. E-mail: andreas.kaufmann@charite.de

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p14^{ARF} is a cell cycle regulator, arresting cell growth at G₁-S and G₂-M¹¹. p14^{ARF} interaction inhibits the Mdm2 oncoprotein, thereby blocking the Mdm2-p53 complex and preventing Mdm2-induced p53 degradation.¹⁰ p14^{ARF} expression is positively regulated by the transcription factor E2F-1.¹¹ HPV E7 inhibits Rb, releasing active E2F-1. Therefore, E7 has the ability to induce p14^{ARF} expression. Additionally, p53 negatively regulates p14^{ARF} mRNA transcription. Consequently, as E6 inhibits p53, it can elevate p14^{ARF} expression.^{11,12}

p16^{INK4A} stops cell cycle progression by binding cyclin-dependent kinase (CDK) 4 and 6, and inhibiting cyclin D. By inhibiting phosphorylation of Rb, p16^{INK4A} promotes the formation of a Rb-E2F repressive transcriptional complex, which also blocks cell cycle progression at the G₁-S restriction point.^{10,13} The HPV E7 protein binds to the phosphorylated Rb. This event releases the transcription factor E2F, and thereby induces uncontrolled cell replication. A positive feedback control loop leads to overexpression of p16^{INK4a} in HPV-transformed cells.^{14,15}

Cervical cancer was shown to be strongly associated with p16^{INK4a} and p14^{ARF} expression by immunohistochemical studies.^{11,16-19} Immunohistochemical staining for p16^{INK4a} became the most widely accepted surrogate biomarker in cervical cancer screening. p16^{INK4a} has the advantage to detect any high-risk HPV infection in conjunction with uncontrolled, malignant cell replication.^{20,21} Furthermore, it has been shown to improve inter-observer agreement on histological diagnosis,²² and commercial kits are widely used to support histological findings.

Apart from HPV-induced expression, p14^{ARF} and p16^{INK4A} also accumulate in many tissues as a function of advancing age as shown in rodents²³ and in human skin.²⁴ Their physiological function is the control of the state of cellular replicative senescence. Such upregulation in older patients may interfere with the high-risk HPV-related upregulation of p14^{ARF} and p16^{INK4A} in cervical intra-epithelial neoplasia.

In contrast to the successful establishment of immunohistochemical staining, very little data exists about the use of CDKN2A-derived mRNA in cervical cancer diagnosis. However, despite RNA derived from exfoliated cervical cells being challenging, other mRNA-based biomarkers such as HPV oncogenes,²⁵ Brn3a,²⁶ or MCM5,²⁷ were successfully tested on cervical smears and biopsies.

We analyzed the mRNA expression levels of p16^{INK4a} and p14^{ARF} in cervical smears from HPV-negative women with normal cytology and compared it with women with high-grade findings (HSIL). We used validated SYBR-Green I assays in a read-out described by Kanellou *et al*.²⁸ Expression was related to β -actin (ACTB) that was used as a housekeeping gene,²⁹ because it turned out to be the most stable reference in exfoliated cervical cells.³⁰

The aim of this study was to (i) study p14^{ARF} and p16^{INK4A} mRNA expression with respect to their

feasibility in cervical cancer diagnosis and (ii) to analyze the influence of age on the expression of p14^{ARF} and p16^{INK4A}. Our results support wariness in interpretation of p16^{INK4a} and p14^{ARF} mRNA expression results from older patients.

Patients and methods

Patients

Patient numbers and characteristics are summarized in Table 1. Pap smears were taken under colposcopic control at the Clinic for Gynecology, Campus Benjamin Franklin, Charité-Universitätsmedizin Berlin, Germany. Two smears were taken, the first for cytology and RNA extraction, and the second for HPV typing. To analyze the effect of age, patients with normal results (cytology and HPV test negative) or with high-grade dysplasia were divided into groups of upper and lower mean-age and analyzed for p16^{INK4a}/p14^{ARF} mRNA expression. Cytological diagnosis was made by cytopathologists blinded to the real-time PCR results. The study was approved by the IRB (EA4 217-20) and patients consented to the scientific use of scrape material.

