Mitosis Targets as Biomarkers in Cervical Cancer

22

Jaime Berumen, Ana María Espinosa, Ingrid Medina, and Mariano Guardado

Contents

Abstract

The effectiveness of a preventive human papillomavirus (HPV) vaccination for reducing the cervical cancer (CC) burden will likely not be known for 30 years. Current screening methods for detecting high-grade cervical intraepithelial neoplasias (CIN2/3) and CC (CIN2+) have low sensitivity (Pap test) or low specificity (HPV tests). Improved procedures for CC screening and treatment are therefore required. Based on comparisons with healthy cervical epithelium,

J. Berumen (✉)
Departamento de Medicina Experimental, Facultad de Medicina, Universidad Nacional Autonoma de Mexico, Mexico city, Mexico

Unidad de Medicina Genomica, Hospital General de Mexico, Mexico city, Mexico
e-mail: jaimeberumen@hotmail.com; jaimeberumen@yahoo.com.mx

A.M. Espinosa • I. Medina • M. Guardado
Unidad de Medicina Genomica, Hospital General de Mexico, Mexico city, Mexico
e-mail: anaesga@hotmail.com; medmarin@unam.com; jos_mariano@hotmail.com

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the genes most upregulated and enriched in CC are those involved in mitosis. Some of these upregulated genes might be good candidates for CC screening or survival markers or as potential therapeutic targets. In this chapter, we analyze the benefits and limitations of current methods used for early CC detection, the evidence that demonstrates that the most enriched genes in CC are those involved in mitosis, the mechanism that regulates mitosis and its relationship with HPV, and our experimental evidence suggesting that some mitosis genes might be good markers for screening and survival in CC. In addition, we discuss the need to develop less expensive and more efficient methods that can be automated for large-scale application in poor and developing countries. We also discuss the potential use of the markers for other types of cancers and as potential therapeutic targets.

List of Abbreviations

APC/C	Anaphase-Promoting Complex
AUC	Area Under the Curve
CC	Cervical Cancer
Cdks	Cyclin-Dependent Kinases
CIN	Cervical Intraepithelial Neoplasia
DNA	Deoxyribonucleic Acid
DSB	Double-Strand Breaks
ELISA	Enzyme-Linked Immunosorbent Assay
FDA	US Food and Drug Administration
FIGO	International Federation of Gynecology and Obstetrics
H&E	Hematoxylin and Eosin Stain
HC2	Hybrid Capture 2 Technology
HPV	Human Papillomavirus
HR	Hazard Ratio
IH	Immunohistochemistry
mRNA	Messenger Ribonucleic Acid
NPV	Negative Predictive Value
Pap	Papanicolaou Test
PPV	Positive Predictive Value
qRT-PCR	Quantitative Reverse-Transcription PCR
RMA	Robust Multi-array Average
ROC	Receiver-Operating Characteristic

Key Facts of Microarray Technology

- A microarray consists of an orderly arrangement of small probes of genetic material that are chemically synthesized on a solid surface. The precise location where each probe is synthesized is called a cell, and a microarray contains thousands of cells. A microarray experiment can be used to explore thousands of genes simultaneously.

- There are different types of microarrays. DNA microarrays are used to explore single-nucleotide polymorphisms (SNPs). RNA microarrays are used to explore gene expression. There are also tissue microarrays for the detection of proteins by immunohistochemistry and immunofluorescence.
- Some applications of microarrays include the identification of differential gene expression, splicing variants, changes in the methylation patterns, germ line and somatic mutations, genotype, and changes in gene copy number.
- Microarray technology is based on hybridization of nucleic acids. In the case of expression microarrays, the experiment starts with the total RNA to be explored. It is then converted to cDNA, transcribed in vitro, fragmented, and labeled. Subsequently, the microarray is hybridized with the labeled material for 16 h at a constant temperature of 45 °C, followed by washing and staining. The intensity signal of each probe is then read with a special fluorescent reader.
- In order to eliminate experimental biases, microarray expression signals are normalized prior to conducting statistical analysis. One of the most commonly used algorithms to accomplish this, is the robust multi-array average (RMA), which is based on equalizing the median signal intensity of each microarray. The accuracy of the algorithm increases with the number of analyzed microarrays.
- One of the objectives of an expression microarray is the identification of genes that are differentially expressed between an experimental condition and a control group. Such genes may be used as biomarkers for screening, diagnosis, prognosis, and treatment decisions.
- Validation of microarray experiments is indispensable. The predominant technology used to validate expression microarrays is quantitative reverse-transcription PCR.

Definitions of Words and Terms

Anaphase Bridges Chromatin fibers that connect two separated chromosomes resulting from the presence of dicentric chromosomes that are formed by the fusion of two telomere-deficient fragments. This phenomenon occurs commonly in cancer because of the lack of ligation of DNA double-strand breaks.

Aneuploidy Structural alterations of the genome characterized by an abnormal number of chromosomes.

Area Under the Curve (AUC) The area under the curve of a receiver-operating characteristic (ROC) graph, which is designed to select the best signal cutoff value for detecting the greatest number of true positive samples (cases, sensitivity) and the lowest number of true negative samples (controls, specificity). The higher the value of the AUC, the higher the discriminative power of the algorithm.

Cervical Intraepithelial Neoplasia (CIN) Noninvasive cervical epithelial lesions characterized by abnormal growth and neoplastic changes in cell morphology.

CINs are classified by the fraction of affected epithelium as measured from the basal to the apical side of the epithelium, CIN1, CIN2, and CIN3 representing 1/3, 2/3, or the full cervical epithelium, respectively.

FIGO Stage A clinical classification system of tumors based on tumor characteristics such as size, degree of invasion, spread to lymph nodes, and metastasis. FIGO was established by the International Federation of Gynecology and Obstetrics.

Hematoxylin and Eosin Stain (H&E) A histological stain that combines a basic (hematoxylin) and an acid (eosin) dye that enables the identification of cellular structures with a light microscope. The stain is used commonly in histologic and pathologic analyses because the procedure is rapidly performed and inexpensive.

Hybrid Capture 2 Technology (HC2) A DNA hybridization technique performed in a liquid solution to detect human papillomavirus DNA obtained from cervical samples, such as exudates and tumor biopsies. It is useful as an initial screening methodology.

Negative Predictive Value (NPV) The probability of being free of the disease if the result of the diagnostic test is negative.

Positive Predictive Value (PPV) The probability of having the disease if the result of the diagnostic test is positive.

Potential Therapeutic Target A deregulated gene that has a high power of discrimination between cancer and control samples and that is extremely important for cancer development. Blocking such a gene might delay or prevent cancer cell proliferation or tumor growth.

Quantitative Reverse Transcription PCR (qRT-PCR) A quantitative DNA technology based on amplification of genomic material by the polymerase chain reaction (PCR) that is used to assess the level of gene expression by measuring the amplified product in each growth cycle with fluorescently labeled probes.

Introduction

The human papillomavirus (HPV) is the main causal factor in the development of invasive cervical cancer (CC), being found in nearly 100 % of these tumors (Schiffman et al. 2011). CC develops through the progression of preinvasive cervical intraepithelial neoplasia (CIN), which is histologically graded as mild (CIN1), moderate (CIN2), or severe (CIN3) dysplasia. CC develops predominately from CIN3 and CIN2 but rarely from CIN1, with estimated progression rates from these lesions to CC of 12 %, 5 %, and 1 %, respectively (Ostor 1993).

Currently, there are vaccines on the market that prevent infection by oncogenic HPV types 16 and 18, which are associated with 65–70 % of CCs worldwide (de Sanjose et al. 2010). These vaccines are highly efficient at preventing infection and high-grade CINs (CIN2/CIN3) (Lehtinen et al. 2012). However, vaccinated women must continue to participate in early detection programs for CC because the vaccines can only protect against certain virus types, and it is not known for how long the immune protection against the targeted virus remains (Romanowski 2011). In many countries, preventive vaccines for HPV16 and HPV18 have been incorporated into a national vaccination program for girls from 9 to 12 years of age (Cuzick 2010; Markowitz et al. 2007). However, because the peak incidence of CC occurs in women 45–50 years old, the effectiveness of these preventive vaccination programs on reducing the incidence of CC will not be known for 30 years. Therefore, it is necessary to improve the procedures for CC screening and treatment. Because each year 530,000 new CC cases and 275,000 CC deaths are reported worldwide, the mortality-to-incidence ratio is approximately 50 % (Hwang and Shroyer 2012; Ferlay et al. 2008).

