Aus der Klinik für Gynäkologie

der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

Molekulare Diagnose von Lymphknotenmetastasen in endoskopisch behandeltem Gebärmutterhalskrebs: Genauigkeit des APTIMA Tests zur Detektion von High Risk Humanen Papillomvirus mRNA in Wächterlymphknoten

zur Erlangung des akademischen Grades

Doctor medicinae (Dr. med.)

vorgelegt der Medizinischen Fakultät

Charité – Universitätsmedizin Berlin

von

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aus Shandong, China

Datum der Promotion: 06.09.2019

1 Foreword—self-plagiarism

Partial results of this thesis were published in:

Köhler C, **Le X**, Dogan NU, Pfiffer T, Schneider A, Marnitz S, Bertolini J, Favero GMolecular Diagnosis for Nodal Metastasis in Endoscopically Managed Cervical Cancer: The Accuracy of the APTIMA Test to Detect High-risk Human Papillomavirus Messenger RNA in Sentinel Lymph Nodes. J Minim Invasive Gynecol.2016; 23(5):748-52.

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2 List of Abbreviations

ASCUS	Atypical squamous cells of uncertain significance
BP	Base pairs
CIN	Cervical intra-epithelial neoplastic
CT	Computed Tomography
DNA	Deoxyribonucleic acid
EBV	Epstein–Barr virus
FIGO	International Federation of Gynecology and Obstetrics
FDA	Food and Drug Administration
HP	Helicobacter pylori
HPV	Human papilloma viruses
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HHV	Human herpes virus
HR-HPV	High-risk HPV
HSV-2	Herpes simplex virus type 2
ITC	Isolate tumor cell
IARC	International Agency for Research on Cancer
LBC	Liquid-based cytology
LR-HPV	Low-risk HPV
MAC	Macrometastases
MIC	Micrometastases
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
NPV	negative predictive value
PPV	positive predictive value
SLN	Sentinel lymph node
SCCs	Squamous cell cervical cancer
TNM	Classification of Malignant Tumors
AdCa	adenosquamous carcinoma
AdSCCa	adenosquamous carcinoma

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5 ABSTRAKT

5.1 Deutsch

Einleitung Metastasen der Beckenlymphknoten sind die wichtigsten negativen Prognoseparameter für Patienten mit Zervixkarzinom im Frühstadium. Mikrometastasen und isolated Tumor Cells (ITC) sind durch Standard Histopathologie schwierig zu erkennen. Obwohl Studien über die Korrelation der Anwesenheit Humaner Papillomvirus-(HPV-) DNA in Beckenlymphknoten mit vorhandener Metastase in Gebärmutterhalskrebspatienten bestehen, sind die Analyse von HPV DNA aus Lymphknotenpräparaten auf Grund des Risikos einer falsch positiven Ergebnisse limitiert. In dieser Arbeit wurde das Vorhandensein von High Risk HR HPV-E6/E7 mRNA in Lymphknotengewebe evaluiert und mit entsprechenden Metastasen assoziiert.

Patienten und Methoden Die Studie beinhaltete 115 Patienten mit Zervixkarzinom im Frühstadium (FIGO IA bis IIB). Cytobrush-Technik wurde zur Probenentnahme aus Lymphknotengewebe angewandt, und diese schließlich pathologisch untersucht. HR HPV-E6/E7 mRNA wurde mittels APTIMA Assay detektiert.

Ergebnisse Das mittlere Patientenalter zum Zeitpunkt der Diagnose betrug 40±11 Jahre (Interquartilsabstand=24-72 Jahre). Vorhandener Gebärmutterhalskrebs war auf das Frühstadium begrenzt (FIGO). HPV-E6/E7 mRNA wurde in Primärmetastasen aller Patienten detektiert, bei 87 Patienten (75,7%) in Zervixmetastasen und in 84 Patienten (73%) in Lymphknotenmetastasen, wie durch abschließende Pathologie bestätigt. 5,6% (16 aus 287) der entnomenen Sentinellymphknoten testete positiv auf HPV-E6/E7 mRNA, entsprechend einer Positivität von 8,7% (10 aus 115) eingeschlossener Patienten. Alle durch Histologie als metastatisch bestätigte Lymphknoten (pelvine- und paraaortal) waren ebenfals HPV-E6/E7 mRNA-positiv. Zusätzlich wurden 8 bzw. 9 histologisch unauffällige Patienten und Lymphknoten positiv auf HPV-E6/E7 mRNA getestet. Die Beziehung von HPV zu histologischem Status zwischen primärer und Lympknotenmetastase, sowie zwischen pelvinen- und paraaortalen Lymphknoten bleIbt schwierig. HPV-Status und Histologie unterscheiden sich nicht zwischen SCC, Adenokarzinom oder Adenosquamösem Karzinom. Die Sensitivität, Spezifizität, NPV, PPV und dIagnostische

Effizienz der Überprüfung auf HPV-E6/E7 mRNA für Patienten mit Metastasen betrugen 100%, 92,4%, 100%, 52,6% bzw. 93%. Die Sensitivität, Spezifizität, NPV, PPV und diagnostische Effizienz der Überprüfung auf HPV-E6/E7 mRNA für Wächterlymphknoten mit Metastasen betrugen 100%, 96.6%, 100%, 67,9% bzw. 96,9%.

Zusammenfassung Das APTIMA HPV Assay ermöglicht die rasche und einfache Detektion von HPV-E6/E7 mRNA in Wächterlymphknoten. Positive Ergebnisse können auf eine Metastase im Frühstadium hinweisen. Die Resultate sollten jedoch durch unabhängige Testverfahren bestätigt werden.

328 Wörter

5.2 English

Introduction Nodal metastasis is the most important negative prognostic parameter for patients with early-stage cervical cancer. However, micrometastasis and isolated tumor cells are difficult to detect by conventional histopathology. Although some studies that focused on the correlation between the human-papillomavirus-(HPV)-DNA presence of pelvic lymph nodes and pathological metastasis in patients with cervical cancer have already been published, HPV DNA analysis of lymph node preparations is limited due to the high rate of false positive results. In the present paper, we evaluated the correlation of the presence of high risk HR HPV-E6/E7 mRNA in the lymph node with histological metastasis.

Patients and Methods The study included 115 patients with stage IA to IIB cervical cancer. The cytobrush technique was used for sample collection from the pelvic lymph nodes before sending them for pathological assessment. HR-HPV-E6/E7 mRNA was detected by APTIMA assay.

Results The median age of the patients at diagnosis was 40±11 years (interquartile range=24-72years). The status of HPV-E6/E7- mRNA was detected in the primary tumor in all patients. We found 87 (75.7%) patients with HPV-E6/E7- mRNA in the lymph nodes, while final pathology confirmed nodal metastases in 84 patients (73%). HPV-E6/E7-mRNA was positive in 5.6% (16/287) of the removed sentinel lymph nodes, representing a positivity of 8.7% (10/115) of the included patients. All the histologically confirmed metastatic lymph nodes (pelvic and paraaortic) were also HPV-E6/E7-mRNA positive. Additionally, pathology confirmed no metastases in 8 patients and 9 sentinel nodes were positive for HPV-E6/E7-mRNA. However, the relationship of HPV and histology status in pelvic and paraarotic lymph node is complicated and the status of HPV and histology is related to the stage but not histology type. The sensitivity, specificity, NPV and PPV of HPV-E6/E7-mRNA for patients with metastases were 100%, 92.4%, 100%, 52.6% and 93%, respectively. And the sensitivity, specificity, NPV and PPV of HPV-E6/E7-mRNA for lymph nodes with metastases were 100%, 96.6%, 100%, 67.9% and 96.9%, respectively.

Conclusion By using the APTIMA HPV assay, it is possible to detect the status of HPV-E6/E7-mRNA in the SLN. And HPV-E6/E7-mRNA is positive could be considered a sign of an early metastasis. Potential advantages of this method are the lower costs and the rapid

assessment to results. However, the result must be further explained carefully.