HPV Typing

Genomic DNA from cervical smears was extracted with the Genomic DNA Mini Kit (Qiagen, Hilden, Germany) according to the standard protocol and eluted in 160 μ l TE buffer. A β -globin PCR was made as described and analyzed on a 2% agarose gel, to monitor DNA quality.³¹ The generic GP5 +/bio6 + primer set was used to amplify a sequence from the L1 gene of a large number of different HPV types.³¹ After PCR amplification, HPV sequences were genotyped by MPG Luminex suspension array technology (Multimetrix, Heidelberg, Germany).³²

RNA Extraction and cDNA Synthesis

Cervical smears were taken and the cyto-brushes were immediately immersed and stored in RNAlater (Qiagen), and transferred to -20°C within 24 h.

Table 1 Patient groups

Groups	N	HPV status (%)	Median age (range)
Cervical cell lines	5	100	NA
All normal women	48	0	38 (21-83)
Younger normal women	25	0	31 (21-38)
Older normal women	23	0	55 (40-83)
All HSIL patients	45	100	35 (21-66)
Younger HSIL patients	24	100	28 (21-35)
Older HSIL patients	21	100	44 (36-66)

Abbreviation: NA, not applicable.

The brush head was placed into a shredder column (Macherey & Nagel, Düren, Germany), filled with 350 μ l lysis buffer RA1 containing 1% mercaptoethanol, and centrifuged at 15 000 r.p.m. for 1 min. Residual material in the RNAlater solution was centrifuged at 12 000 r.p.m. after adding one volume of PBS. The pellet was combined with the flow-through from the shredder column, reloaded to the column, and centrifuged again. From the lysed material, RNA was extracted with the Macherey & Nagel RNA II Mini Isolation Kit (Macherey & Nagel) according to manufacturer instructions, with DNA digest using recombinant DNase. RNA was eluted with 36 μ l RNase free water. RNA concentration was measured by Nanodrop (PqLab, Erlangen, Germany). RNA integrity was analyzed for representative samples using Agilent Bioanalyzer 2100 (Agilent Santa Clara, USA). The extracted RNA was stored at -80°C . The cDNA synthesis was done with the Quantitect cDNA Kit (Qiagen) according to manufacturer instructions, using 0.25 μ l/reaction RNase inhibitor (Sigma Aldrich, St Louis MO, USA), and up to 2 μ g RNA per reaction. The cDNA was diluted 1:2 with RNase-free water and used as template in subsequent real-time reverse transcriptase PCR assays.

Real-Time PCR

All real-time PCR experiments were performed on a Chromo4 real-time detector (Bio-Rad, Hercules, USA). All quantitative PCRs were prepared in a dedicated facility in a laminar flow benchhood, using aerosol-resistant pipette tips.

Specific p16^{INK4a} and p14^{ARF} expression was quantitated using a real-time reverse transcriptase PCR assay with SYBR-Green I. The specific primer pairs for p16^{INK4a} and p14^{ARF}, and the temperature were described by Kanellou *et al.*²⁸ For normalization, we used ACTB as an internal control. ACTB primers were described by Kreuzer *et al.*²⁹ All primer sequences are listed in Table 2. The temperature protocol was 15 min at 95 $^{\circ}\text{C}$, followed by 40 cycles of 30 s at 95 $^{\circ}\text{C}$, 1 min at 60 $^{\circ}\text{C}$ and 30 s at 72 $^{\circ}\text{C}$. Melting curves were made between 50 and 95 $^{\circ}\text{C}$ with a temperature increment of 0.2 $^{\circ}\text{C}$. cDNA (1 μ l) was amplified in 1 \times Qiagen multiplex mastermix containing 1 \times SYBR-Green I, and 300 nM of each primer.