For many years, the Papanicolaou (Pap) test has been the most important screening procedure for early detection of CC, and its massive application in developed countries has decreased the incidence of CC by more than 50 % in the last 40 years (McCrory et al. 2009). Women with abnormal Pap test results are referred for colposcopy to confirm, reject, or clarify the diagnosis with histopathological analysis. Unfortunately, the average sensitivity of cytology for detection of CIN lesions is only 50–60 %, although the specificity is very high at approximately 90 % (Wright 2007). Since HPV is essential for the development of CC, several procedures to detect the HPV genome have been incorporated into CC screening. Hybrid Capture 2 technology (HC2) is the methodology most frequently used for screening, particularly for measuring high-risk virus. This method, approved by the Food and Drug Administration (FDA) in the United States, demonstrates higher sensitivity but lower specificity than conventional cytology for detecting CIN2 lesions or higher (CIN2+). HC2 has an average sensitivity of 95 % (range, 62–98 %) for detecting high-grade lesions and invasive cancer. However, HC2 has low specificity for CIN2+, especially in young women, because the majority of detected infections are not associated with neoplastic lesions (Leinonen et al. 2009; Whitlock et al. 2011). In women over the age of 30 years, the specificity is higher, but it varies among studies because of its partial dependence on the prevalence of HPV in the study population (Giorgi-Rossi et al. 2012). In most studies, the positive predictive value (PPV) is less than 20 %, which is consistent with the percentage of infected women having high-grade lesions. The high sensitivity and high negative predictive value (NPV) of HPV DNA tests for the detection of CIN2+ lesions suggest that this test could be used to extend screening intervals. However, the low specificity of HPV DNA tests would increase the number of follow-up tests and colposcopy referrals, thereby increasing the cost of screening (Leinonen et al. 2009).

Therefore, there is an obvious need to develop new methods for early detection of CC with high sensitivity and specificity. Multiple tumor markers associated with

CIN2+ have been identified, in particular *CDKN2A*, *TOP2A*, and *MCM2*. However, these markers have been proposed not for screening but for diagnosis, prognosis, or clinical management (Natunen et al. 2011). Although several studies have used microarrays to identify genes associated with cervical cancer (Narayan et al. 2007; Gius et al. 2007; Zhai et al. 2007; Pyeon et al. 2007; Biewenga et al. 2008; Buitrago-Pérez et al. 2009), most have been insufficient for identifying screening markers because they employed an insufficient design, such as heterogeneous samples positive for different or undetermined HPV types and/or a small number of tumors and controls.

It is important to emphasize that the primary value of CC biomarkers and the goal of developing procedures for cervical screening are to improve the specificity rather than the sensitivity relative to HPV testing (Hwang and Shroyer 2012). Primary HPV DNA screening with cytology triage increases the specificity to an extent similar to that of conventional cytology (Markowitz et al. 2007; Leinonen et al. 2009). However, use of this procedure in developing countries creates logistical problems because a high percentage of women who test positive for HPV do not return for a cytological follow-up or because of the handling difficulties associated with a sample taken for cytology at the first visit. In addition, the procedure appears to be impractical because it cannot be automated. The simultaneous use of HC2 for high-risk viruses with a molecular method that can distinguish CIN2+ from CIN1− would increase the specificity and PPV and would provide the advantages of speed and automation potential compared to triaged cytology.

Use of p16 for Clinical Diagnosis but not for Screening Cervical Cancer

Of the markers associated with CC, the tumor suppressor protein p16 has been the most widely studied (Hwang and Shroyer 2012). This protein accumulates in the nucleus and cytoplasm of cells transformed by high-risk HPVs and is usually detected by immunohistochemistry (IH). The amount of p16 is related to the severity of cervical neoplasia and is considered to be a marker of CIN2+. p16 has been used successfully to classify HPV-related diseases. Lower interobserver variations have been reported for IH detection of p16 in punch and cone biopsies than for diagnosis with hematoxylin and eosin (H&E)-stained sections. Recently, p16 has emerged as a sensitive and specific diagnostic tool to detect CIN2+ lesions in cervical cytology specimens (Hwang and Shroyer 2012). p16 consistently exhibits high sensitivity (80–95 %) for detection of CIN2+, but its specificity is lower (ca. 50 %) than that of cytology (Tsoumpou et al. 2009). The low specificity is because p16 is expressed in approximately 38 % of low-grade CIN lesions, i.e., those infected with high-risk HPV types (Tsoumpou et al. 2009). The relatively low specificity and the need for a pathologist to interpret the IH results are the major reasons contributing to the fact that p16 has not been widely adopted for primary screening. Recently, Wentzensen et al. developed enzyme-linked immunosorbent

assay (ELISA) method to detect p16 protein in cell lysates of cervix exudates, which provides a sensitivity and specificity for the identification of high-risk lesions of 84–87 %, respectively (Wentzensen et al. 2006).

Mitosis is the Main Phase of the Cell Cycle Altered in Cervical Cancer

The cell cycle is the primary process altered in CC; it is ranked highest in all published CC studies that have analyzed biological processes (Buitrago-Pérez et al. 2009). Similarly, in two studies of CC, in which we analyzed the expression of 8,638 genes with the HG-Focus microarray (Espinosa et al. 2013) or 21,034 genes with the HG 1.0 ST microarray from Affymetrix, the cell cycle process was the most enriched and appeared at the top of the lists of gene datasets that were analyzed with the Database for Annotation, Visualization and Integrated Discovery (DAVID) tool at medium stringency (Table 1). However, in both datasets, the M-phase processes were the most enriched (labeled in red in Table 1) when the analyses were conducted at high stringency, suggesting that the M phase is the main altered cell cycle phase in CC. According to the IH data, approximately 30 % of tumor cells in CC might be in the M phase (Espinosa et al. 2013). These findings are consistent with the in vitro alterations in the cell cycle and mitosis caused by HPV (Teissier et al. 2007; Moody and Laimins 2010), and they correlate with other CC studies (Teissier et al. 2007). The E6 and E7 oncoproteins of high-risk HPVs induce numerous mitotic defects, including multipolar mitoses, chromosomal missegregation, anaphase bridges, and aneuploidy. Although cells with abnormal mitosis are normally targeted for cell death, E6 and E7 act cooperatively to allow cells with abnormal centrosomes to accumulate by relaxing the G2/M checkpoint response and inhibiting apoptotic signaling (Moody and Laimins 2010). In agreement with these data, the canonical pathways G2/M DNA damage checkpoint regulation and role of CHK proteins in cell cycle checkpoint control were ranked at the second and fifth positions, respectively, of the altered canonical pathways in CC (Espinosa et al. 2013). Furthermore, E6 and E7 induce mechanisms that also avoid the mitosis checkpoint. The E6 and E7 genes have been shown to induce overexpression of CDC20 and UBCH10, which activates the APC/C ubiquitin ligase complex (Patel and McCance 2010). The finding of the enrichment of positive regulation of ubiquitin–protein ligase activity during the mitotic cell cycle in CC is consistent with the in vitro results (Espinosa et al. 2013).

A total of 128 genes of the mitosis phase were identified in two series of CCs studied in Mexico: 72 with HG-Focus microarray, 114 with HG 1.0 ST microarray, and 58 genes shared with both. The non-supervised hierarchical clustering performed with both sets of gene expression values clearly separated the cancer samples from those of the control group (Fig. 1). Interestingly, all but four genes involved in mitosis were upregulated (Fig. 2). Eleven of those genes (*CCNB2, CDK1, CDC20, CDKN3, CKS2, MKI67, NUSAP1, PRC1, SMC4, SYCP2,* and *ZWINT*), together with some genes used previously as markers for CC (*CDKN2A,*

Table 1 DAVID functional annotation cluster analysis at medium stringency of genes deregulated in cervical cancer. The table shows the 10 most enriched biological processes in the set of deregulated genes in cervical cancer obtained both with HG-Focus (panel A) and HG 1.0 ST (panel B) microarrays and analyzed with the DAVID tool

