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Cervical Intraepithelial Neoplasia-Predictive Molecular Growth Factors in Natural History

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Abstract

There is considerable controversy regarding the possible over-treatment of patients with mild Cervical Intraepithelial Neoplasia (CIN), with lesions being often excised or ablated. Thus, identifying the markers of potentially malignant lesions would be of a great prognostic value. In the current study, we hypothesized that using colposcopic, cytological and histological findings together with assessing the expression of molecular growth factors can predict CIN outcome. The study group consisted of 285 women between 19 and 81 years of age (median age, 37,8 years). The follow up were 60 months and considered 138 women: 50 women with Subclinical Papillomavirus Infection (SPI), 50 women with CIN1 and 38 women with CIN2.

All patients underwent cytology, colposcopy, and sampling for subsequent testing for HPV. In cases in which colposcopy suggested the presence of suspicious lesions, biopsy specimens were taken. HPV DNA was genotyped for HPV types 16, 18, 31, 33, and 45 by multiplex PCR. Transcripts of HR HPV types 16, 18, 31, 33, and 45 were detected by the NucliSens EasyQ HPV assay. The VEGF expression was analyzed with immunohistochemistry, RNA extraction, cDNA synthesis and RT-PCR analysis and Western blot.

We found that so called lymphangiogenetic switch (over expression of VEGF C and VEGFR-2) appears already in CIN 2, which is a rare observation, Persistent HPV HR infection is not only a trigger but also a maintenance factor in the cervical carcinogenesis. CIN2/3 and cervical cancer is in high percentage associated with the presence of HR DNA HPV as well as E6/E7 DNA mRNA.

In CIN2/3 and cervical cancer VEGF and its receptor expression correlate with the stage of cervical carcinogenesis.

Progression of cervical intraepithelial neoplasia occurs when co expression of all: HR DNA HPV, E6/E7 HR HPV mRNA and VEGF is present.

Keywords: CIN (Cervical intraepithelial neoplasia); Cervical cancer; VEGF; VEGFR; DNA HPV; mRNA E6/E7 HPV

Introduction

Cervical cancer is the second most common cancer in women and the leading cause of cancer-related death in females from underdeveloped countries. Each year, approximately 500,000 cases of cervical cancer are diagnosed worldwide.

Routine screening has decreased the incidence of invasive cervical cancer in the United States, where approximately 13,000 cases of invasive cervical cancer and 50,000 cases of cervical carcinoma in situ (i. e. true precancer) are diagnosed annually [1].

Cervical cancer arises from the metaplastic epithelium of the Transformation Zone (TZ) (squamocolumnar junction) and develops slowly through progressive dysplastic changes to Carcinoma In Situ (CIS) and invasive cancer. Cervical Intraepithelial Neoplasia (CIN) is divided into three stages according to the degree of epithelial dysplasia and differentiation. Lesions are accessible to colposcopic evaluation and biopsy, which makes monitoring disease progression relatively easy. Low grade lesions (i.e. CIN1) and, in some cases CIN2 may spontaneously regress or not progress further, while the malignant potential of CIN 3 is 36% over 20 years [2].

There is considerable controversy regarding the possible over-treatment of patients with mild cervical abnormalities, with lesions being often excised or ablated. Thus identifying the markers of potentially malignant lesions would be of great prognostic value [3].

The most common types of Human Papillomavirus (HPV) found in CIN and cancer patients are types 16, 18, 31, 33, and 45 [4]. Persistent infection with these types is regarded as the earliest carcinogenesis stage [5]. The role of HPVs in the etiology of cervical cancer is tightly correlated with the overexpression of two oncogenes (E6 and E7) due to a specific opening in the E2 open reading frame in the integrated viral genome [6]. Studies of cervical cancer cell lines and cancer biopsy specimens have shown that the continuous expression of these genes is a necessary condition for the transformation and maintenance of neoplastic and dysplastic cells [7-10].

In recent years, many studies have shown that testing for HPV DNA can improve the detection of High-Grade Squamous Intraepithelial Lesions (HSILs) and cervical cancer [8-12]. This suggests that DNA testing can make a useful contribution to the triage of women with

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an equivocal cytology finding and for follow-up after the treatment of precursor lesions. However, the high prevalence of transient and asymptomatic HPV infections means that DNA tests have low specificities. Identification of the persistent infections likely to produce high-grade lesions currently requires repeated monitoring of the HPV DNA types. Commercial nucleic acid sequence-based amplification in a real-time format allows the reliable type-specific detection of E6 and E7 mRNA from HPV types 16, 18, 31, 33, and 45. Several authors have thus suggested that RNA-based assays could be more effective than DNA testing in risk assessment [13-18].