338 words

6 Introduction

6.1 LITERATURE REVIEW

Currently, cervical cancer is still a lethal disease that poses a threat to the health of a great number of young women. Worldwide, the occurrence rate of cancers is still high, exacerbated by problems such as gradual aging of the population, smoking, infection, environmental pollution, dietary structure, and other issues. The situation regarding the diagnosis and treatment of cancer continues to be extremely grim. Currently, there is a consensus that only through early detection, early intervention, and early treatment can the survival rates and the quality of life of cancer patients be improved. Therefore, scientists are continually searching for new methods that could be used for accurate diagnosis even before clinical signs or symptoms appear.

Nowadays, advances in molecular biology allow for the integration of laboratory methods into the diagnosis and monitoring of tumors. The development of molecular cancer diagnostics is becoming an important area of research. Recently, the molecular diagnostics of tumors have progressed greatly and are used for the following: early diagnosis and differential diagnosis; definition of tumor grade, stage and prognosis; and detection of metastasis in lymph nodes. The potential capabilities of the method also drive the discovery of new biological markers.

Although the mechanisms leading to tumor genesis are being intensively studied, key elements that lead to cancer are still waiting to be found and understood. Regarding biological factors, the relationship between viruses and human tumors was not understood for a long time. However, since the discovery of the Rous sarcoma virus in 1909, it has been recognized that the occurrence of tumors is often associated with infections by certain viruses. Currently, there is some epidemiological evidence showing that infections with persistent pathogens are strongly associated with cancer. It has been estimated that about 15%–20% of the global cancer burden is correlated with infectious agents[1].

The confirmation of the relationship between viruses and carcinogenesis opens new perspectives for cancer prevention, control, and treatment. As viruses can be detected more easily and accurately with the implementation of molecular biology techniques, tumors can potentially be diagnosed at an earlier stage by identifying the virus. Moreover, viral detection is more precise and less dependent on the pathologist than conventional histopathology. For this reason, some

types of viruses are used as markers in clinical practice. Infectious agents have different biological behaviors, and they should be better investigated to improve cancer prevention.

Cervical cancer is one of the types of cancer induced by viruses; the association between human papillomavirus (HPV) infection and the development of cervical cancer is well-established. By using modern technologies, one can detect the presence of HPV in 99% of primary cervical carcinomas[2]. Moreover, recently, HPV detection has been used as an important screening method for cervical cancer both in the United States and Western Europe. However, the incidence and mortality rate of cervical cancer are still increasing in some developing countries due to the lack of molecular tests [3].

Up to 15% of early-stage cervical cancer patients will develop recurrent cancer even after adequate primary therapy, such as surgery, chemotherapy, or radiotherapy[4]. It is believed that recurrence is due to the presence of cancer cells in the lymphatics or connective tissue, where the cells are undetected by conventional histopathologic analysis[5]. The survival rate of patients after cancer recurrence is significantly lower[6]. There is no doubt that cervical cancer is still a threat to women's health and quality of life worldwide. Therefore, it is important to find more reliable and effective methods to diagnose cervical cancer metastasis and micrometastasis.

In recent years, faster and more effective HPV tests have become commercially available. Isolated or combined with cervical cytology, the HPV test is an integral part of the cervical cancer screening program in many places of the world[7]. The widespread use of the HPV test provided the inspiration for examining the utility of HPV-detection techniques as markers for nodal metastases and micrometastases.

6.1.1 Cervical cancer

6.1.1.1 The status of the lymph node is important for fertility-sparing surgery

The overall incidence rate of cervical cancer has markedly decreased in developed countries due to the implementation of effective screening programs, demonstrating the importance of early diagnosis of and therapy for non-invasive diseases. Thus, in those countries, cervical cancer is detected considerably more frequently in the earlier stages than in other parts of the world. Approximately half of all cervical cancer cases are diagnosed at stage 1, and more than

three-fourths of cases are diagnosed by stage 2 or earlier[8]. In the past decade, the mortality of cervical cancer has decreased significantly compared with the 1970s. Currently, cervical cancer is only the 16th most common cause of cancer death among European women[9]. However, the incidence of cervical cancer among younger women is increasing. In Germany, 8.2 out of 100,000 women between the ages of 20 and 40 are diagnosed with cervical cancer every year[10]. Cervical cancer thus affects many women at child-bearing age, which, combined with a social trend of delaying childbearing, results in a large number of women who desire to have children. Therefore, cervical cancer represents a considerable physio-social burden for the affected women. Several studies suggest that cervical cancer survivors have significantly more reproductive concerns (such as the inability to bear children and not being able to talk openly about fertility[11]) compared to age-matched controls. Thus, preservation of fertility and reproductive function is a major concern for these young women with regard to the effects of treatment for cervical cancer. Less aggressive and fertility-sparing treatments need to be available to women with early-stage disease, with more options for family planning, while allowing them to maintain an acceptable quality of life. Over the past 15 years, gynecologic oncologists have sought ways to preserve female fertility when treating invasive cervical cancer. Radical vaginal trachelectomy with laparoscopic lymph node dissection for early cervical cancer was first introduced and described by Daniel Dargen[12]. Nowadays, this treatment for early cervical cancer has been accepted by most gynecologists in the world. The primary intention of the procedure was to preserve fertility while treating the cancer. The surgical procedure begins with a laparoscopic pelvic lymphadenectomy to exclude nodal metastasis, as the fertility-sparing surgery cannot be safely offered to patients with lymph node involvement.

6.1.1.2 Retroperitoneal lymphadenectomy is not a good method to evaluate the status of lymph node

Although the status of pelvic lymph nodes is not included in the FIGO stage[13], it is a crucial prognostic factor for initial assessment of cervical cancer. Accurate prediction of lymph node status is of extreme importance for the correct planning of treatment in patients with early cervical cancer. This cancer normally spreads in a continuous manner, initially involving the lower pelvic lymph nodes and then progressing to the higher pelvic lymph nodes, including the

common iliac nodes, followed by the para-aortic nodes[14]. Radical hysterectomy with retroperitoneal lymphadenectomy remains the gold standard to assess the nodal status[15]. However, besides extending the surgical procedure, potential complications, such as increased blood loss, neurovascular injury, infection, and lymphoceles, associated with lymphadenectomy, can lead to loss of reproductive capacity in young women[16, 17, 18]. Furthermore, the majority of patients with early-stage cervical cancer undergoing a pelvic or para-aortic lymphadenectomy in this setting will ultimately be found to have disease-free lymph nodes and thus will not benefit from lymphadenectomy, yet are subject to the morbidity of lymphadenectomy[19]. Thus, in most patients with cervical cancer, lymph node dissection could be omitted.

These observations have prompted some authors to recommend a less radical surgery or to question the necessity of complete lymphadenectomy. Also, there is a great interest in developing novel nodal assessment techniques that can minimize morbidity and maximize the detection of lymphatic metastases.