Cluster	Enrichment score[a]	Biological process	No. of genes	*p*-value	Fold change[b]
A. Deregulated genes in 43 CC identified with HG-Focus ($n = 997$)					
1	19.92	Cell cycle	140	2.1E-30	2.8
		M phase	72	2.0E-20	3.4
		Mitosis (1)	51	1.4E-15	3.6
2	11.59	DNA metabolic process	99	4.3E-24	3.1
		DNA repair	51	4.0E-11	2.8
3	9.53	Regulation of ubiquitin–protein ligase activity during mitotic cell cycle (2)	30	6.3E-17	6.6
		Anaphase-promoting complex-dependent proteasomal ubiquitin-dependent protein catabolic process	28	4.5E-16	6.8
4	7.97	Response to chemical stimulus	150	9.8E-14	1.8
5	7.26	Spindle organization	21	6.8E-13	7.3
		Cytoskeleton organization	57	3.7E-07	2.1
6	5.53	Developmental process	268	5.6E-08	1.3
		Cell differentiation	129	7.1E-03	1.2
7	5.5	Negative regulation of nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process	51	1.6E-03	1.6
8	5.38	Interphase	24	1.7E-07	3.6
		G1/S transition of mitotic cell cycle	13	1.7E-04	3.6
9	5.23	Embryonic development	66	2.7E-06	1.8
10	4.89	Regulation of cell cycle	61	1.1E-13	2.9
		Regulation of mitosis	13	1.7E-04	3.6
B. Deregulated genes in 48 CC identified with HG 1.0 ST ($n = 1{,}812$)					
1	29.47	Cell cycle	200	4.3E-41	2.7
		M phase	114	8.5E-36	3.6
		Mitosis (1)	83	4.9E-29	3.9
2	9.19	DNA metabolic process	112	1.9E-17	2.3
		DNA repair	63	4.4E-10	2.3
3	8.06	Chromosome segregation	36	2.0E-15	4.6
		Mitotic sister chromatid segregation	19	7.3E-10	5.5
4	7.62	Microtubule cytoskeleton organization	39	9.2E-09	2.8
		Cytoskeleton organization	54	3.6E-08	2.2

(*continued*)

Table 1 (continued)

Cluster	Enrichment score[a]	Biological process	No. of genes	*p*-value	Fold change[b]
5	6.87	DNA packaging	45	2.3E-16	4.0
		Chromosome organization	92	2.4E-10	2.0
6	6.74	Meiosis	31	4.9E-09	3.3
		Meiotic cell cycle	31	8.3E-09	3.2
7	5.31	Developmental process	387	1.3E-08	1.3
		Cell differentiation	196	5.9E-04	1.2
8	4.73	Response to chemical stimulus	172	3.1E-06	1.4
9	4.17	Regulation of cell cycle	69	1.0E-09	2.2
		Regulation of mitosis	16	1.9E-04	3.0
10	3.94	Angiogenesis	31	6.1E-05	2.2

[a]Enrichment score is the -log10 of the average *p*-value of the terms in the cluster. [b]Fold change is the ratio of the proportion of genes in the tested list versus the Human Gene Reference database. The clusters in red were enriched in a functional annotation cluster analysis at highest stringency

MCM2, *TOP2A*, and *PCNA*), were validated with quantitative reverse-transcription polymerase chain reaction (qRT-PCR) mostly in HPV16-positive CC and healthy cervical epitheliums (Espinosa et al. 2013). The box plots (Fig. 3) clearly show the difference in gene expression between the cancer and control groups ($p < 1 \times 10^{-15}$ for all genes, Mann–Whitney *U* test).

The mitosis genes identified in both series of tumors participate in several of the subprocesses of mitosis, primarily formation and control of mitotic use, regulation of the metaphase-to-anaphase transition, chromosome segregation, cytokinesis, and mitotic entrance/exit (Fig. 4). While activation of cyclin-dependent kinases (Cdks) drives cells into mitosis, mitotic exit depends on inhibition of Cdk activity and dephosphorylation of proteins phosphorylated by Cdks. The activity of Cdks is inhibited primarily through degradation of mitotic cyclins by the anaphase-promoting complex (APC/C) and accumulation of Cdk inhibitor proteins. Five (CDK1, CCNB2, CDC20, CDKN3, and PRC1) of the eleven mitosis proteins validated in a previous paper (Espinosa et al. 2013) appear to be essential in these processes. Cyclin B2 (CCNB2) and cyclin B1 (CCNB1) bind to CDK1 (CDC2) to form the complex M-CDK, which is essential for control of the cell cycle at the G2/M transition. However, while cyclin B1-CDK1 causes chromosome condensation, reorganizes microtubules, and disassembles the nuclear lamina and the Golgi apparatus, cyclin B2-CDK1 is restricted to the cytoplasm and disassembles the Golgi apparatus during mitosis (Gong and Ferrell 2010). Consistent with these data, cyclin B2 was localized exclusively in the cytoplasm of the CCs examined in our study (Espinosa et al. 2013). Interestingly, the expression of cyclin B1 in these tumors did not differ from that in the control samples. Degradation of cyclin B1 by APC/C, a key regulator of the metaphase-to-anaphase transition, allows progression of mitosis from metaphase to anaphase (Gong and Ferrell 2010). While *CCNB2* has been scantily associated with CC (Buitrago-Pérez et al. 2009), it has also been

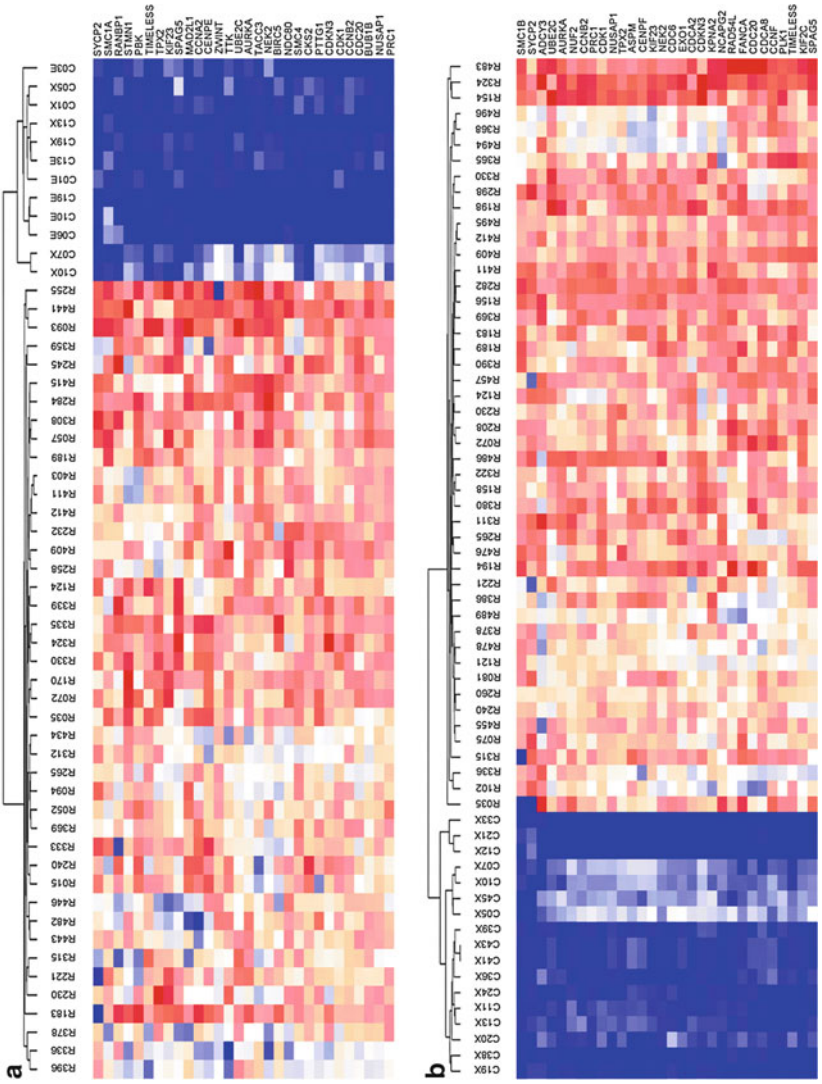

Fig. 1 (continued)

reported to be associated with other types of cancer. For example, it is upregulated in cancers of the colon (Park et al. 2007), lung, and digestive tract (Mo et al. 2010).