Each sample was run in duplicate and each run contained two 'no template controls' for each gene.

Expression analysis and statistical evaluation was made using the pair-wise fixed re-allocation randomization test by the Qiagen REST 2009 Software, V2.0.13.³³ Additionally, relative copy numbers were calculated for each patient.

For the purpose of quantification, and to show efficiency and sensitivity, plasmid standard curves were made by cloning the p14^{ARF}, p16^{INK4a}, and ACTB amplicons into pCR2.1, using the TOPO TA cloning system (Invitrogen, Groningen, The Netherlands), according to the manufacturer's instructions. Plasmids were verified by sequencing, and serial dilutions of 10⁷–10² plasmid copies containing the PCR inserts were made. Standard curves were generated by amplified plasmid DNA serial dilutions.

Results

The efficiency of the real-time PCR assays was tested by evaluating the results of serial template dilutions of plasmid DNA containing the respective amplicons. The efficiency of all assays was high, and all assays had a dynamic range between at least 10²–10⁷ (Figure 1a). Highly specific melting curves are shown for representative samples (Figure 1b). RNA integrity number (RIN) was analyzed in each of five cervical smears and cervical cell lines (CasKi, HeLa, SiHa and the recently developed HPV16-positive cell lines, Goe and Marq). As expected, RNA isolates from smears were highly degraded (RIN_(mean) = 4.6, range = 2.4–6.6). RNA from cervical cancer cell lines showed less degradation (RIN_(mean) = 8.5, range = 5.1–9.1), although it was exposed to the same storage and extraction procedure. The cervical cancer cell lines were used as positive controls to estimate the expression level of samples with good RNA quality and 100% transformed cells.

The p14^{ARF} and p16^{INK4a} expression levels relative to ACTB were significantly upregulated in HSIL patients. A significant age-related bias was observed. The results are summarized in Table 3. The data show the *N*-fold upregulation that was calculated in 45 high-risk HPV-positive tested women with HSIL diagnosis compared with 48 normal women with no detectable HPV infection. Both groups were divided at their median age into four subgroups (Table 1). To analyze the sensitivity and specificity of these tests, we calculated the individual p14^{ARF} and p16^{INK4a} copy numbers in relation to ACTB. The respective cut-offs were defined as the median value for normal patients, plus one s.d. For p16^{INK4a}, we calculated a sensitivity to detect HSIL positive patients of 79% (95% CI = 65–88%) and a specificity of 83% (95% CI = 67–92%). For p14^{ARF}, we calculated a sensitivity to detect HSIL-positive patients of 74% (95% CI = 60–84%) and a

Table 2 Primer sequences

Primer/probe	Sequence	Author
P14 ^{ARF} F	5'-ccctcgtgctgatgctactg-3'	Kanellou <i>et al.</i> ²⁸
P14 ^{ARF} R	5'-catcatgacctggctcttagaa-3'	Kanellou <i>et al.</i> ²⁸
P16 ^{INK4a} F	5'-ggggccaccagaggcagt-3'	Kanellou <i>et al.</i> ²⁸
P16 ^{INK4a} R	5'-ggttggtggcggggcagtt-3'	Kanellou <i>et al.</i> ²⁸
ACTB F	5'-agcctgcctttgccga-3'	Kreuzer <i>et al.</i> ²⁹
ACTB R	5'-ctgggcctggggcg-3'	Kreuzer <i>et al.</i> ²⁹