6.1.1.3 Sentinel lymph node biopsy could be a new choice if diagnostic accuracy could be improved further

Sentinel lymph node (SLN) biopsy has been suggested to substitute for lymphadenectomy[20]. The sentinel lymph node is the first one to receive the cancer cells along the route of lymphatic vessels from the primary tumor, while secondary spread to more distal lymph nodes or hematogenic metastasis may occur later. Theoretically, a histologically negative sentinel lymph node would predict the absence of tumor metastases in the other non-sentinel lymph nodes. A positive SLN biopsy result indicates that cancer is present in the SLN and may be present elsewhere. Currently, SLN mapping is part of the surgical management of selected cancer types, such as breast cancer, and it is widely recommended in gynecological oncology practices worldwide. Because the incidence of nodal metastases is relatively low in patients with early-stage cervical cancer[21], many patients with negative nodes will risk unnecessary morbidity if lymphadenectomy is performed; however, SLN biopsy can avoid unnecessary lymph node (LN) dissection and decrease the rate of related morbidity. Conventional imaging techniques (lymphangiography, computed tomography (CT), and magnetic resonance imaging (MRI)) notoriously fail to identify lymph node metastases accurately[22]. Additionally, the cervix has

complex lymphatic drainage due to its midline position. Anatomists have shown that lymph node drainage is predictable, but not uniform, so that identification of the SLN as the indicator of a cervical metastatic disease would be useful for improving the detection of cancer cell spread, and reducing morbidity from the method of detection. In addition, one of the important advantages of SLN biopsy is the identification of the key node(s), which enables the pathologist to evaluate the node more accurately or to subject the nodes to immunostaining against cytokeratin or molecular quantification by reverse-transcriptase PCR to identify micrometastases, thus increasing the reliability of lymph node surgical staging.

Therefore, there has been an increasing number of studies on sentinel node investigations in cervical cancer. Usually, the uterine cervix has copious lymphatic drainage consisting of primary and secondary groups of lymph nodes[23]. Localization of the SLN in cervical cancer has shown that the group of primary nodes is mainly located in iliac blood vessels (including internal, external) and obturator regions. The feasibility of SLN identification in cervical carcinoma is well-documented[24]. The sensitivity, defined as the detection of metastatic disease in the SLN, is reported to be high in some publications. The false-negative rates are acceptably low, and the negative predictive value in the literature can reach up to 97%; this means that the risk that any other pelvic lymph node is positive is as low as 0%–3% if the SLNs on both sides of the pelvis are negative. An SLN procedure will reduce the pelvic lymph node dissection rate from 80% to 10% with an acceptable risk of occult metastases of only 0.08%[25]. Thus, SLN mapping has been recognized as an appropriate alternative to surgical nodal assessment in patients with initial cervical cancer. Currently, the SLN biopsy technique has been used for selecting patients for surgery or radiotherapy and identifying candidates for fertility-sparing treatment. The SLN biopsy has the potential to change the current surgical treatment principles of cervical cancer.

However, up to 15% of initially lymph node-negative early-stage cervical cancer patients will develop recurrent disease, which is mainly due to the fact that micrometastases and isolated tumor cells (ITC) present in lymphatic and connective tissue are rarely identified by conventional histology analysis[5]. Although some proteins such as cytokeratin could be used as markers to label micrometastases or ITCs for detecting cervical cancer cells, they proved to be non-specific; hence, more advanced and less expensive diagnostic methods are needed.

6.1.2 HPV

6.1.2.1 Introduction

Currently, human papillomavirus (HPV) infection has become the most common sexually transmitted disease worldwide. The prevalence of HPV infection among sexually active adolescents and young adults is high. Genital HPV prevalence reaches a peak in women aged 16–25 years old. Almost half of women of reproductive age and nearly 80% of teenagers and young adults have the potential to develop chronic infections by different types of HPV. Another peak of infection among women is between 55 and 64 years of age according to some epidemiological studies[26]. Fortunately, most of the infections caused by HPV are of benign evolution, and 80%–90% are temporary, especially among women under 30 years of age, which is basically due to the action of the immune system. Despite that, HPV still causes approximately 5% of the cancer cases.

HPV is a large and diverse group of viruses. In recent years, more sensitive detection methods have allowed for the identification of multiple sub-types of HPV, and the number of newly characterized types is rapidly increasing. To date, approximately 200 types of HPV have been identified, and 150 variants have been completely sequenced. They are classified as high-risk and low-risk groups, based on their potential for carcinogenesis. According to the International Agency for Research on Cancer (IARC)[27], fourteen types are in the high-risk groups (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 66, 68, and 59) which contribute to the initiation and progression of genital high-grade dysplasia, carcinomas in situ, and invasive carcinomas[28].

The recognition that infection is an underlying cause of associated diseases, especially certain kinds of squamous cell carcinomas, has opened the door for the scientific community to gain extensive knowledge about HPVs. Clarifying the interactions of the virus with host cells, tissues, and the immune system has made it possible to implement strategies for prophylactic vaccination against HPV infections as well as develop increasingly sensitive and specific molecular diagnostic tools.

6.1.2.2 Molecular Structure

HPV are small non-enveloped viruses with a diameter of 52–55 nm, surrounded by a proteinaceous coat, which forms an icosahedral capsid. It has a genome of about 8000 base pairs

(bp) consisting of a covalently closed circular double-stranded DNA molecule. According to the protein expression during a viral cycle, the HPV coding region contains the early genes, E1, E2, E4, E5, E6, and E7, which are the most important ones for the transactivation of transcription, transformation, and replication, as well as viral adaptation to different cellular milieus, and both E6 and E7 are key requirements for productive replication and cell transformation.

However, only in a small proportion of high-risk HPV (HR-HPV) infection cases, is viral DNA inserted into the host genome. Once the viral genome is integrated, its DNA is damaged, and the expression of the E7 and E6 genes is no longer controlled by the virus. In these cases, the cell often displays a strong expression of E6 and E7 genes. The E6 and E7 proteins from high-risk HPV are sufficient for the induction and maintenance of cell transformation[29]. These two oncoproteins are responsible for the immortalization and malignant transformation of normal cells by different mechanisms. The overexpression of E6 and E7 within the proliferating cells is considered to be tumorigenic. The deregulated expression of these two genes is directly responsible for the accumulation of genetic errors in the infected cell and once the eventual integration of viral episomes into the host cell chromosome has taken place, a lot of the key cellular processes such as proliferation, senescence, apoptosis, differentiation, and immune response are altered. The HPV replication cycle depends on different HPV oncogenes and oncoproteins, the E6 and E7 genes certainly play causative roles and the increase of the severity of the cervical lesions was accompanied by the elevation of HPV E6 / E7 mRNA copies [30]. In fact, E6 and E7 have multiple interactions with important cell pathways of the host cells and this may represent a potential targets for the development of specific therapeutic alternatives for HPV-induced cancers. The E6 and E7 oncogenes are the most important factors for clarifying the status of HPV replication and cellular transformation in the tissues. Thus, probably the detection of the viral E6/E7 oncogenes from carcinogenic HPV types might serve as a potentially risky evaluation factor.

6.1.2.3 Detection assays

As a DNA virus, HPV can be detected by different assays, such as immunohistochemistry, in situ hybridization (ISH), RNA-Seq, and RT-PCR. However, all of these methods are technically demanding and time-consuming, which limits their use in clinical practice. Currently, they are employed mainly for basic science research or for detecting HPV in specimen tissues, especially

by immunohistochemistry.

Currently, there are approximately 148 different HPV tests commercially available worldwide. They can be divided into different groups according to their functions, for example some assays test for the presence of a pool of carcinogenic HPV types, while others provide information on individual genotypes. Some assays can detect HPV DNA, while others detect HPV mRNA. A selected number of tests have been clinically validated, and only a minority has been approved by the Food and Drug Administration (FDA). There are only four HPV DNA assays approved by the FDA[31]: (1) the Hybrid Capture 2 (HC2) (QiAgen), detecting 13 HR-HPV types; (2) Cervista HPV HR (Hologic), targeting 14 high-risk HPV types; (3) Cervista HPV 16/18 (Hologic), specifically designed to identify HPV16 and 18; and (4) Cobas 4800 HPV (Roche Diagnostics), targeting 14 high-risk types HPV types. However, methods used for diagnosing HPV infections have the L1 region as the target, a highly conserved viral genome region; such methods detect the presence of viral DNA only, but neither provide information on possible viral activity or productivity nor identify the infections that pose the highest risk of progressing to squamous dysplasia or neoplasia. Thus, novel HPV RNA-based assays are becoming popular because of their increased specificity compared to HPV DNA assays, including genotyping. There are two HPV mRNA tests currently approved by the FDA[32], the mRNA-based APTIMA HPV16-18/45 Genotype Assay (Hologic), and an HR-HPV E6/E7 mRNA-based screening test (APTIMA HPV Assay; (Hologic). The Aptima HR-HPV assay (AHPV; Hologic, Bedford, MA, USA), is a qualitative test for E6/E7 RNA for a pool of 14 HR-HPV genotypes (i.e., types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68). This assay is based on target capture following cell lysis, with subsequent transcription-mediated amplification and probe hybridization protection for the detection of E6/E7 mRNA expression in one measurement. The APTIMA assay can also be used for genotyping HPV 16 alone and HPV 18 and 45 together but requires a separate APTIMA 16, 18/45 genotype assay.