The development of cervical cancer as well as other malignant tumors has conventionally been considered to follow a pre-vascular phase, where the growth of the primary tumor is restricted to a few millimeters in diameter due to the diffusion limit of oxygen [19]. Vascular Endothelial Growth Factor (VEGF) is one of the most specific and strongest growth factors for the endothelial cells among all the factors that have been discovered so far. VEGF, a multifunctional cytokine, stimulates angiogenic activity by increasing vascular permeability and acting as an endothelial cell mitogen. The protein family includes the following: VEGF- A [14], -B [15], -C [16], -D [17], -E [18] and placenta growth factor (PIGF) [20].

In this study, we evaluated the correlation between VEGF-C and VEGFR-2 and clinicopathologic parameters in cervical cancer.

In the current study, we hypothesized that using colposcopic, cytological and histological findings together with assessing the expression of molecular growth factors can predict CIN outcome.

Materials and Methods

Cervical specimens were collected from October 2006 to December 2007 from patients admitted for secondary screening to the Colposcopy Outpatient Service and the Gynecological Oncology Unit (Jagiellonian University Medical College, Krakow, Poland). The study group consisted of 285 women between 19 and 81 years of age (median age, 37,8 years).

In Poland the natonal screening for cervical cacner is an organized call- recall system with conventional Pap test as a screening tool. The triage for positive subject is colposcopy. The target population is women starting from 25 years of life, with 3 years interval. There are 16 regional centers serving as the colposcopy clinics, where colposcopy is performed. After colposcopy the subject is reffered to treatment or is referred back to her primary physician. The database of the screening program is centralized and linked to the national healthcare system.

The follow up period was 60 months and considered only 138 women of whom the informed consent was obtain for this type of observation. The distribution of the observed women was: 50 women with SPI, 50 women with CIN1 and 38 women with CIN2. The Ethics Committee of Jagiellonian University Medical College approved the study protocol recommending surgery instead of follow-up of women diagnosed with CIN3. Written informed consent was obtained from all participants. All participants received a self-administered questionnaire requesting personal data, a gynecologic history, and information on exposure to risk factors. Women undergoing previously treatment for invasive cervical cancer were excluded. All patients underwent cytology, colposcopy, and sampling for subsequent testing for HPV. In cases in which colposcopy suggested the presence of suspicious lesions, biopsy specimens were taken.

Follow up visits comprised of gynecological evaluation, Pap test, HPV sampling and colposcopy with cervical biopsy for histologic evaluation. The interval of visits was 6 months. The call-recall system was used. The SPI (sublinical papilloma infection) is a colposcopic term describing productive HPV infection, in which cytology presents koilocytosis or ASCUS results, and histology reveals no CIN but koilocytosis-presence of koilocytic epithelial cells with perinuclear halo containing HPV particles. The complete remission was defined as return to normal epithelium (no disease). The partial remission was defined as a step back in the sequence: normal, SPI, CIN1, CIN2 cervical tissue. The stationary state was defined as a persistency of SPI, CIN1 or CIN2 diagnosis during observation. The progression was defined as any step forward in the sequence: normal, SPI, CIN1, CIN2, CIN3, cervical cancer. All diagnosis i.e. SPI, CIN1 and CIN2 were based on histologic evaluation. Cytology was based on a conventional Pap smear. The cytological diagnosis was made by specialized cytopathologists using the Bethesda classification system. Colposcopy was performed by specialized gynecologists. The results were reported following guidelines issued by PSCCP (The Polish Society of Colposcopy and Cervical Pathophysiology), a member of EFC (European Federation of Colposcopy) and IFCPC (International Federation of Cervical Pathology and Colposcopy). Histology was performed with specimens collected by colposcopy-directed biopsy (traditional punch biopsy specimens) and/or cone specimens collected by the loop excision procedure. Histology results were obtained for all 138 patients.

Part of each tissue specimen was collected during surgery, one part for mRNA analysis - immediately frozen in RNAse later solution (Qiagen, Hilden, Germany), second part for protein analysis, both stored at -80°C until used for study. Cancer tissue preparation was done as reported by Molden et al. [21].

Cervical specimens for nucleic acid analyses were collected with a cervical brush by standard procedures. The material was preserved in PreservCyt/ThinPrep solution. Analyses were performed by the Virology Laboratory at the University Hospital, Krakow, Poland.

HPV DNA was genotyped for HPV types 16, 18, 31, 33, and 45 by multiplex PCR.

Samples were analyzed for HPV E6 and E7 mRNA by real-time multiplex nucleic acid sequence-based amplification. Transcripts of HR HPV types 16, 18, 31, 33, and 45 were detected by the NucliSens EasyQ HPV assay (bioMerieux, Poland), according to the manufacturer's instructions. The VEGF expression was analyzed with immunohistochemistry, RNA extraction, cDNA synthesis and RT-PCR analysis and Western blot analysis.