An increased amount of CDC20, a key regulatory protein of the APC/C complex during anaphase, could explain the absence of cyclin B1. UBE2C (also known as UBCH10) was also found to be increased in CC (Espinosa et al. 2013). Both CDC20 and UBE2C are required for full ubiquitin ligase activity of the APC/C complex and may confer substrate specificity upon the complex. CDC20 is negatively regulated by MAD2L1 and BUB1B (also known as BUBR1). In metaphase, the MAD2L1-CDC20-APC/C ternary complex is inactive, whereas in anaphase, the CDC20-APC/C binary complex is active in degrading substrates. Interestingly, the MAD2L1 and BUB1B transcripts were also increased in CC (Espinosa et al. 2013), suggesting that the corresponding proteins could be increased, thereby preventing activation of APC/C. However, part of the CDC20 protein could remain free to bind and activate APC/C, as has been shown in transfected cells expressing E6 and E7 proteins (Patel and McCance 2010). Upregulated CDC20 has been found in lung, pancreatic, and gastric cancers (Nakayama and Nakayama 2006), as well as in CC (Teissier et al. 2007).

CDKN3 is a dual-specificity protein phosphatase of the Cdc14 phosphatase group that interacts with CDK1 (CDC2) and inhibits its activity (Demetrick et al. 1995). CDKN3 and other Cdc14 phosphatases have not been well studied, but they appear to be essential for antagonizing Cdk activity in late mitosis, allowing cells to exit mitosis and enter telophase. Regulation of cytokinesis may be the one conserved function of the Cdc14 phosphatases. Although overexpression of CDKN3 has been associated with inhibition of cell proliferation in colon cancer cell lines (Galamb et al. 2010), it has been found to be overexpressed in breast, prostate, and lung cancers (MacDermed et al. 2010; Julien et al. 2011; Taylor et al. 2010). In the previous report of the association of *CDKN3* with cervical cancer, *CDKN3* was shown to behave as an oncogene (Espinosa et al. 2013). However, it has been proposed that CDKN3 is a tumor suppressor of mitosis control (Nalepa et al. 2013).

PRC1 is essential for control of the spatiotemporal formation of the midzone and successful cytokinesis (Subramanian et al. 2010). It is required for kinesin family member 14 (KIF14) (Gruneberg et al. 2006) and polo-like kinase 1 (PLK1) (Lens et al. 2010) localization to the central spindle and midbody. Suppression of PRC1 blocks cell division. Transcription of *PRC1* is repressed by p53 and is one of the routes by which p53 stops the cell cycle at the G2/M checkpoint (Li et al. 2004).

Fig. 1 Segregation of tumor and control samples according to the mitosis gene expression profile. Unsupervised hierarchical cluster analysis using the expression values of the top 30 genes deregulated from mitosis as identified with either the HG-Focus (**panel a**: 43 CCs and 12 healthy cervical epitheliums) or the HG 1.0 ST (**panel b**: 48 CCs and 17 healthy cervical epitheliums) microarray. Each row represents a gene, and each column represents a sample. The length and the subdivision of the branches represent relationships among the samples based on the intensity of gene expression. The cluster is color-coded using *red* for upregulation, *blue* for downregulation, and *white* for unchanged expression

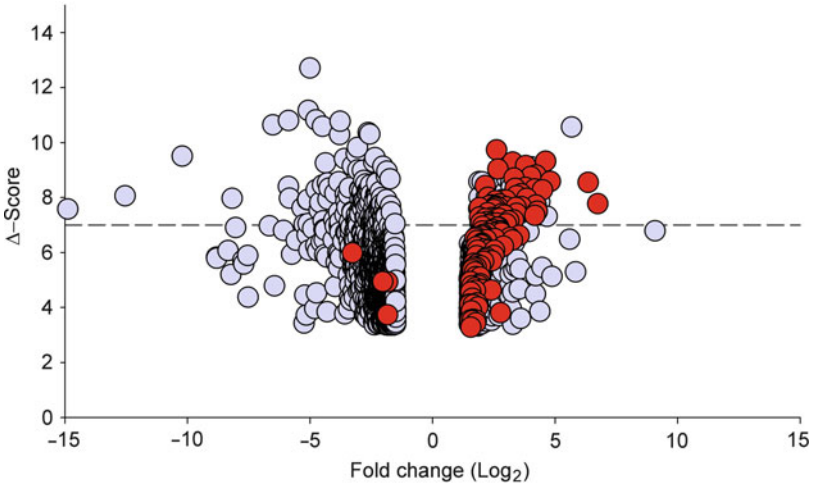

Fig. 2 Distribution of deregulated genes involved in mitosis according to fold change (FC) and Δ-score. The 1812 deregulated genes (circles) found in tumors of cervical cancer with the HG 1.0 ST microarray are shown in a volcano plot. The x-axis represents the FC of gene expression (tumor/control) expressed in $\log_2$ values, and the y-axis shows the absolute value of the Δ-score, which is equivalent to the t value in a student's t-test, calculated by the significance analysis of microarrays (SAM) method. A higher value of the Δ-score correlates with greater statistical significance. Log_2 FC values were positive in overexpressed genes and negative in underexpressed genes. The *black dashed lines* show the cutoff point for the most significant genes. The 128 genes of mitosis identified with HG-Focus and HG 1.0 ST microarrays are shown as *red circles*. The *gray circles* in the background represent deregulated genes not involved in mitosis

Because the E6 oncoprotein of HPV16 induces degradation of p53 in proteasomes, it is likely that in cervical carcinomas PRC1 is overexpressed via this mechanism. PRC1 has been reported to be associated with liver cancer (Wang et al. 2011) and CC (Zhai et al. 2007).

NUSAP1 is a nucleolar-spindle-associated protein that plays a role in spindle microtubule organization. The gene for NUSAP1 has not been associated with CC but has been found to be upregulated in breast cancer and melanoma (Kretschmer et al. 2011).

SYCP2 is a major component of the synaptonemal complex, which promotes the repair of double-strand breaks (DSBs) by the homologous recombination pathway in meiosis (Li et al. 2011). The high levels of *SYCP2* expression in the CCs examined in our previous work (Espinosa et al. 2013) suggest that DSBs are very common in some CC samples and that SYCP2 might be involved in DSB repair via the stimulation of the homologous recombination pathway. Interestingly, *SYCP2* has been found to be upregulated in CC (Buitrago-Pérez et al. 2009) and oropharyngeal squamous cell carcinomas positive for HPV16 but not in HPV-negative carcinomas (Martinez et al. 2006).

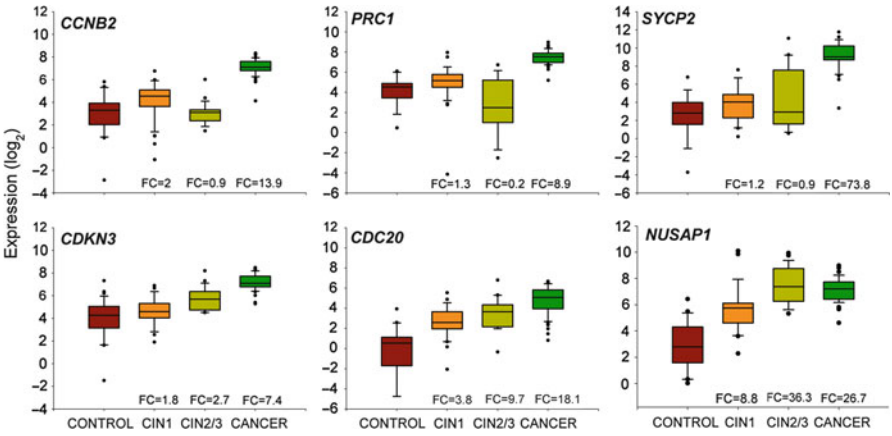

Fig. 3 Validation of gene expression of six genetic markers by qRT-PCR. The intensity of gene expression, expressed in $\log_2$ values, is shown in box plots. Expression of the six genes (*CCNB2*, *PRC1*, *SYCP2*, *CDKN3*, *CDC20*, and *NUSAP1*) is compared among four groups: healthy cervical epitheliums (normal, $n = 25$), low-grade CIN (CIN1, $n = 29$), high-grade CIN (CIN2/3, $n = 21$), and invasive CC (cancer, $n = 44$). The upper and lower boundaries of the boxes represent the 75th and 25th percentiles, respectively. The *black line* within the box represents the median value, and the whiskers represent the minimum and maximum values that lie within 1.5× the interquartile range from the end of box. Values outside this range are represented by *black circles*. The fold change (FC) in expression was calculated by dividing the median of each pathological group by the median of the control group

Mitosis as a Source of Targets for Screening and Survival in Cervical Cancer

The genes involved in mitosis were not only the most enriched in CC but also the most different in terms of expression fold change and Δ-score (statistical significance) when compared with control samples (red circles in Fig. 2). Therefore, these genes are good candidates to be tested as markers for screening and survival or as potential therapeutic targets. Furthermore, several of the 15 markers validated in our previous study either have not been identified previously (*CDKN3, NUSAP1*, and *SMC4*) or have been identified rarely in other studies (*CCNB2, CDC20, CKS2, PRC1*, and *SYCP2*). By contrast, genes not associated with mitosis have been identified in many studies (*CDKN2A, MCM2, TOP2A*, and *PCNA*) (Buitrago-Pérez et al. 2009).