An ideal HR-HPV test for identification of HPV-associated disease that needs treatment must combine high clinical sensitivity with high clinical specificity. Compared to the DNA test, it is reported that AHPV has similar sensitivity to the HC2 assay, but is of higher specificity and higher predictive value (PPV) for high-grade neoplasms. Thus, it is noteworthy that the Aptima mRNA test may represent a novel diagnostic strategy and a very attractive option.

6.1.3 Cervical cancer and HPV

6.1.3.1 Persistent high-risk HPV infection is a necessary risk factor for the development of cervical cancer

In 1983, Prof. von zur Hausen demonstrated that HPV is the underlying cause of cervical cancer and this initiated a new era in the management of cervical cancer. Nowadays, persistent HPV infection with high-risk genotypes is widely accepted as the principal risk factor that is also necessary for the development of intraepithelial dysplasia and invasive cervical cancer[33]. Persistent HPV infection induces secondary genetic changes caused by the viral oncoproteins in normal cervical cells which finally induces a tumor.

Cervical cancer usually arises at squamous metaplastic epithelium in the transformation zone of the cervix when infected by one or more of the oncogenic HR-HPV types. HR-HPV infection in the basal cell layer of the cervical epithelium, which is often exposed by micro-abrasions on the cervical surface, can be initiated through micro-lesions of the tissues and cells, because active cell division, which occurs during wound healing, is necessary for the entry of viral genome into the nucleus. At some loci, HPV can multiply in an episomal state, with its replication cycle being closely linked to the differentiation of the infected cell. Also, the susceptibility of the cervical transformation zone to cancer progression may be linked to an increased likelihood of infection, particularly at puberty when metaplastic cells are present at this site 34. (The transformation zone is the area of the cervix where the columnar epithelium meets the squamous epithelium in the endocervix).

The HPV life cycle is divided into a productive phase and an abortive phase[35]. In the productive phase, viral genomes are maintained as low-copy-number episomes in the lower layers of the cervical epithelium. The episomes are amplified as the infected cells migrate toward the epithelial surface during cellular differentiation. During the abortive phase, HPV presents as a persistent infection with low copy numbers of HPV-DNA.

The viral products detected in the infected cervix depend on how complete the productive life cycle is, which potentially determines the severity of the cervical dysplasia (CIN). The E6 and E7 proteins are key regulators of cell cycle progression. In cervical disease, it is thought that the

level of expression of E6 and E7 closely correlates with the progression towards malignancy. Increased levels of E6 and E7 activity are thought to underlie the development of neoplasms. Unbalanced expression of high-risk E7 proteins can produce genome instability in the host cell through deregulation of the centrosome cycle, while deregulated expression of E6 contributes to the accumulation of mutations by compromising the DNA repair activity of p53. In low-grade CIN, these two viral proteins are expressed only in the basal and para-basal layers, but in high-grade neoplasms, such as CIN3 and SCC, they are found in all epithelial layers[36]. Moreover, viral E6 and E7 can contribute to enhanced resistance to chemotherapy and radiation therapy[37].

Following viral genome replication and cell division, one of the daughter cells migrates away from the basal layer and initiates a process of differentiation. However, unlike normal cells, HPV-infected cells undergo differentiation but remain active in the cell cycle. This proliferative activity is one of the reasons why approximately one-third of those with CIN-3 will progress to invasive cervical cancer in 10 to 20 years.

Currently, there are two major incidence peaks of HPV infection; in women under 30 years of age and in perimenopausal women. HPV tends to infect younger women because of their cervix is more frequently injured, and, unlike adults, the epithelium has larger areas of immaturity, characterized by a predominance of columnar and metaplastic cells [38]. During puberty, the cervix undergoes cellular changes, known as ectopy, at the transformation zone. The common presence of blood when cervical smears are obtained from these areas proves this area is fragile. As long as ectopy is present, the cervical cells may not only be more susceptible to HPV infection, but they are also more prone to develop persistent HPV infection[39].

Regarding the second incidence peak, there are two main theories: 1) a new sex partner or increased numbers of sex partners; and 2) immune senescence that allows a latent infection to emerge[40]. Thus, although vaccination can help prevent some percentage of cervical occurrence, diagnosis and therapy of cervical cancer is still an important issue. As the induction of cervical carcinogenesis by persistent HR-HPV infection is a multistep process consisting of persistent infection, different stages of CIN lesions, and ultimately, cervical cancer. This process develops slowly and may take up to 10 to 20 years; at the same time, this fact makes this type of cancer easily preventable, detectable, and treatable[32].

6.1.3.2 The significance of HPV for the diagnosis and prediction of cervical cancer.

I. HPV and cervical cancer primary lesion

A. HPV can be used for diagnosing cervical cancer

The use of biomarkers in cytological and histological samples has significantly improved the results of cervical cancer screening. One of the promising methods is based on the detection of excessive cellular protein synthesis that is directly or indirectly activated by deregulated expression of viral oncogenes E6 and E7. Unfortunately, this technology is used only for research purposes and not in clinical practice.

Given the strong association between HR-HPV infection and cervical cancer, HR- HPV testing is considered as an alternative to cytology-based cervical cancer screening. One study has demonstrated that the use of HPV DNA testing once in life reduces mortality from invasive cervical cancer by approximately 50%[41]. New cervical cancer screening guidelines adopted in the United States that are about to be adopted in many European countries recommend that women be tested only for HPV. Compared to the conventional Pap smear or the liquid-based cytology methods, HPV DNA detection has both higher sensitivity and a negative predictive value. Numerous randomized controlled clinical trials have demonstrated that the HPV DNA test can detect approximately 50% more high-grade cervical dysplasia than conventional cytology[42]. However, DNA-based assays provide information exclusively about the presence of the virus, but cannot define the state of infection. This fact leads to a significant reduction in test-specificity due to a large number of transient HPV infections, especially among the younger population. The use of the HPV DNA test exclusively in a screening program would increase costs without proven benefits and cause an excessive number of unnecessary examinations and treatments.

Moreover, potential overtreatment can increase the risk of preterm delivery and other obstetrical complications[43]. These considerations are particularly important for adolescents and young women since the age of first pregnancy is often postponed in developed countries. Thus, it is crucial to find new biological markers which can 1) anticipate the emergence of high-grade lesions; 2) improve screening programs; and 3) decrease the number of women referred to unnecessary colposcopy.

E6/E7 oncogenes are expressed through mRNA. The messenger RNAs for E6/E7 may be

promising alternatives to test for because E6/E7 mRNA expression occurs only in actively infected cells and gross transcript levels increase during the development of cervical dysplasia and progression towards invasive disease; thus, the false-positive rates are decreased in this test[44]. It was hypothesized that HPV mRNA might be more specific but less sensitive than DNA-based tests. Hence, positive results for HPV were expected to be lower with an mRNA test targeting oncogenic expression than with a DNA test that detects the HPV presence non-specifically. This question has been addressed in some publications, where it was found that only a small proportion of HPV DNA-positive women with normal, atypical squamous cells of uncertain significance (ASCUS) or low-grade squamous intraepithelial lesions have detectable mRNA expression. Thus, mRNA, as a triage test, could reduce excessive exposure to diagnostic procedures as well as treatment, and consequently, reduce the psychological burden associated with HPV-DNA testing.

