New methods for the diagnosis of cervical cancer and cervical premalignant lesions

PhD Thesis

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1. Introduction

According to the Globocan 2018 database cervical cancer is the fourth most common cancer in women regarding both incidence and mortality. Although in recent years mortality has decreased in developed countries, incidence is on the increase, especially in the less developed countries. Widespread use of cervical cytology screening („Pap test"), identification of the human papilloma virus (HPV) as a pathogen, as well as introduction of HPV vaccination at population level in several countries around the world, have altogether led to significant results in the early recognition of cervical cancer and premalignant lesions. It bears significance therefore that the first Hungarian HPV Center was established at the 2nd Department of Obstetrics and Gynecology of Semmelweis University, in which I was able to participate from the very beginning (Galamb et al. 2011).

The results and observations obtained at our HPV Center, however, raised new questions and problems to be faced by the gynecologist, which were mostly related to early diagnosis. The cytology test was found to have high specificity but low sensitivity. On the contrary, detection of high risk HPV types (hrHPV) by molecular pathology techniques showed the reverse, with the promise of becoming high sensitivity, low specificity methods suitable for diagnosis of cervical cancer. Based on these findings, there have been several attempts to introduce „complementary" tests with the aim to improve the above shortcomings. Highlighted among these are the CINtec® and CINtec® PLUS tests, which have recently become widely known. These tests help to determine the degree and severity of cervical squamous intraepithelial lesions in cytological smears, which is essential for both gynecologist and patient in order to decide the type of therapy to be administered, i.e. whether surgical intervention is necessary. Many research groups have developed additional tests to help resolve doubtful cases and select the most appropriate treatment. We also started our work with this goal in mind.

In their earlier work, my supervisor Dr. Gábor Sobel and his coworkers (2005) proved that certain cell junction proteins, primarily claudin-1 (CLDN1), show increased expression in cervical cancer and premalignant lesions. Based on their

findings, we started to examine the presence and enhanced expression of certain CLDN proteins in histological as well as cytological specimens, with the aim to help choose the right treatment option.

Regarding further possibilities of this new approach, we also studied certain epigenetic alterations. It is already known that the expression of the so-called *mikroRNAs* (miR), these small, endogeneous, non-coding ribonucleic acid (RNA) molecules, undergoes changes during the development and progression of several tumors, as well as on the effect of HPV infection, and we ourselves were among the first in Hungary to call attention to this (Galamb et al. 2011). The changes in miR expression have been well observed in both tissue and blood samples taken from tumors of several organs. Altered miR expression profiles in normal and tumorous cervical epithelial tissues have been the subject of several studies, in the majority of which however, no clear conclusion could be drawn, moreover, only a fraction of studies included observations on the changes taking place in premalignant lesions.

Based on the above, our objectives were directed towards the early detection of cervical cancer and premalignant lesions, and their association with HPV infection.

The studies involving cytology, histology and immunohistochemistry were conducted in collaboration with the 2nd Department of Pathology of Semmelweis University in laboratory settings, as well as with Dr. Márta Benczik and dr. Adrienn Kocsis, who performed the HPV testings.

2. Aims

2.1. Examination of HPV infection and distribution of HPV types among women examined in the first Hungarian HPV center.

2.2. Comparison of the evaluation results of „conventional" smear samples and liquid-based cytology (LBC) samples.

2.3. Assessment of the diagnostic value of cell junction protein claudin-1 (CLDN1) in parallel liquid-based cytology (LBC) samples and conventional cytological smears and to compare it with $p16^{INK4a}$ immunoreactions.

2.4. Improving the diagnostic value of increased CLDN1 expression using other biomarkers (Ki67 proliferation marker) in cervical cytology and histological (conization) specimens, with the aim to enhance the sensitivity and specificity of the method.

2.5. Comparison of the results of CLDN1/Ki67 double staining technique with the CINTec®PLUS test on liquid-based cytology (LBC) samples and histological samples obtained by conization.