Markers for Cervical Cancer Screening

In order to establish the potential value of these genes as markers in CC by defining a line of separation between cancer and control group signals, cutoff values were established by analyzing receiver-operating characteristic (ROC) curves.

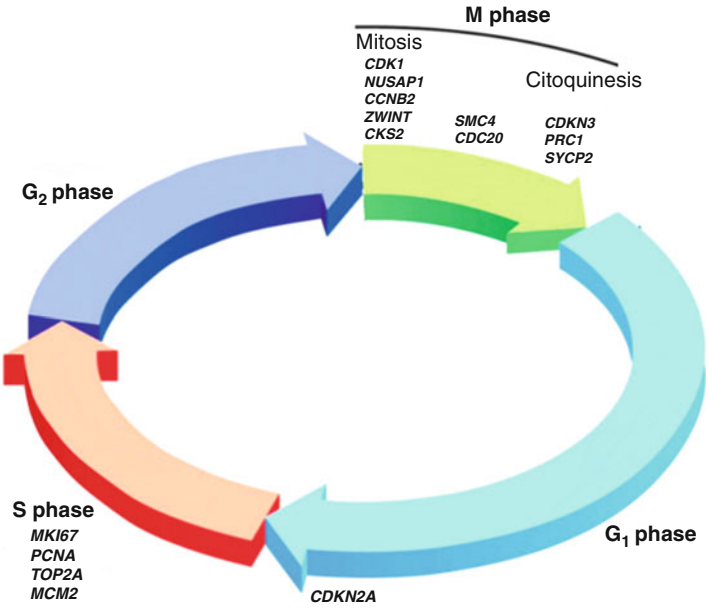

Fig. 4 Localization of the 15 genes described in this chapter in the cell cycle phases. The cell cycle phases and the genes altered in cervical cancer described in this chapter. All but four genes (*TOP2A, PCNA, MCM2*, and *CDKN2A*) participate in mitosis

In general, ROC curves with an area under the curve (AUC) ≤ 0.75 are not clinically useful, whereas an AUC of 0.97 has very high clinical value (Fan et al. 2006). The AUC for 10 genes (*CDKN2A*, *MKI67*, *PRC1*, *CDC2*, *CCNB2*, *SYCP2*, *PCNA*, *NUSAP1*, *CDC20*, and *CDKN3*) was ≥ 0.97 (Table 2). In fact, most of these genes had a sensitivity and specificity greater than 95 %, suggesting that they might be good screening markers that could distinguish healthy samples from invasive cancers. Interestingly, this subset included six genes involved in mitosis that had not been reported to be associated with CC (*NUSAP1* and *CDKN3*) or that had been only rarely reported to be associated with CC (*PRC1*, *SYCP2*, *CCNB2*, and *CDC20*).

For screening tests, it is important to detect not only CC but also high-grade lesions (CIN2/3) and to distinguish them from low-grade CIN lesions (CIN1) and healthy controls. To investigate whether these genes can differentiate CIN2+ from CIN1−, their expression was analyzed in two additional groups of samples: 29 low-grade CINs and 21 high-grade CINs (Espinosa et al. 2013). According to the median and distribution of the data shown in the box plots of Fig. 3, the six markers could be classified into two groups. The first group included markers linked exclusively (*CCNB2* and *PRC1*) or mostly (*SYCP2*) to invasion, which clearly differentiated invasive tumors from high-grade CIN, low-grade CIN, and normal cervices. The specificity for detecting only CC, and no other lesions, ranged from 0.85 (*SYCP2*) to 0.98 (*CCNB2*). The second group included the other three genes

Table 2 ROC analysis and calculus of sensitivity, specificity, and predictive values. The table shows calculations of predictive values, specificity, and sensitivity for 15 genes validated in cervical tumors and healthy controls. Eleven of the genes are involved in mitosis

Genes	AUC	Cutoff value[a]	Controls ($n = 25$)		Cervical cancer ($n = 44$)		p-value[b]	Sensitivity	Specificity	PPV	NPV	Youden index[c]
			FPF	TNF	TPF	FNF						
CDKN2A	0.996	18	0	25	42	2	$<1 \times 10^{-10}$	0.95	1	100	92.6	0.95
CCNB2	0.995	58	0	25	43	1	$<1 \times 10^{-10}$	0.98	1	100	96.2	0.98
MKI67	0.995	79	0	25	43	1	$<1 \times 10^{-10}$	0.98	1	100	96.2	0.98
PRC1	0.995	80	0	25	43	1	$<1 \times 10^{-10}$	0.98	1	100	96.2	0.98
CDK1	0.995	85	0	25	42	2	$<1 \times 10^{-10}$	0.95	1	100	92.6	0.95
SYCP2	0.992	115	0	25	42	2	$<1 \times 10^{-10}$	0.95	1	100	92.6	0.95
NUSAP1	0.99	48	1	24	43	1	$<1 \times 10^{-10}$	0.98	0.96	97.7	96	0.94
PCNA	0.99	100	0	25	42	2	$<1 \times 10^{-10}$	0.95	1	100	92.6	0.95
CDC20	0.971	3	3	22	42	2	$<1 \times 10^{-10}$	0.95	0.88	93.3	91.7	0.83
CDKN3	0.97	83	1	24	41	3	$<1 \times 10^{-10}$	0.93	0.96	97.6	88.9	0.89
SMC4	0.96	431	1	24	40	4	$<1 \times 10^{-10}$	0.91	0.96	97.6	85.7	0.87
TOP2A	0.866	128	5	20	43	1	$<1 \times 10^{-10}$	0.98	0.8	89.6	95.2	0.78
MCM2	0.846	121	4	21	40	4	2.5×10^{-9}	0.91	0.84	90.9	84	0.75
ZWINT	0.827	59	7	18	39	5	1.1×10^{-6}	0.89	0.72	84.8	78.3	0.61
CKS2	0.815	239	5	20	35	9	5×10^{-6}	0.8	0.8	87.5	69	0.6

AUC area under the curve, *FPF* false-positive fraction, *TNF* true-negative fraction, *TPF* true-positive fraction, *FNF* false-negative fraction, *PPV* positive predictive value, *NPV* negative predictive value

[a]Optimal cutoff values (ng/ml) were selected according to the ROC analysis

[b]Chi-square test

[c]J= sensitivity - specificity - 1

(*CDC20*, *NUSAP1*, and *CDKN3*), the expression of which tended to increase from the control to the CC group (*CDC20* and *CDKN3*) or the high-grade CIN group (*NUSAP1*). These three genes could distinguish CIN2+ lesions from CIN1− lesions ($p < 1 \times 10^{-15}$, Mann–Whitney *U* test [MW]). It is clear that genes in the first group would not be good markers for screening since they could not distinguish high-grade CIN and CC lesions from low-grade CIN lesions and control samples. Therefore, ROC analysis was performed to explore the potential of the genes in the second group (*CDC20*, *CDKN3*, and *NUSAP1*) together with *CDKN2A* as markers for screening. None of them had AUC values equal to or greater than 0.97; the highest AUC value was obtained with *CDKN2A* (0.92), followed by *NUSAP1* (0.917), *CDKN3* (0.91), and *CDC20* (0.86). However, the new markers (*NUSAP1* and *CDKN3*) showed slightly greater sensitivity than *CDKN2A* but lower specificity (Fig. 5). The sensitivity and specificity did not increase significantly when data for the individual markers were combined (Espinosa et al. 2013).

NUSAP1, CDKN3, or CDKN2A might be good candidates to use with HC2 as a first-line strategy in a screening program. The goal of our previous study (Espinosa et al. 2013) was to perform a feasibility evaluation of mRNA levels of novel genes in cervical samples as diagnostic markers to identify high-grade CIN or invasive lesions with high sensitivity and specificity. However, the sensitivities reported in that analysis were probably greater than those likely to be found in clinical practice because patients with CIN2+ have a higher proportion of cervical cancer (which is easy to identify) than would be expected in any screening setting. By contrast, the specificity appears to be underestimated, given that a large proportion of CIN1− had CIN1. Therefore, we did not expect to obtain conclusive data on the sensitivity, specificity, or predictive values of the assays. Additional studies of a screening population are needed to determine the levels of *CDKN3*, *NUSAP1*, or *CDC20* mRNA or protein in cervical samples, to obtain information about the predictive values, and to define the optimal trade-off between sensitivity and specificity for the detection of CIN2+.