In conclusion, type-specific HR-HPV E6/E7 mRNA, as a marker of productive and persistent infection, might serve as a better risk evaluation factor for monitoring HPV DNA positive women, by predicting high-grade cervical intraepithelial neoplasia and invasive cervical cancer more accurately than DNA tests. mRNA testing represents a new challenge, but with the promising possibility of being integrated into the pool of valuable molecular tools that may lead to the elimination of invasive cervical cancer in women in the near future.

B. HR-HPV-mRNA is a good indicator of recurrence and persistence of lesion

The recurrence or persistence of CIN after surgical treatment is an important risk factor for progression to invasive cancer. These can occur at frequencies ranging from 5% to 53%. Approximately 16% of diagnosed invasive cervical cancers have previously been treated as intraepithelial neoplasia[45]. The reason is that there is a residual disease in these patients' cervix due to incomplete removal of the primary lesion and persistent infection of HR-HPV remaining after treatment. Some authors observed that HPV DNA persistence strongly correlated with residual or recurrent lesion and abnormal cervical cytology during follow-up after treatment. It is more frequently detected in patients with positive HR-HPV infection after conization than in patients testing negative for HR-HPV infection[46]. However, no recurrent or residual disease was found among women whose test results were negative for type-specific HPV. Indeed, a positive test for HR-HPV during follow-up is the most significant independent predictor of recurrent

disease and the most powerful predictor of progression to invasive disease. Patients who test negative after treatment could be safely removed from clinical surveillance. Conversely, patients who remain infected may not have had their lesions fully removed and would require more frequent and comprehensive surveillance. Compared to cytology, HPV DNA testing allows quicker identification of residual/recurrent CIN and has a higher sensitivity and better negative predictive value (NPV)[47].

However, the HR-HPV test has lower specificity than cytology in distinguishing a persistent disease from a relapse, because DNA-based assays cannot distinguish between persistent infections and new transient ones. Moreover, recent studies suggest that the level of HR-HPV-mRNA tests, which show higher specificity and NPV than DNA-based tests, may be better indicators of the risk of persistent disease or relapse in women than DNA-based tests.

In conclusion, because of the sensitivity, negative predictive value, and optimal reproducibility of HPV testing, HR-HPV-testing can be used as an accurate indication of disease clearance. HR-HPV-testing is currently considered a powerful tool in clinical practice to improve the management of patients with cervical dysplasia.

II. HPV could be used in predicting lymph node metastasis

Up to 15% of the initially lymph node-negative early-stage cervical cancer patients may develop recurrent disease[48]. Since micrometastasis and ITC are rarely identified by conventional histology, one may suppose that recurrence cases may be linked to an incorrect diagnosis. Regional and systemic cervical cancer metastatic cells exhibit the transformed genotype induced by HPV in 99% of the cases. Thus, detection of actively transcribed nucleic acids of HPV oncogenes in the epithelial cells of lymph nodes indicates the presence of metastatic cancer cells. Previous studies[49, 50] have already investigated the applicability of testing for HPV-DNA in predicting nodal metastasis. The authors observed a very high sensitivity, but the specificity was very low, leading to an unacceptable rate of false positive results. Since HPV-E6/E7-mRNA is directly linked only to active forms of the virus, some authors[51, 52] have focused on the expression of E6/E7-mRNA in the lymph node and have found that it has a similar sensitivity but higher specificity than DNA-based tests. However, there is only one related paper that used a complex methodology which limits the clinical application of the method. Thus, the

implementation of easier, faster, and more convenient diagnostic methods is urgently needed to detect MIC and ITC in the lymph nodes.

6.2 Objective

In the above setting, we used APTIMA assays to observe the relationship between the status of HR-HPV E6/E7 mRNA in the SLN and histological evidence of metastasis to evaluate the predictive value of HR HPV-E6/E7 mRNA for the metastatic involvement of SLN.

7 Methods:

7.1 Patients and Methods

Following ethics board review approval (Hamburg ethics committee PV5000), we conducted a prospective study of women diagnosed with cervical cancer (FIGO stage I-II) submitted to laparoscopic retroperitoneal lymphadenectomy and/or sentinel node biopsy between November 2014 and November 2016. The trial was conducted in the Asklepios Hospital, Hamburg-Harburg, Germany. Cytology detected was performed in the Asklepios Hospital, Hamburg-Harburg, Germany. And HPV-mRNA testing was performed by the Institute for Cytology and Dysplasia, Berlin, Germany.

Inclusion criteria were as follows: (a) histologically and molecularly confirmed HPV-associated cervical cancer, (b) age older than 18 and younger than 80 years, and (c) no evidence of extra-pelvic disease on initial imaging staging.

Exclusion criteria were as follows: (a) no written informed consent, (b) clinical or surgical contraindication for surgery, (c) evidence of peritoneal or distant metastasis detected preoperatively or during surgery, (d) molecularly confirmed HPV-negative cancer. All women eligible for the study underwent laparoscopic identification and harvesting of SLN alone or followed by comprehensive endoscopic retroperitoneal lymphadenectomy.

The primary objective of this study was to evaluate the feasibility and accuracy of the commercially available HR-HPV-E6/E7-mRNA (APTIMA® HPV Assay, Hologic, Bedford, Massachusetts, USA) for the detection of metastasis in the sentinel lymph nodes of patients with HPV-positive CC.

We determined the sensitivity and false-negative rates of the method. The definitive histopathological result of systematic lymphadenectomy was considered as the criterion standard parameter of comparison.

The following information was collected: age of the patients at diagnosis, histological type, extension of nodal dissection, final pathology and the result of HR-HPV-E6/E7-mRNA detection.

7.2 The SLN Biopsy Technique:

At the beginning of surgery, the patients were placed in a lithotomy position and injected with 4 cc of Patent Blue into the cervix. Once the administration of the labeling substance was finished, the patients underwent laparoscopic sentinel node biopsy and retroperitoneal lymphadenectomy. The first part of the procedure is consist of a careful inspection of the abdominal cavity and peritoneal washing, followed by a laparoscopic visual identification of the SLN. After locating the transperitoneal blue spots, the retroperitoneum was accessed and the spaces were opened. Each colored lymph node was separately excised. In patients with tumors >2cm or if there was no agreement for sentinel concept, transperitoneal bilateral pelvic and, if indicated, paraaortic lymphadenectomy were systematically performed following the technique that has already been described.

7.3 Sample collection for molecular test

Prior to surgery, samples for the HPV-mRNA test from the primary tumor were collected by using the cytobrush technique and cells were suspended in the PreservCyt transport medium (**Figure 1**).

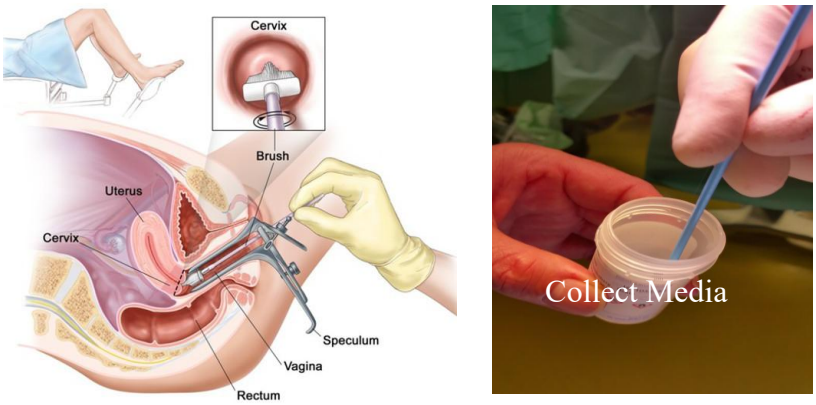

Figure 1: Collection of samples from primary tumor before surgery

Each harvested sentinel lymph node was cut lengthwise and a smear was taken by using a cytobrush from the cut plane of both halves of the sentinel lymph node. To avoid contamination, a fresh scalpel was used for the transection of each individual sentinel lymph node. The cytobrush was immersed in PreservCyt transport medium and sent for HPV mRNA analysis (**Figure 2**).