Using the Shapiro–Wilk test, the distributions of countinous variables in the examined groups of women were analyzed and the results were presented as median values (minimum–maximum). U Mann-Whtney test or Kruskal–Wallis analysis of variance, with post hoc test (when appropriate) were used as distribution of analyzed variables differed from the normal ones or were quantitive data were analyzed. A p value of 0.05 was accepted as statistically significant. All calculations were carried out with the use of STATISTICA software v.9.0. (StatSoft, USA 2009)

Result

Subclinical papilloma infection (SPI) and CIN outcome

(Table 1) 4–5-years clinical follow-up (cytologic-colposcopic) did not reveal statistically important differences in remission percentage of SPI women in comparison to CIN1 women. While remission was

Lesion	N	Complete remission		Progression	
		n	%	n	%
SPI*	50	38	76,0	6	12,0
CIN 1	50	26	52,0	9	18,0
CIN 2*	38	14	36,8	12	31,6
Σ	138	78	56,5	27	19,6

*statistical significance at p=0,006

Table 1: Comparison of SPI, CIN 1 and CIN 2 complete remission and progression.

statistically more often observed (p=0,038) in both SPI and CIN1 cases comparing to CIN2.

The stationary status was statistically more frequently observed (p=0,050) in women with CIN1 comparing to SPI women. Analogically, in cases of CIN2 the stationary status was observed more often (p=0,022) comparing to SPI women. There were no statistically important differences between stationary status of women with CIN1 and CIN2. This may be the proof of non-stable, dynamic SPI characteristic, influenced by immunological factors. Such a concept may be also confirmed by higher SPI remission percentage in comparison toCIN1 andCIN2. Number of SPI, CIN1 and CIN2 was calculated in complete remission group and compared with the progression group (Yeats ch^2 (7,46) ; p=0,024). There were no statisticall difference between CIN 1 and CIN 2, between SPI and CIN, but there was statistically important difference between SPI and CIN 2 (p=0,006).

SPI, CIN 1 and CIN2 outcome and HPV DNA type

During prospective, clinical observation, the highest remission percentage was noted in SPI (76,0%) women and the lowest one in CIN2 (36,8%) group of women. In these lesion, the low risk (LR) DNA HPV was noted in 78,2% cases, high risk (HR) and LR DNA HPV–in12,8% cases, and in 9% of women no DNA HPV was detected using HC2 test. While, the progression rate was the lowest in SPI (12,0%) women, and the highest in CIN2 (31,6%) cases, the progression in 96,3% of cases was accompanied with HR HPV DNA and only in one case (3,7%) with LR HPV DNA. These observations confirmed the importance of HR HPV DNA in cervical carcinogenesis. In the Table 1 the remainder was the cases of stationary state.

SPI, CIN 1 and CIN2 outcome and E6/E7 mRNA HPV expression

The analysis on SPI, CIN 1 andCIN 2 outcome in relation to E6/E7 HPV 16, 18, 31, 33 and45 mRNA expression revealed that in 138 women who were observed presented that progression occurred overall in 20/22 (90.9%) mRNA (+) and 7/83 (8.4%) mRNA [-]. The remission rate was be 91.6% for mRNA [-] and 9.1% for mRNA [+] (Table 2).

The progression/remission analysis revealed that CIN 1 and CIN 2 remission in 55/60 (91,6%) occurs when there are not E6 and E7 HPV mRNA expression. The progression occurred in 9/11 (91.6%) of cases of SPI andCIN1 and 11/11 (100%) of cases of CIN 2, in which the E6/E7 HPV mRNA was detected. Persistent HR HPV infection is an important factor in cervical carcinogenesis.

SPI, CIN 1 and CIN2 outcome and VEGF expression

Assuming this is the Mann-Whitney U test, there was a significant difference in the VEGF expression between the groups of women who regressed compared with those who progressed. The median VEGF expression in group of SPI, CIN 1 and CIN 2 women which spontaneously regressed was 18,98 pg/ml. This expression was statistically lower than median VEGF expression of SPI, CIN 1 and CIN 2 women, which progressed. In these cases the median VEGF expression was 34,72 pg/ml. The lowest value of VEGF expression (29,45 pg/ml) was reported it CIN2 cases which progressed to CIN3 and CIS (Table 3).

Normal cervical epithelium did not express VEGF-C or VEGFR-2 in the samples analyzed. Significant differences were found between CIN1-2 and CIN3, but not between CIN3 and cervical cancer. VEGFR-2 expression was the strongest in the CIN3 samples, while in the group of CIN1, it was comparable to the control group.