Markers for Cervical Cancer Survival

One way to investigate whether or not these molecular targets are associated with CC progression is to conduct a survival study. Therefore, a survival analysis using qRT-PCR expression values of the 15 validated markers and International Federation of Gynecology and Obstetrics (FIGO) staging was conducted on 42 patients with HPV16-positive CC whose progress was followed for at least 3.5 years after diagnosis and initial treatment (Espinosa et al. 2013). This sample included individuals at FIGO stages IB1 ($n = 16$), IB2 ($n = 14$), IIA ($n = 1$), IIB ($n = 9$), and IIIB ($n = 2$). The overall survival rate for the whole sample was 79.6 % and for FIGO stages IB1, IB2, IIA, IIB, and IIIB was 100 %, 69.2 %, 0 %, 85.7 %, and 0 %, respectively. These differences were statistically significant ($p < 0.001$, log-rank test, Fig. 6). Of the 15 genes analyzed using Kaplan–Meier survival curves, only *CDKN3* was associated with poor survival ($p = 0.004$, log-rank test; Fig. 6).

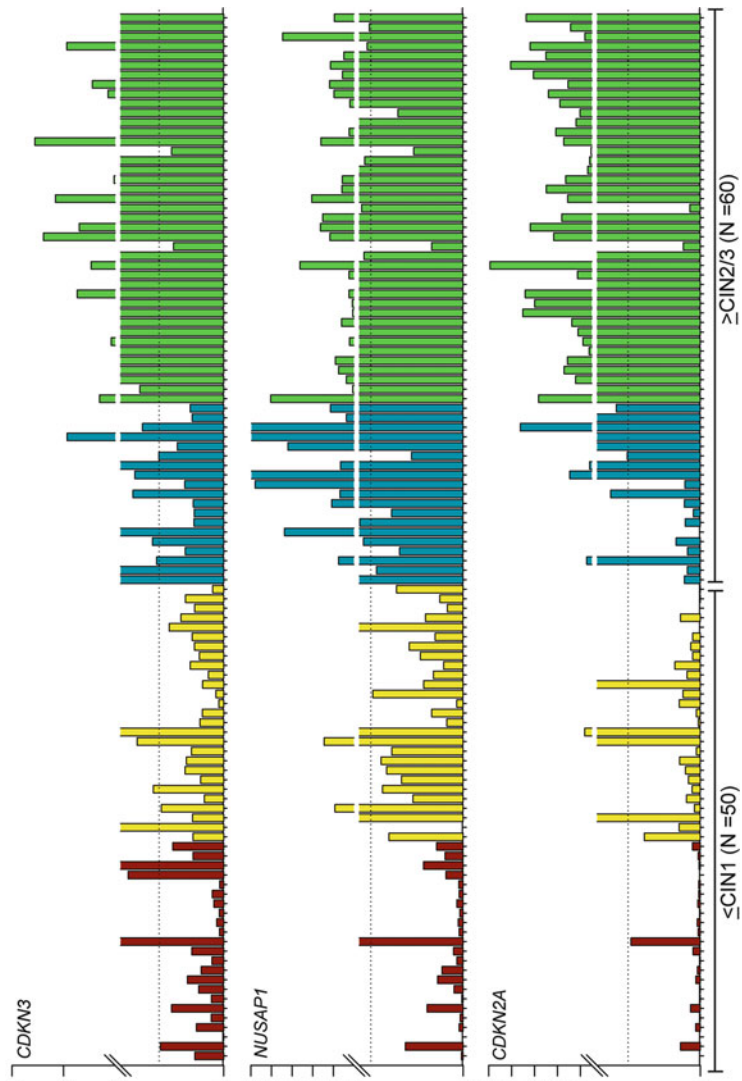

Fig. 5 Cutoff values of gene expression intensity for *NUSAP1*, *CDKN3*, and *CDKN2A*. Gene expression intensity of the individual samples and the cutoff values (*dashed lines*) calculated by using the ROC method. The first group (CIN1-, $n = 50$) included 23 healthy cervical epitheliums (*red bars*) and 27 low-grade lesions (*yellow bars*), and the second group (CIN2+, $n = 60$) included 19 high-grade lesions (*blue bars*) and 41 CCs positive for HPV16 (*green bars*). Markers *CDKN3* and *NUSAP1* showed slightly greater sensitivity than *CDKN2A*, but the opposite was true for specificity. Graphed values represent the median intensity of triplicate experiments normalized with an internal control (*GAPDH*)

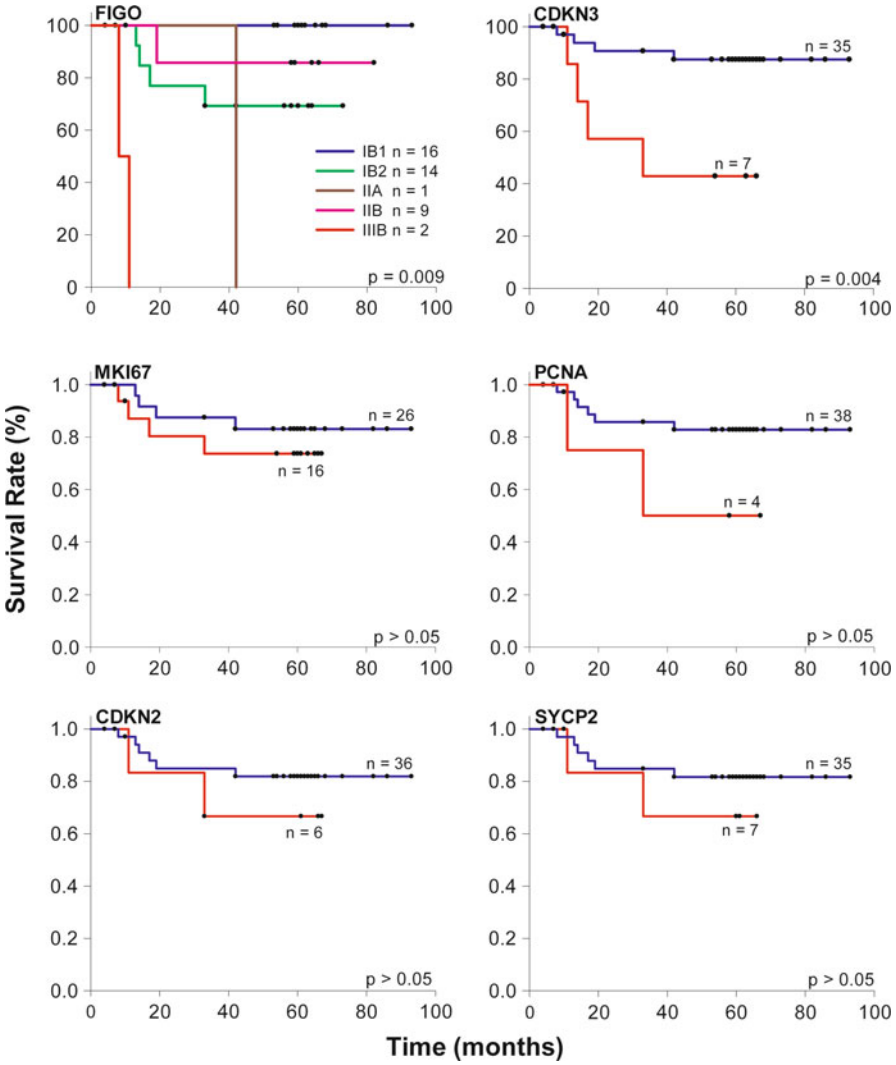

Fig. 6 The Kaplan–Meier survival curves for FIGO staging and *CDKN3, MKI67, PCNA, CDKN2*, and *SYCP2* genes. Patients were followed for at least 42 months. For gene expression, cancer patients with higher (*red line*) and lower (*blue line*) expression fold-change values were compared. The cutoff values were calculated with the ROC method. The *p*-values were calculated by comparing the curves with the log-rank test. Censored patients are labeled with *black dots*, but only four of them were censored before the minimal period of follow-up (42 months)

The overall survival rate of patients with higher levels of *CDKN3* (fold change $>$ 15) was 42.9 %, and the median survival time was 22 months. By contrast, those with lower levels of *CDKN3* had an overall survival rate of 87.5 %. FIGO staging and *CDKN3* expression were analyzed together using Cox proportional hazard models,

and *CDKN3* remained invariably significant with a hazard ratio (HR) of 5.9 (95 % confidence interval (CI), 1.4–23.8, $p = 0.01$). These results suggest that *CDKN3* could be a prognostic factor for survival that is independent of FIGO staging. However, a larger sample size is needed to confirm these results.