2.6. Examination of miR expression changes taking place in premalignant cervical lesions observed in conization material.

3. Patients, materials and methods

The studies were performed with approval from Semmelweis University Regional and Institutional Committee of Science and Research Ethics (TUKEB #148/2012) and were supported by grants #KMR_12-1-2012-0032 and #OTKA PD105019 (Dr. Gábor Sobel).

Patients: In each study the cytological and histological samples were evaluated in different numbers of patients. First, the samples taken from the patients for cytological analyses were processed using both conventional cytology and liquid-based cytology

(LBC), then the latter method was evaluated, which was then followed by further analyses

(immunoreactions). The histological samples were obtained by conization, based on clinical indication.

HPV determination*:* HPV was determined from cervical samples obtained by the LBC method at GenoID Molecular Diagnostic Laboratory (Budapest) using CONFIDENCE HPV^{TM} under the guidance of Dr. Márta Benczik and dr. Adrienn Kocsis Adrienn. The results obtained were evaluated at the Laboratory.

Cytological and histological samples: Alltogether 9847 samples were used, which are recorded in the induvidual studies. The samples were sent to the 2nd Department of Pathology, Semmelweis University for further processing, then to the GenoID Laboratory for HPV determination. The smears were evaluated according to the Bethesda system, with initial evaluations performed by cytology-scrrening assistants, followed by diagnosis of expert cytologists. The cervical samples for LBC testing were prepared by an assistant using ThinPrep® 2000 Processor, Hologic^{TM} Inc. instrument, according to manufacturer instructions. The formalin-fixed, paraffin-embedded (FFPE) conization specimens were evaluated by pathologists, following which the samples were subjected to further tests.

Immunocytochemistry/immunohistochemistry: For the immune reactions, anti-CLDN1 antibody was used at 1:100x dilution provided by Zymed (San Francisco, CA, USA), Cell Marque (Roclin, CA, USA) as well as Dako (Glostrup, Denmark) (detailed description is given in our manuscripts). The reactions were performed using Ventana ES automated immunostainer (Ventana Medical System Inc., Tucson, AZ, USA) at the 2nd Department of Pathology, Semmelweis University. CINtec®PLUS reactions were performed manually by immunohistochemical assistant according to manufacturer instructions (Hoffmann-La Roche, Basel, Switzerland). The reactions were evaluated with multi-discussion microscope by cytologist/pathologist. Based on the company descriptions, those sample were considered positive in which both brown cytoplasmic staining for p16^{INK4a} and red nuclear staining for Ki67 appeared in the same cell. Using the same evaluation principles, the CLDN1/Ki67 reactions were performed on parallel slides produced from each LBC sample.

microRNA determination: Formalin-fixed paraffin-embedded (FFPE) samples were used for the determination of miR by means of the TaqMan MicroRNA Assay (Life Tech Thermo Fischer Sci Inc). Reverse transcription (RT) and quantitative PCR reaction was performed with the appropriate kit and Light Cycler 480 Instrument II (Roche Diagnostic) was used for the amplification (2nd Department of Pathology, Semmelweis University).

Statistical evaluation: The statistical analyses were performed with the help of Tímea Szekerczés, bioengineer, PhD student, with whom I am coauthor in several publications. For
the evaluations, 2-way Contingency Table Analysis was used based on Java Statistics (http://statpages.org/ctab2x2.html). Further analyses were performed using the Yates-corrected chi-square, the Mantel-Haenszel chi-square and the Fisher's exact test.

4. Results

4.1. The data of 1155 patients examined between 2007-2011 at the first Hungarian HPV Center revealed that **HPV 16** is the most common type of HPV, followed by types **HPV51 and HPV31**.

4.2. Studying a total of **687 parallel samples, no significant differences were found** between the results of **liquid based cytology (LBC) and conventional cytology samples** evaluated according to the Bethesda system.