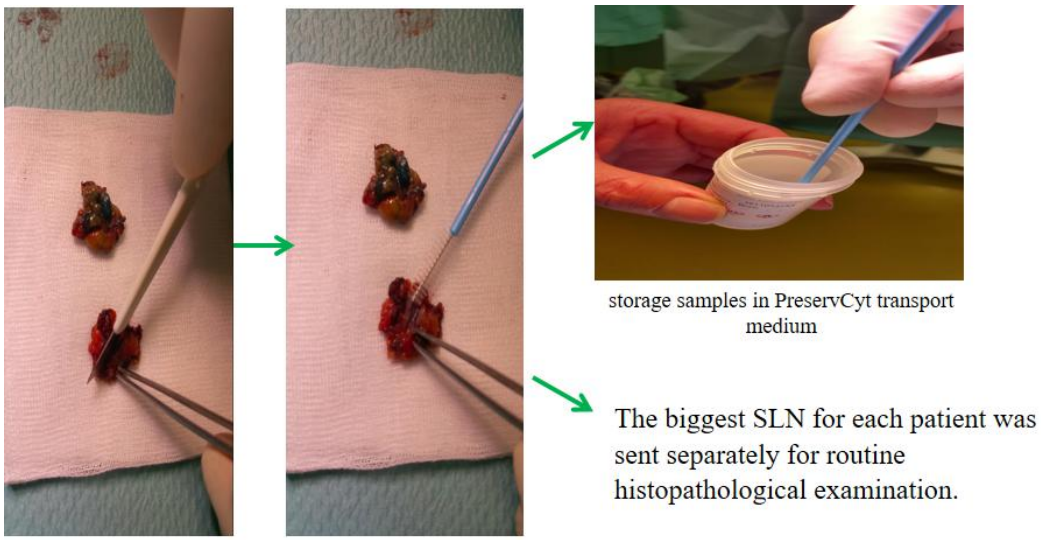

Figure 2: Collection of samples from lymph node during surgery

7.4 Detection of HPV-E6/E7-mRNA in the primary tumor and in the sentinel nodes

APTIMA® HPV assay (AHPV) is a qualitative test for detecting mRNA expressed from 14 different types of high-risk HPV viral E6/E7 oncogenes (16/18/31/33/35/39/45/51/52/56/58/59/66 and 68). The procedure was carried out according to the manufacturer's instructions. Briefly, a 1 mL aliquot of each of the PreservCyt samples was transferred to 2.9 mL of buffered detergent solution. A 400 µL aliquot of this mix was then tested on a semiautomatic Direct Tube Sampling system (Gen-Probe). Assay results were determined on the basis of the signal-to-cutoff ratio (S/CO) for the analysis. Specimens with an S/CO value of ≥1.0 were considered positive. The results were obtained within approximately 20 minutes during diagnostic HPV detection.

7.5 Sentinel Node Processing and Histopathological Analysis:

Sentinel lymph nodes were either sent to AK perform frozen section or fixed in neutral buffered formaldehyde for approximately 24 hours. Following fixation, lymph nodes were cut perpendicular to the long axis into slices 0.2 cm thick and embedded in a paraffin block. Multiple sections were prepared from each block. A set of three 4 µm thick sections was cut every 250 µm and stained with hematoxylin-eosin. Detection of tumor cells defined a positive SLN. If the SLN is negative, then ultrastaging and IHC will be done. All non-sentinel nodes were processed identically by the pathologists, cut into 3 to

4 mm sections, and submitted for routine staining (hematoxylin-eosin) and evaluation.

7.6 Statistical Analysis:

Continuous variables are presented as means and standard deviations. Categorical variables are presented as numbers of cases or percentages. The false negative rate was defined as the number of procedures with a negative SLN divided by the number of procedures in which the sentinel node was identified and a positive lymph node was found. Fisher, Kruskal-Wallis and Linear-by-Linear Association were used to compare differences between groups. A ROC curve was used to assess the diagnostic accuracy (sensitivity and specificity) of E6/E7 mRNA. The significance of the ROC analysis was based on the calculated area under the curve, with a corresponding 95% confidence interval. The predictive power of the assessed examination was described by sensitivity, specificity, negative and positive predictive value, overall accuracy, as well as ROC-derived area under curve. All tests were two-sided and the p-value < 0.05 was considered as the cut-off level for statistical significance for all analyse. Statistical analyses were performed using SPSS 20.0 for Windows software.

8 Results

8.1 Basic information of patients

During the period of the study, 115 patients with cervical cancer were initially selected to participate in the study. One patient was subsequently identified as not meeting the inclusion criteria and was excluded from the trial: this patient had an HPV-negative adenocarcinoma of gastric type. All 115 women included in the study underwent laparoscopic SNB, followed by endoscopic pelvic +/-PA lymphadenectomy (21 cases). The median age of the patients was 40 +11 years (interquartile range = 24-72 years). Histology revealed squamous-cell carcinoma (SCC) in 81 cases (70%), adenocarcinoma in 32 cases (27.8%), whereas adenosquamous carcinoma was found in the other 2 patients (1.7%). The distribution of the stages according to the revised 2009 FIGO staging system for cervical cancer was as follows: IA, n =30 (26.1%); IB1, n = 64 (55.7%); IB2, n = 10 (8.7%); IIB, n = 11 (9.6%) (Epidemiological, histological and stage features of the included patients are summarized in **Table 1**.)

Table 1: Epidemiological and histological features of the patients with CC included in the study

	Sum	***age***	***Histology type***			***Stage***				
			SCC	AdCa	AdSCCa	IA	IB1	IB2	IIA	IIB
Whole group	115	40±11	81	32	2	30	64	10	0	11

AdCa: adenosquamous carcinoma, SCC: squamous cell cervical cancer, AdSCCa: adenosquamous carcinoma.

8.2 The status of HPV-E6/E7- mRNA and histology

We found 87 (75.7%) patients with HPV-E6/E7- mRNA in the cervix, while final pathology confirmed nodal metastases in 84 patients (73%). There were two patients with HPV-E6/E7-mRNA but their histology confirmed negative (Table 2). And 87 (75.7%) patients with HPV-E6/E7- mRNA in the cervix, while final pathology confirmed nodal metastases in 84 patients (73%).

Table 2: Association of the HPV-mRNA status with final conventional histopathology and HPV mRNA status in the cervix

		Histology		
		Positive	Negative	Sum
HPV	Positive	84	3	87
	Negative	0	28	28
	Sum	28	86	115

Sentinel nodes were identified in all patients. All women had at least 1 sentinel node in each hemi-pelvis and 21 (18.2%) patients had also a paraaortic SLN. A total of 287 SLNs (264 pelvic and 23 PA) were removed, and the mean number of SLNs per patient was 2.5 (range, 2-4). The results of the final pathology confirmed nodal metastases in 19(6%) SLNs from 13 different patients (11%). HPV-E6/E7-mRNA was positive in 16/287 (5.6%) of the removed sentinel lymph nodes, representing a positivity of 10/115 (8.7%) of the included patients. The status of histology and HPV both in the cervix and lymph node are summarized in the **Tables 3 and 4**, **Figures 3 and 4**.

Table 3: The status of histology and HPV in both primary and lymph nodes for patients.

	Sum	***Cervical status***				***Pelvic Lymph node status***				***Paraarotic Lymph node status***			
		Histology		HPV		Histology		HPV		Histology		HPV	
		+	-	+	-	+	-	+	-	+	-	+	-
Patients involved number	115	84	31	87	28	10	105	16	99	3	18	4	17

+: Positive, -: Negative.