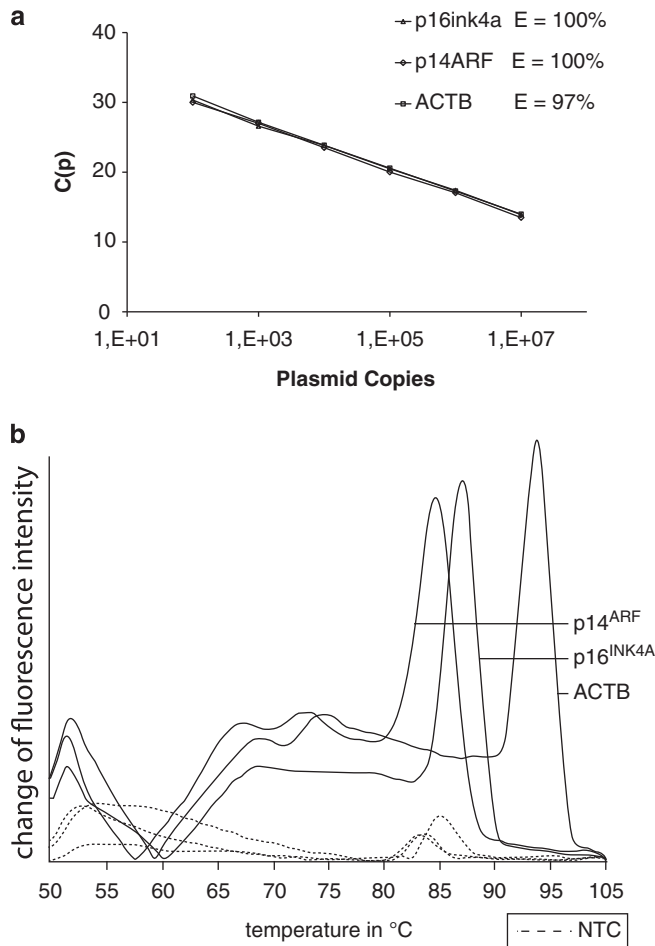


Figure 1 Sensitivity and specificity of real-time PCR assays. (a) Dynamic range and efficiencies of P14^{ARF} and P16^{INK4a}. (b) Melting curve analysis of SYBR-Green I assays. E = efficiencies, C(p) = crossing point, NTC = no template control.

Table 3 Relative p14^{ARF} and p16^{INK4A} expression in relation to dysplasia and age

Control	Case	Transcript	N-fold expression	s.e.	95% CI	P
Normal all	HSIL all	p16	6.27	1.32–29.84	0.25–137.92	<0.001
Normal younger	Normal older	p16	2.88	0.79–9.96	0.19–107.17	<0.001
HSIL younger	HSIL older	p16	2.54	0.59–13.29	0.117–42.767	0.006
Normal younger	HSIL younger	p16	6.73	1.25–35.21	0.25–216.99	<0.001
Normal olderer	HSIL older	p16	5.94	1.65–18.56	0.49–51.14	<0.001
Normal all	Ca cell lines	p16	15.70	4.58–50.57	1.73–274.37	<0.001
Normal younger	HSIL older	p16	17.12	3.59–70.34	0.71–488.03	<0.001
Normal older	HSIL younger	p16	2.34	0.58–9.45	0.15–30.45	0.007
Normal all	HSIL all	p14	4.83	0.93–21.31	0.27–89.62	<0.001
Normal younger	Normal older	p14	1.90	0.40–8.80	0.12–24.45	0.046
HSIL younger	HSIL older	p14	2.02	0.47–8.3	0.14–26.59	0.015
Normal younger	HSIL younger	p14	4.72	0.85–24.13	0.20–81.38	<0.001
Normal older	HSIL older	p14	5.04	1.31–16.87	0.46–45.78	<0.001
Normal all	Ca cell lines	p14	4.48	1.22–16.57	0.45–54.61	0.008
Normal younger	HSIL older	p14	9.55	1.86–53.48	0.37–176.05	<0.001
Normal older	HSIL younger	p14	2.49	0.72–7.87	0.26–25.27	<0.001

Abbreviations: s.e., standard error; CI, confidence interval; P, significance.
 Ca cell lines: CaSki, HeLa, SiHa, Goe, Marq.

specificity of 83% (95% CI = 67–93%). A total of 7 out of 48 normal women had an elevated p16^{INK4a} value, and 6 out of 48 normal women had an

elevated p14^{ARF} value. Of the 45 HSIL patients, 11 had p16^{INK4a} and 15 had p14^{ARF} mRNA levels below the defined cut-off.