4.3. 502 parallel LBC sliedes were used for **CLDN1 and p16^{INK4a} immunocytochemistry** According to statistical analysis, the sensitivity of CLDN1 reaction was slightly higher than that of p16^{INK4a} immunoreaction [77.3% (68.7 – 84.6%) vs. 69.3% (60.9 – 76.3)], the specificity, however was lower [60.9% (53.5 – 67.2) vs. 80.5% (73.2 – 86.5)]. Data proved that the sensitivity of LC-CLDN1 is the highest among the three tests (cytology, IC-CLND1, IC-p16^{INK4a}) , but the specificity is lower than the cytology.

4.4. Double immunoreaction with claudin-1 (CLDN1) and Ki67 was developed (CLDN1/Ki67) in cytological and histological specimens and compared with the results of CINtec®PLUS reaction. Atogether 2907 LBC smears were analysed, (in total 121 HSIL, 339 LSIL and 51 ASC-US samples, 2380 normal cases and 16 samples

that could not be evaluated). CINtec®PLUS reaction was performed in 1596 cases, out of it with 1386 cases with valuable results. The CLDN1/Ki67 reaction was performed in 1358 cases, out of it 1159 with acceptable results. The comparison of the two tests were possible in 1097 LBC samples. 840 smaples were negative and 163 positive equally by both methods ((κ=0,724, 95% CI from 0,672 til 0,776). The sensitivity of CLDN1/Ki67 reactions in HSIL lesions was 76,00%, sepcificity 85,67%, positive predictive value 20,21%, negative predictive value 98,6%. The sensitivity of CINtecPLUS in HSIL was 74,00%, specificity 81,38%, positive predictive value 15,98%, negative predictive value 98,50%. Using ROC (Receiver Operating Chracteristics) analysis, **there was no significant difference between the two tests (p=0,177)**.

4.5. In the 63 **histological samples** obtained by conization (7 carcinoma in situ, 30 HSIL, 3 LSIL cases and 23 specimens showing no dysplasia) the CLDN1/Ki67 reaction showed positivity in the abnormal samples (CIS, HSIL, LSIL cases), whereas the specimens showing no dysplasia proved to be negative.

4.6. RNA was isolated from 22 paired paraffin embedded samples obtained by laser microdissection. **miR-20b was signidicantly increased in the HSIL** samples as compared with the non-tumorous epithelium (p<0,0001).

5. Conclusions

5.1. Based on data from the first Hungarian Human Papilloma Virus Center, in the studied population the most common is **HPV type 16, followed by HPV types 51 és 31**. These data were later confirmed by others.

5.2. No difference was found in the diagnostics between the results of liquid based cytology (LBC) and conventional cytology tests. The **LBC method**, however, is more suitable for preparing multiple, single-cell layer slideswhich allowed analysis for further immunohistochemical reactions.

5.3. By claudin-1 (CLDN1) immunocytochemical reaction, the abnormal epithelial/tumor cells were detectable in cytological samples as well, similarly to histological sections. Statistical evaluation showed the sensitivity of the CLDN1 reaction to be higher, however the specificity did not reach that of the cytological tests. These findings demonstrate that **CLDN1 reaction can be used to detect premalignant and malignant cervical epithelial cells/tumor cells,** though this needs to be futher developed and associated with other biomarkers.

5.4. The use of the Ki67 protein as a proliferation marker following CLDN1 reaction (**CLDN1/Ki67** double immunocytochemical reaction) produced similar results regarding both sensitivity and specificity as did the CINtec®PLUS reaction. This proves that **CLDN1/Ki67 double immunoreaction is suitable for the detection of transformed cervical cells and shows similar sensitivity and specificity as the CINtec®PLUS test used as a comparison.**

5.5. Studies on the changes in microRNA expression in cervical premalignant and malignant lesions show contradictory data in the literature. Based on our results, **miR-20b revealed significantly increased expression** in CIN2-3 cases (HSIL) as

compared to the surrounding normal epithelium ($p<0,05$). Based on these data, miR20b could possibly be used as a biomarker in the future.