Table 4: **The status of histology and HPV in the lymph node.**

		Sum	*Lymph node status*			
			Histology		HPV	
			+	-	+	-
Lymph node	Pelvic	264	16	248	23	241
	Paraarotic	23	3	20	4	19
	Sum	287	19	268	27	260

+: positive, -: negative.

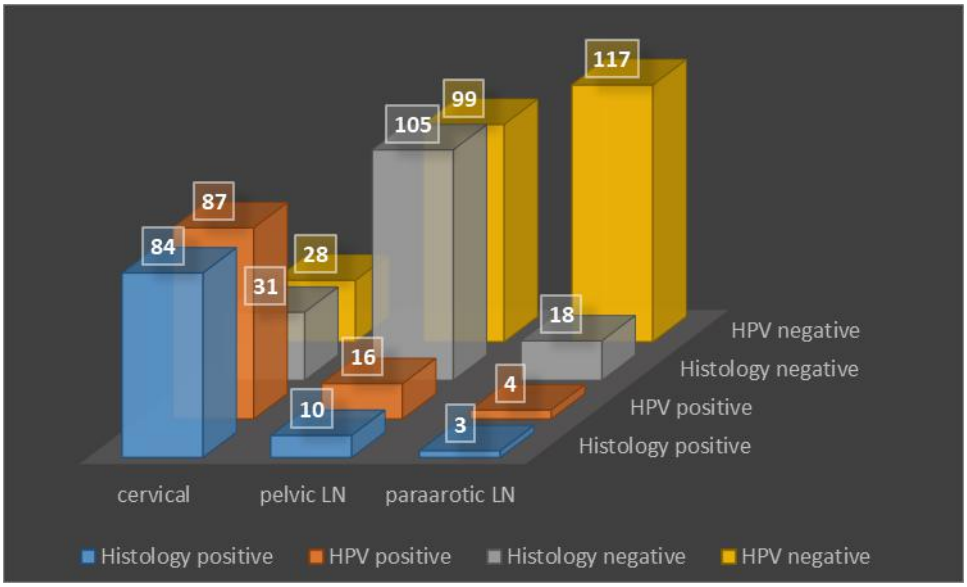

Figure 3: The status of histology and HPV in both primary and lymph nodes of patients. The status of histology and HPV of patients are summarized based on cervical, pelvic LN and paraarotic LN.

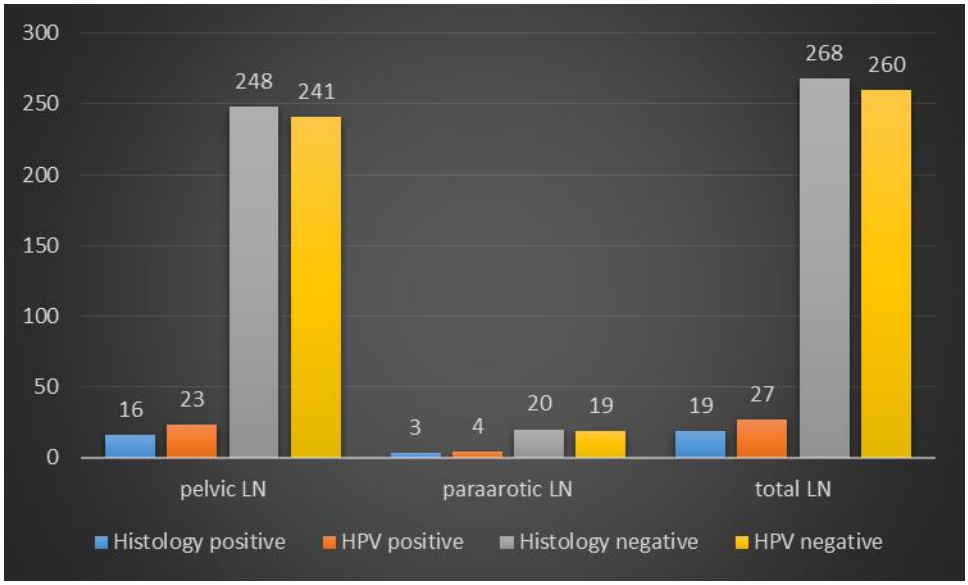

Figure 4: The status of histology and HPV in the lymph node.The status of histology and HPV of LN are summarized based on pelvic LN，paraarotic LN and total LN.

Further comparing basic information between patients groups with or without metasitasis and HPV in the lymph node ,it is found that metastasis and HPV could be detected in the lymph node only related to the FIGO stage ($p \leq 0{,}001$) but not age or histology type($p \geq 0{,}05$). Details can be seen in **Table 5**.

Table 5: Basic information on patient groups with or without metastasis and HPV in the lymph node.

		Histology and HPV status				*Sum*	*P Value*
		HPV(+) Histology(-)	HPV(+) Histology(+)	HPV(-) Histology(-)	HPV(-) Histology(+)		
Age		47±12	38±9	40±12	-	40±11	0.300
Stage	IA	1	1	28	0	30	0.00
	IB1	3	2	59	0	64	
	IB2	2	1	7	0	10	
	IIA	0	0	0	0	0	
	IIB	0	6	5	0	11	
	Sum	6	10	99	0	115	
Histology type	SCC	3	9	69	0	81	0.39
	Adenocarcinoma	3	1	28	0	32	
	Adenosquamous carcinoma	0	0	2	0	2	
	Sum	6	10	99	0	115	

SCC: squamous cell cervical cancer.

8.3 Comparison the status of HPV with histology

The sentinel node biopsy had a correlation rate of 100% with the status of other removed nodes (no case of false negativity). All the histologically confirmed metastatic lymph nodes (pelvic and paraaortic) were also HPV-E6/E7-mRNA positive. Additionally, 8 patients and 9 sentinel nodes were histologically negative, while HPV-E6/E7-mRNA were positive. The results are summarized in the **Tables 6 to 9**. For detailed information on patients with HPV-E6/E7-mRNA positive in the lymph node, see **Table 10 and Table 11**.

Table 6: Comparison of patient's status of histology and HPV in the lymph node.

patient		***Histology***		
		Positive	Negative	Sum
HPV	Positive	10	8	18
	Negative	0	97	97
	Sum	10	105	115

Table 7: Comparison of lymph node's status of histology and HPV.

Total lymph node		***Histology***		
		Positive	Negative	Sum
HPV	Positive	19	9	28
	Negative	0	259	259
	Sum	19	268	287

Table 8:**Comparison of pelvic lymph node's status of histology and HPV**.

pelvic lymph node		***Histology***		
		Positive	Negative	Sum
HPV	Positive	16	8	24
	Negative	0	240	240
	Sum	16	248	264

Table 9: **Comparison the status of histology and HPV in the paraaortic lymph node.**

paraaortic lymph node		*Histology*		
		Positive	Negative	Sum
HPV	Positive	3	1	4
	Negative	0	19	19
	Sum	3	20	23