Potential Applications to Diagnosis, Prognosis, and Other Diseases or Conditions

A system for early detection of CC must be efficient and inexpensive. The markers proposed in our previous work (Espinosa et al. 2013) and this chapter (CDKN3, NUSAP1) have a sensitivity and specificity of approximately 90 %. However, the initial discoveries were made by analyzing mRNA of tumors and controls with traditional qRT-PCR, which is performed in four steps and costs approximately 50 USD. Because analysis of these markers with traditional qRT-PCR would be neither inexpensive nor easy, cheaper, easier, and high-performance screening methods that can be automated must be developed. Because our previous work using IH demonstrated that proteins encoded by the genes proposed as markers were overexpressed in tumors, ELISA methodology meets these requirements. However, it must be shown that the measurement of CDKN3 and NUSAP1 proteins by ELISA has a sensitivity and specificity similar to that of qRT-PCR analysis of mRNA. Another possibility would be to develop a cheaper qRT-PCR procedure that can be automated, for example, the use of a one-step qRT-PCR procedure that would allow simultaneous synthesis of cDNA and quantitative PCR in the same tube. Three markers labeled with different fluorochromes could be used simultaneously: one to detect high-grade lesions (NUSAP1 or CDKN3), another to detect exclusively invasive lesions (PRC1 or CCNB2), and an internal control. This methodology, besides to detect the CIN2+ lesions (NUSAP1 or CDKN3), could detect already invasive lesions (PRC1 or CCNB2), which deserve an immediate clinical attention. The cost of such a system, including RNA purification, produced in high volume would be approximately 5–10 USD. A low-cost system with high sensitivity and specificity could be easily adopted for the early detection of cervical cancer in poor and developing countries. However, validation of the system would require comparison with cytology and HPV testing in a large sample (ca. 5,000) of unselected women who regularly attend an early cancer detection program.

The association of CDKN3 with lower survival of CC patients clearly indicates that this gene is involved in the progression of CC. CDKN3 mRNA measurements might serve as a good predictive marker to assess patient survival and tumor aggressiveness. Depending on the clinical stage of the disease, invasive CC is treated currently with surgery, chemotherapy, radiotherapy, or a combination of these therapies. The success of conventional therapies and patient survival diminishes as the disease progresses to more advanced stages (Andrae, et al. 2012). In fact, the percentage of women who survive 5 years decreases from 93 % for stage IA to 15 % for stage IVB (www.cancer.org). The level of CDKN3 mRNA could be used to select women who need to be treated more aggressively with additional

neoadjuvant chemotherapy. Appropriate clinical trials should investigate the clinical value of measuring CDKN3 mRNA as an indicator for additional specific cancer therapy.

In contrast to other types of cancer, for which several specific molecular drugs have been developed (Torti and Trusolino 2011), there are no specific molecular-targeted therapies for CC. The majority of specifically targeted cancer drugs are directed toward mutated proteins, especially protein kinases (Knight et al. 2012), but some drugs also target normal proteins that are overexpressed, such as HER2/neu in breast cancer (Saxena and Dwivedi 2012). The first step in developing a specific molecular drug is identifying universal molecular targets that are present in patients with CC and absent in healthy women. Inhibition of mitosis is a well-known strategy to combat cancers. Drugs that perturb the process of cell division have proven to be effective anticancer therapies. Well-known examples of these drugs, such as taxanes and vinca alkaloids, perturb the formation of the mitotic spindle. However, they have remarkably low therapeutic indices and narrow therapeutic windows. Their efficacy is restricted because they also perturb the microtubule network of nondividing cells, causing neurotoxic effects and affecting endothelial cell function. To resolve these issues, a new generation of antimitotic agents has been developed that target kinesins and kinases that play unique roles in mitosis, such as KIF11, PLK1, and aurora kinase A (AURKA) (Lens et al. 2010). Interestingly, the transcripts of these three genes were upregulated in the CCs evaluated; *AURKA* ranked in 19th place, *KIF11* ranked in 72nd place, and *PLK1* ranked in 263rd place (Espinosa et al. 2013). Therefore, the new generation of antimitotic drugs could be tested for treating cervical cancer. In addition to testing exiting drugs, several factors make CDKN3 a potential target for treating CC. First, CDKN3 is involved in mitosis; second, it is overexpressed in CC, averaging seven times higher than expression in the healthy cervix; and third, it is associated with low survival in CC patients, suggesting that it is associated with aggressive tumors. However, it remains to be demonstrated that CDKN3 is indispensable for tumor growth and that small drugs that inhibit CDKN3 function in tumor cells can be discovered.

Consistent with our data, *CDKN3*, along with other genes, has been found to be associated with lower survival of patients with lung adenocarcinomas (MacDermed et al. 2010; Tang et al. 2013). In addition, *CDKN3* has been shown to be essential for in vitro neoplastic growth of cells derived from hepatocellular carcinoma (Xing et al. 2012). Therefore, the clinical utility of *CDKN3* as a potential drug target and as a diagnostic tool to evaluate survival and select patients for more aggressive treatments could also be evaluated in lung cancer and hepatocellular carcinoma.

Summary Points

- The effect of preventive human papillomavirus (HPV) vaccination on the reduction of the cervical cancer (CC) burden will not be known for 30 years. Current methods for screening have low sensitivity (Pap test) or low specificity

(HPV tests) for the detection of high-grade cervical intraepithelial neoplasias (CIN2/3) and CC (CIN2+). Therefore, it remains necessary to improve the procedures for CC screening and treatment.

- The tumor suppressor protein p16 is one of the most widely studied markers associated with CC. The amount of p16 is related to the severity of cervical neoplasia and is considered a marker of CIN2+. However, the relatively low specificity of this marker and the need for a pathologist to interpret the test results are the major reasons that this marker has not been adopted for primary screening.
- The most upregulated and enriched genes in cervical cancer, compared with healthy cervical epithelium, are those involved in mitosis. Some mitotic genes might be good candidates as markers for screening or survival or as potential therapeutic targets.
- Six genes from mitosis were recently discovered to be associated with CC (*CCNB2*, *PRC1*, and *SYCP2*) and also with CIN2/3 (*CDC20*, *NUSAP1*, and *CDKN3)*.
- The sensitivity and specificity of *CDKN3* and *NUSAP1* to detect CIN2+ was approximately 90 %. Therefore, they may be potential targets for the development of novel screening methods.
- *CDKN3* was also associated with poor CC patient survival, and it was independent of clinical stage. Therefore, *CDKN3* might serve as both a screening tool and a survival marker.

References

Andrae B, Andersson T, Lambert P, et al. Screening and cervical cancer cure: population based cohort study. BMJ Br Med J. 2012;344:e900.

Biewenga P, Buist M, Moerland P, et al. Gene expression in early stage cervical cancer. Gynecol Oncol. 2008;108:520–6.

Buitrago-Pérez A, Garaulet G, Vázquez A, et al. Molecular signature of HPV-induced carcinogenesis: pRb, p53 and gene expression profiling. Curr Genomics. 2009;10:26–34.

Cuzick J. Long-term cervical cancer prevention strategies across the globe. Gynecol Oncol. 2010;117:S11–4.

de Sanjose S, Quint W, Alemany L, et al. Human papillomavirus genotype attribution in invasive cervical cancer: a retrospective cross-sectional worldwide study. Lancet Oncol. 2010;11:1048–56.

Demetrick D, Matsumoto S, Hannon GJ, et al. Chromosomal mapping of the genes for the human cell cycle proteins cyclin C (CCNC), cyclin E (CCNE), p21 (CDKN1) and KAP (CDKN3). Cytogenet Cell Genet. 1995;69:190–2.

Espinosa A, Alfaro A, Roman E, et al. Mitosis is a source of potential markers for screening and survival and therapeutic targets in cervical cancer. PLoS One. 2013;8:e55975.