6. List of publications

6.1. Publications related to the thesis

1. **Galamb A**, Pajor A, Langmár Z, Sobel G. (2011) Results of the first human papilloma virus center in Hungary (2007-2011) [Az első magyarországi humán papillomavírus központ tapasztalatai (2007-2011)]. Orv Hetil, 152:1804-1807.

2. Benczik M, **Galamb Á**, Zinner B, Mikó M, Ács N, Jeney Cs, Sobel G. (2013) New molecular biology approaches and biological markers in cervical cancer screening. [Új molekuláris biológiai jelzők a méhnyakrák szűrésében]. Nőgyógy Onkol, 18:63-67.

3. **Galamb Á,** Benczik M, Zinner B, Vígh E, Baghy K, Jeney Cs, Kiss A, Lendvai G, Sobel G. (2015) Dysregulation of microRNA expression in human cervical preneoplastic and neoplastic lesions. Pathol Oncol Res, 21:503-508. **IF: 1,940**

4. Benczik M, **Galamb Á**, Koiss R, Kovács A, Járay B, Székely T, Szekerczés T, Schaff Zs, Sobel G, Jeney Cs. (2016) Claudin-1 as a biomarker of cervical cytology and histology. Pathol Oncol Res, 22:179-188. **IF: 1,736**

5. Szekerczés T, **Galamb Á**, Kocsis A, Benczik M, Takács T, Martonos A, Járay B, Kiss A, Jeney Cs, Nyíri M, Schaff Zs, Sobel G. (2019) Dual-stained cervical cytology and histology with Claudin-1 and Ki67. Pathol Oncol Res, 25: 477-486. *(shared first authorship)* **IF: 2,433**

6. **Galamb Á,** Szekerczés T, Benczik M, Schlachter K, Kocsis A, Kiss A, Schaff Zs, Lendvai G, Jeney Cs, Sobel G. (2020) MicroRNA expression in high-grade cervical intraepithelial neoplasia. Pathol Oncol Res, prepared forfor publication, February-March 2020. **IF: 2,433**

6.2. Lectures related to the thesis

1. **Galamb Á,** Sobel G, Ács N: New molecular biology methods and biology markers in cervical cancer screening. [Új molekuláris biológiai módszerek és biológiai jelzők a méhnyakrák szűrésében]. Fiatal Nőorvosok Jubileumi Kongresszusa, Mátraháza, október 0-12, 2014.

2. Sobel G, Vígh E, Benczik M, **Galamb Á**, Koiss R, Szekerczés T, Járay B, Schaff Zs, Ács N, Jeney Cs: Experiences with CINtecPlus (Roche) test in the evaluation of smears. [A CINTecPlus (Roche) teszttel szerzett tapasztalatok a cervicalis kenetek értékelésében]. Magyar Méhnyakkórtani és Kolposzkópos Társaság IV. Kongresszusa. I. Inter-diszciplináris HPV-kongresszus, Budapest, március 20-21, 2015.

3. Szekerczés T, Vígh E, Dóra R, Kocsmár É, Benczik M, **Galamb Á**, Koiss R, Kiss A, Lotz G, Járay B, Schaff Zs, Jeney Cs, Sobel G: Experiences with CINtec®Plus (Roche) test in the evaluation of smears. [A CINtec®Plus (Roche) teszttel szerzett tapasztalatok a cervicalis kenetek értékelésében]. 72. Pathológus Kongresszus, Hajdúszoboszló, szeptember 24-26, 2015.

4. Szekerczés T, Vígh E, Dóra R, Kocsmár É, Benczik M, **Galamb Á,** Koiss R, Kiss A, Lotz G, Járay B, Schaff Zs, Jeney Cs, Sobel G: Claudin-1 based double-labeling test for cervical samples. [Claudin-1 fehérjén alapuló, kettős jelölésű teszt kialakítása cervicalis mintákon]. 72. Pathológus Kongresszus, Hajdúszoboszló, szeptember 24-26, 2015.