Discussion

In this paper in the women with histologically diagnosed Subclinical Papillomavirus Infection (SPI), CIN1, CIN2, CIN3 and cervical cancer the epidemiological, morphological and molecular factors impacting cervical carcinogenesis were evaluated. In the subgroup of 138 women with SPI, CIN1 and CIN2 prospectively observed for 4-5 years, the importance of DNA HPV, E6/E7 HPV mRNA expression and VEGF expression as potential predictive factors were measured.

Despite of the fact, that the triggering factors in cervical carcinogenesis are pretty well studied [21] there are still gaps in the understanding of the mechanism, in which the CIN spontaneously regress, persists or progress into invasive cancer.

This is of special importance, because CIN in most cases affects women in their procreative life time. Own experience [22] and many other authors observations [23-28], revealed that SPI in nearly 80%, CIN1 in 40–60%, and CIN2 in 30–35% of cases spontaneously regress. So in these cases therapeutic approach is unnecessary. Similar findings reported Cox and Schifman in ALTS study [29]. S^{240}. In their study the DNA HPV, especially HR was related to higher percentage of SPI, CIN 1 and CIN2 progression to HSIL (CIN3+) (10%, 12,5% and 30,2% respectively). Similar findings reported Goldie et al. [30], Castle et al. [31], Insinga et al. [32] and Moore et al. [33].

According to many clinicians and molecular biologists E6/E7 mRNA HPV expression is the sign of persistent HPV infection [21,27,28]. Moscicki et al. [34] and Castle et al. [31] state, that HPV-16 persistency increases the risk of CIN3 development of 40% within 5 years. Both transcriptors E6 andE7 HPV HR are stimulating cell cycle. They inactivate p53 andpRB, and stimulate replication and activate telomerase.

Lesion	n	Remission					Progression				
		mRNA (+)		mRNA (–)			mRNA (+)		mRNA (–)		
		n	%	n	%	Σ	n	%	n	%	Σ
SPI	50	1	2,6	37	97,4	38	4	66,7	2	33,3	6
CIN 1	50	1	3,8	25	96,2	26	5	55,6	4	44,4	9
CIN 2	38	0	0,0	14	100,0	14	11	91,7	1	8,3	12
Σ	138	2	2,6	76	97,4	78	20	74,1	7	25,9	27

Table 2: SPI, CIN 1 and CIN 2 remission and progression and E6/E7 HPV 16, 18, 31, 33 and 45 mRNA expression.

Lesion	n	remission			progression		
		VEGF (pg/ml) expression			VEGF (pg/ml) expression		
		Min	Max	Me	Min	Max	Me
SPI	50	16,00	18,24	16,76$^{*,\#}$	17,11	29,99	28,42$^{*,\#\#}$
CIN 1	50	17,12	19,11	17,98**	21,89	31,21	29,12**
CIN 2	38	20,99	22,45	21,13$^{***,\#}$	29,45	72,00	36,12$^{***,\#\#}$
Σ	138	16,00	22,45	18,98$^{\$}$	17,11	72,00	34,72$^{\$}$

p <0,001*p<0,001;**p<0,001; ***p<0,001; $^{\$}$p<001; #p0=,008; ##p=0,039

Table 3: VEGF (pg/ml) expression and SPI, CIN 1 and CIN 2 outcome.

The important issue in carcinogenesis studies is examining the neoangiogenesis. We found that so called lymphangiogenetic switch (over expression of VEGF C andVEGFR-2) appears already in CIN2, which is an original observation. In fact Van Trappen et al. [35] fund in 78% CIN3 lesions moderate to strong expression of VEGF-C and VEGF-D, but in CIN1 and CIN2 only in single cases. In our study we stated that VEGF expression May be useful as a progression indicator in CIN2 to CIN3. This is one of the first reports of such an observation confirmed in prospective observations.

These observations confirm that VEGF is an important angiogenic factor in progression of cervical carcinoma and suggest molecular growth stimulation via VEGFR-2 in cervical carcinogenesis. Our results show that the switch to the lymphangiogenic phenotype occurs prior to the stage of invasion and probably between CIN2 and CIN3, and blocking VEGFR-2 signaling may represent a novel therapeutic approach to the treatment of a subset of cervix cancer and cervical intraepithelial neoplasia.

Conclusions

Persistent HPV HR infection is not only a trigger but also a maintenance factor in the cervical carcinogenesis.

In high percentage of CIN2/3 and cervical cancer cases HR DNA HPV and E6/E7 DNA mRNA are expressed.

In CIN2/3 and cervical cancer VEGF and its receptor expression correlate with the stage of cervical carcinogenesis.

Progression of cervical intraepithelial neoplasia occurs when co expression of at least one of factor: HR DNA HPV, E6/E7 HR HPV mRNA or VEGF is present. It seems that the more than one of these factors are expressed the trend of progression is stronger.

We are aware that there are many reasons, some related to the process of screening why alternative explanation for the findings, including bias other than those we postulate could explain the findings.

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