Fan J, Upadhye S, Worster A. Understanding receiver operating characteristic (ROC) curves. CJEM. 2006;8:19–20.

Ferlay J, Shin H, Bray F, et al. Cancer Incidence and Mortality Worldwide: IARC Cancer Base No. 10. International Agency for Research on Cancer. 2008; 2010. http://globocan.iarc.fr

Galamb O, Spisák S, Sipos F, et al. Reversal of gene expression changes in the colorectal normal-adenoma pathway by NS398 selective COX2 inhibitor. Br J Cancer. 2010;102:765–73.

Giorgi-Rossi P, Franceschi S, Ronco G. HPV prevalence and accuracy of HPV testing to detect high-grade cervical intraepithelial neoplasia. Int J Cancer. 2012;130:1387–94.
Gius D, Funk M, Chuang E, et al. Profiling microdissected epithelium and stroma to model genomic signatures for cervical carcinogenesis accommodating for covariates. Cancer Res. 2007;67:7113–23.
Gong D, Ferrell J. The roles of cyclin A2, B1, and B2 in early and late mitotic events. Mol Biol Cell. 2010;21:3149–61.
Gruneberg U, Neef R, Li X, et al. KIF14 and citron kinase act together to promote efficient cytokinesis. J Cell Biol. 2006;172:363–72.
Hwang S, Shroyer K. Biomarkers of cervical dysplasia and carcinoma. J Oncol. 2012;2011:507286.
Julien S, Dubé N, Hardy S, et al. Inside the human cancer tyrosine phosphatome. Nat Rev Cancer. 2011;11:35–49.
Knight Z, Lin H, Shokat K. Targeting the cancer kinome through polypharmacology. Nat Rev Cancer. 2012;12:130–7.
Kretschmer C, Sterner A, Siedentopf F, et al. Identification of early molecular markers for breast cancer. Mol Cancer. 2011;10:15.
Lehtinen M, Paavonen J, Wheeler C, et al. Overall efficacy of HPV-16/18 AS04-adjuvanted vaccine against grade 3 or greater cervical intraepithelial neoplasia: 4-year end-of-study analysis of the randomised, double-blind PATRICIA trial. Lancet Oncol. 2012;13:89–99.
Leinonen M, Nieminen P, Kotaniemi L, et al. Age-specific evaluation of primary human papillomavirus screening vs conventional cytology in a randomized setting. J Natl Cancer Inst. 2009;101:1612–23.
Lens S, Voest E, Medema R. Shared and separate functions of polo-like kinases and aurora kinases in cancer. Nat Rev Cancer. 2010;10:825–41.
Li C, Lin M, Liu J. Identification of PRC1 as the p53 target gene uncovers a novel function of p53 in the regulation of cytokinesis. Oncogene. 2004;23:9336–47.
Li X, Bolcun E, Schmenti J. Genetic evidence that synaptonemal complex axial elements govern recombination pathway choice in mice. Genetics. 2011;189:71–82.
MacDermed D, Khodarev N, Pitroda S, et al. MUC1-associated proliferation signature predicts outcomes in lung adenocarcinoma patients. BMC Med Genomics. 2010;3:16.
Markowitz L, Dunne E, Saraiya M, et al. Quadrivalent human papillomavirus vaccine: recommendations of the Advisory Committee on Immunization Practices (ACIP). MMWR Recomm Rep. 2007;56:1–24.
Martinez I, Wang J, Hobson K, et al. Identification of differentially expressed genes in HPV-positive and HPV-negative oropharyngeal squamous cell carcinomas. Eur J Cancer. 2006;43:415–32.
McCrory D, Matchar D, Bastian L, et al. Evaluation of cervical cytology. Evid Rep Technol Assess (Summ). 2009;1999:1–6.
Mo M, Chen Z, Li J, et al. Use of serum circulating CCNB2 in cancer surveillance. Int J Biol Markers. 2010;25:236–42.
Moody C, Laimins L. Human papillomavirus oncoproteins: pathways to transformation. Nat Rev Cancer. 2010;10:550–60.
Nakayama K, Nakayama K. Ubiquitin ligases: cell-cycle control and cancer. Nat Rev Cancer. 2006;6:369–81.
Nalepa G, Barnholtz-Sloan J, Enzor R, et al. The tumor suppressor CDKN3 controls mitosis. J Cell Biol. 2013;201:997–1021.
Narayan G, Bourdon V, Chaganti S, et al. Gene dosage alterations revealed by cDNA microarray analysis in cervical cancer: identification of candidate amplified and overexpressed genes. Genes Chromosomes Cancer. 2007;46:373–84.
Natunen K, Lehtinen J, Namujju P, et al. Aspects of prophylactic vaccination against cervical cancer and other human papillomavirus-related cancers in developing countries. Infect Dis Obstet Gynecol. 2011;2011:675858.

Ostor A. Natural history of cervical intraepithelial neoplasia: a critical review. Int J Gynecol Pathol. 1993;12:186–92.
Park S, Yu G, Kim W, et al. NF-Y-dependent cyclin B2 expression in colorectal adenocarcinoma. Clin Cancer Res. 2007;13:858–67.
Patel D, McCance D. Compromised spindle assembly checkpoint due to altered expression of Ubch10 and Cdc20 in human papillomavirus type 16 E6- and E7-expressing keratinocytes. J Virol. 2010;84:10956–64.
Pyeon D, Newton M, Lambert P, et al. Fundamental differences in cell cycle deregulation in human papillomavirus–positive and human papillomavirus–negative head/neck and cervical cancers. Cancer Res. 2007;67:10163–72.
Romanowski B. Long term protection against cervical infection with the human papillomavirus: review of currently available vaccines. Hum Vaccin. 2011;7:161–9.
Saxena R, Dwivedi A. ErbB family receptor inhibitors as therapeutic agents in breast cancer: current status and future clinical perspective. Med Res Rev. 2012;32:166–215.
Schiffman M, Wentzensen N, Wacholder S, et al. Human papillomavirus testing in the prevention of cervical cancer. J Natl Cancer Inst. 2011;103:368–83.
Subramanian R, Wilson E, Arthur C, et al. Insights into antiparallel microtubule crosslinking by PRC1, a conserved non motor microtubule binding protein. Cell. 2010;142:433–43.
Tang H, Xiao G, Behrens C, et al. A 12 gene set predicts survival benefits from adjuvant chemotherapy in non-small lung cancer patients. Clin Cancer Res. 2013;19:1577–86.
Taylor K, Sims A, Liang L, et al. Dynamic changes in gene expression in vivo predict prognosis of tamoxifen-treated patients with breast cancer. Breast Cancer Res. 2010;12:R39.
Teissier S, Ben J, Mori M, et al. New E6/P63 pathway, together with a strong E7/E2F mitotic pathway, modulates the transcriptome in cervical cancer cells. J Virol. 2007;81:9368–76.
Torti D, Trusolino L. Oncogene addiction as a foundational rationale for targeted anti-cancer therapy: promises and perils. EMBO Mol Med. 2011;3:623–36.
Tsoumpou I, Arbyn M, Kyrgiou M, et al. p16(INK4a) immunostaining in cytological and histological specimens from the uterine cervix: a systematic review and meta-analysis. Cancer Treat Rev. 2009;35:210–20.
Wang S, Ooi L, Hui K. Upregulation of Rac GTPase-activating protein 1 is significantly associated with the early recurrence of human hepatocellular carcinoma. Clin Cancer Res. 2011;17:6040–51.
Wentzensen N, Hampl M, Herkert M, et al. Identification of high-grade cervical dysplasia by the detection of p16INK4a in cell lysates obtained from cervical samples. Cancer. 2006;107:2307–13.
Whitlock E, Vesco K, Eder M, et al. Liquid-based cytology and human papillomavirus testing to screen for cervical cancer: a systematic review for the U.S. Preventive Services Task Force. Ann Intern Med. 2011;155:687–97.
Wright Jr T. Cervical cancer screening in the 21st century: is it time to retire the PAP smear? Clin Obstet Gynecol. 2007;50:313–23.
Xing C, Xie H, Zhou L, et al. Cyclin-dependent kinase inhibitor 3 is overexpressed in hepatocellular carcinoma and promotes tumor cell proliferation. Biochem Biophys Res Commun. 2012;420:29–35.
Zhai Y, Kuick R, Nan B, et al. Gene expression analysis of preinvasive and invasive cervical squamous cell carcinomas identifies HOXC10 as a key mediator of invasion. Cancer Res. 2007;67:10163–72.