Discussion

Despite the successful reduction of cervical cancer deaths by Pap cytology screening, a reliable biomarker-based screening method that verifies high-grade disease is highly desirable to support cytological findings.

The most popular biomarker for the detection of cervical intraepithelial neoplasia is the immunohistochemical staining for p16^{INK4a}. Rare data exist about p16^{INK4a} mRNA quantification. Our aim was to explore the transcripts p14^{ARF}/p16^{INK4a} with respect to their usability in cancer screening, because higher levels of these mRNAs may indicate HPV type independently the upregulation of E6 and E7 oncogene expression that in turn indicates malignant transformation. However, p14^{ARF}/p16^{INK4a} expression was also described to be a biomarker of age in rodents and in human skin.^{23,24} The influence of age in p16-based cervical cancer screening has not been described yet, and may help to improve data interpretation.

We compared normal HPV-negative patients ($n=48$) with high-risk HPV-positive patients referred as HSIL ($n=45$) with regard to their p14^{ARF} and p16^{INK4a} expression. We used published SYBR-Green I assays and related the expression to the housekeeping gene *ACTB*. As expected, we found that relative p16^{INK4a} and p14^{ARF} transcript frequencies increased significantly in samples of patients with HSIL diagnoses. We also found a significant correlation between the patient age and the increased expression of p16^{INK4a} and p14^{ARF} that interfered with HPV-induced upregulation.

Overall, p16^{INK4a} expression was higher than the p14^{ARF} expression in HSIL patients. In women with equal diagnosis, both transcripts were significantly upregulated in older patients compared with younger patients (see Table 3). This age-dependent upregulation can strongly influence the HPV oncogene-related upregulation of p14^{ARF} and p16^{INK4a}. For p16^{INK4a}, the comparison between younger normal women with older HSIL patients would result in an upregulation of 17.12-fold. In turn, older normal women compared with younger HSIL would result in 2.34-fold higher expression only. Therefore, age induces a bias concerning p14^{ARF} and p16^{INK4a} expression.

Patients in the HSIL group were younger compared with the normal group, because of the higher HPV prevalence in young women. Although the age difference of normal women was wider compared with patients with HSIL diagnosis, the age-related upregulation was in the same range.

The quantification of mRNA using real-time PCR has the advantage to be independent from subjective visual sample assessment, as well as being high-throughput compatible. However, the use of mRNA from exfoliated cervical cells is challenging, because cervical scrapes consist of cervical mucus, cellular debris and mRNA is mostly degraded. This critical

fact affords small PCR amplicons, good storage solutions and a rapid sample processing. We tested the RNA integrity number in a subset of patients and found mRNA from cervical smears to be highly degraded. Indeed, some samples may be not suitable for mRNA-based analysis. A total of 11 and 15 out of 45 HSIL patients did not reach sufficient p16^{INK4a} and p14^{ARF} levels, respectively. This may be due to insufficient RNA quality. To investigate whether degradation occurred *in situ* or during storage and extraction, we validated our sampling and extraction procedure by simultaneous treatment of cervical cell lines. The high RNA integrity numbers in the cell lines verified our appropriate extraction and storage procedure. We suggest that beside RNA quality, it may be advantageous to extract the relevant material to reduce the ratio of healthy to dysplastic tissue. Our samples were taken under colposcopic control. This may have enhanced the quality of sampling. Tumor cell enrichment through techniques such as laser capture microdissection was not investigated in this study, as the design aimed to analyze the feasibility of CDKN2A/B mRNA analysis in a standard clinical setting. Our results warrant further analysis of mRNA expression of p16^{INK4a} and p14^{ARF} from cervical smears.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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