Table 10: **Detailed information on patients with HPV-E6/E7-mRNA positive**

Pathology and HPV positive patients' information													
No.	age	Stage	Histo-logy type	Cervical status		Pelvic LN				Paraaortic LN			
				Histo-logy	HPV	Detecti-on SLN number	Histolo-gy status	HPV status	Consistency of Histology and HPV	Detection SLN Number	Histolo-gy status	HPV	Consistency of Histology and HPV
1	45	IIB	SCC	+	+	3	**No.1 +** **No.2 +** **No.3 -**	**No.1 +** **No.2 +** **No.3 -**	Consistent	0	No	No	No
2	27	IA	AdCa	-	-	2	**No.1 +** **No.2 -**	**No.1 +** **No.2 -**	Consistent	0	No	No	No
3	35	IIB	SCC	+	+	2	**No.1 +** **No.2 -**	**No.1 +** **No.2 -**	Consistent	1	+	+	Consistent
4	46	IIB	SCC	+	+	2	**No.1 +** **No.2 +**	**No.1 +** **No.2 +**	Consistent	1	+	+	Consistent
5	29	IB2	AdSCCa	+	+	2	**No.1 +** **No.2 -**	**No.1 +** **No.2 -**	Consistent	1	-	-	Consistent
6	29	IIB	SCC	+	+	3	**No.1 +** **No.2 +** **No.3 -**	**No.1 +** **No.2 +** **No.3 -**	Consistent	1	-	-	Consistent
7	54	IIB	SCC	+	+	2	**No.1 +** **No.2 +**	**No.1 +** **No.2 +**	Consistent	1	+	+	Consistent
8	42	IB1	SCC	+	+	2	**No.1 +** **No.2 -**	**No.1 +** **No.2 -**	Consistent	1	-	-	Consistent
9	30	IIB	SCC	+	+	3	**No.1 +** **No.2 -** **No.3 -**	**No.1 +** **No.2 -** **No.3 +**	Inconsistent	1	-	-	Consistent

AdCa: adenosquamous carcinoma, No.: number, SCC: squamous cell cervical cancer, AdSCCa : adenosquamous carcinoma; +: positive, -: negative.

Table 11: **Detailed information on patients with HPV-E6/E7-mRNA positive**

Pathology negative and HPV positive patients' information													
No.	age	Stage	Histo-logy type	Cervical status		Pelvic LN				Paraarotic LN			
				Histo-logy	HPV	Detecti-on SLN number	Histolo-gy status	HPV status	Consistency of Histology and HPV	Detection SLN Number	Histolo-gy status	HPV	Consistency of Histology and HPV
10	39	IB1	SCC	+	+	3	**No.1 +** **No.2 +** **No.3 -**	**No.1 +** **No.2 +** **No.3 -**	Consistent	1	-	+	Inconsistent
11	36	IB2	SCC	+	+	2	**No.1 -** **No.2 -**	**No.1 +** **No.2 -**	Inconsistent	0	-	-	Consistent
12	53	IB2	AdCa	+	+	3	**No.1 -** **No.2 -** **No.3 -**	**No.1 +** **No.2 -** **No.3 +**	Inconsistent	1	-	-	Consistent
13	55	IB1	SCC	+	+	2	**No.1 -** **No.2 -**	**No.1 +** **No.2 -**	Inconsistent	1	-	-	Consistent
14	61	IB1	AdCa	+	+	2	**No.1 -** **No.2 -**	**No.1 +** **No.2 -**	Inconsistent	1	-	-	Consistent
15	46	IA	SCC	+	+	3	**No.1 -** **No.2 -** **No.3 -**	**No.1 -** **No.2 -** **No.3 +**	Inconsistent	0	-	-	Consistent
16	30	IB1	AdCa	+	+	2	**No.1 -** **No.2 -**	**No.1 +** **No.2 -**	Inconsistent	0	-	-	Consistent

AdCa: adenosquamous carcinoma, No.: number, SCC: squamous cell cervical cancer, AdSCCa : adenosquamous carcinoma; +: positive, -: negative.

We further compared basic information among four groups: HPV(+) and histology(+), HPV(+) and histology(-),HPV(-) and histology(+), HPV(-) and histology(-) and found that there is a significant difference only between HPV(+) and histology(+) group and three other groups related to stage ($p \leq 0{,}001$) but not age or histology type($p \geq 0{,}05$). Details could are shown in Table 12.

Table 12: Comparison of age, stage and histology type of different groups, subvided by the status of HPV and histology.

		Histology		*Sum*	*P Value*	*HPV*		*Sum*	*P Value*
		+	-			+	-		
Age		38±9	41±12	40±11	0.386	41±11	40±12	40±11	0.935
Stage	IA	1	29	30	0.000	2	28	30	0.00
	IB1	2	62	64		5	59	64	
	IB2	1	9	10		3	7	10	
	IIA	0	0	0		0	0	0	
	IIB	6	5	11		6	5	11	
	Sum	10	95	115		16	99	115	
Histology type	SCC	8	72	8	0.767	12	69	81	0.837
	AdCa	2	31	33		4	28	32	
	AdSCCa	0	2	2		0	2	2	
	Sum	10	105	115		16	99	115	

AdCa: adenosquamous carcinoma, SCC: squamous cell cervical cancer, AdSCCa: adenosquamous carcinoma.

In addition, the relationship between HPV and histology status between pelvic and paraarotic lymph nodes was: 1) 11 patients with negative HPV and histology status both in the pelvic and paraarotic lymph nodes; 2) 3 patients with positive HPV and histology status both in the pelvic and paraarotic lymph nodes; 3) 3 patient with positive HPV and histological in the pelvic lymph node, while the paraarotic lymph node was HPV positive without histology confirmation; 4) 3 patients with positive HPV but negative histology in the pelvic lymph node, while HPV and histology status were both negative; 5) 1 patient with positive HPV and histology in the pelvic

lymph node, while her paraaortic was HPV positive without confirmed histology.(Details in the table 13)

Table 13: the correlation rate of HPV and histology between pelvic and paraaortic lymph nodes.

		Pelvic lymph node				
		HPV(+) Histology(-)	HPV(+) Histology(+)	HPV(-) Histology(-)	HPV(-) Histology(+)	Sum
Paraaotic Lymph Node	HPV(+) Histology(-)	0	1	0	0	1
	HPV(+) Histology(+)	0	3	0	0	3
	HPV(-) Histology(-)	3	3	11	0	17
	HPV(-) Histology(+)	0	0	0	0	0
	Sum	3	7	11	0	21

The status of HPV and histology is not different among patients with SCC, adenocarcinoma or adenosquamous carcinoma (see Table 14).

Table 14: **HPV and histology expressed in the lymph nodes based on different histology type.**

		Histology and HPV status				***sum***	***P Value***
		HPV(+) Histology(-)	HPV(+) Histology(+)	HPV(-) Histology(-)	HPV(-) Histology(+)		
Age		47±12	38±9	40±12	-	40±11	0.300
Stage	IA	1	1	28	0	40	<0.01 1
	IB1	3	2	59	0	64	
	IB2	2	1	7	0	10	
	IIA	0	0	0	0	0	
	IIB	0	6	5	0	11	
	Sum	6	10	99	0	115	
Histology type	SCC	3	9	69	0	81	0.39
	AdCa	3	1	28	0	32	
	AdSCCa	0	0	2	0	2	
	sum	6	10	99	0	115	

AdCa: adenosquamous carcinoma, SCC: squamous cell cervical cancer, AdSCCa: adenosquamous carcinoma.

8.4 The sensitivity, specificity, NPV and PPV of HPV-E6/E7-mRNA diagnosis histology

The sensitivity, specificity, NPV and PPV of HPV-E6/E7-mRNA for patients with metastases were 100%, 92.4%, 100%, 52.6% and 93%, respectively. And the sensitivity, specificity, NPV and PPV of HPV-E6/E7-mRNA for lymph nodes with metastases were 100%, 96.6%, 100%, 67.9% and 96.9%, respectively(as the tables 15 and 16, Figures 5 and 6 shown).

Table 15: **Sensitivity, specificity, NPV and PPV of HPV-E6/E7-mRNA identify lymph node metastasis status of patients.**

patients	*Sensitivity*	*Specificity*	*NPV*	*PPV*	*Diagnostic efficacy*
outcoming	100%	92.4%	100%	52.6%	93 %

NPV: negative predictive value, PPV: positive predictive value

Table 16: **Sensitivity, specificity, NPV and PPV of HPV-E6/E7-mRNA identify metastases status of lymph node.**

Lymph node	*Sensitivity*	*Specificity*	*NPV*	*PPV*	*Diagnostic efficacy*
outcoming	**100%**	**96.6%**	**100%**	**67.9 %**	**96.9%**

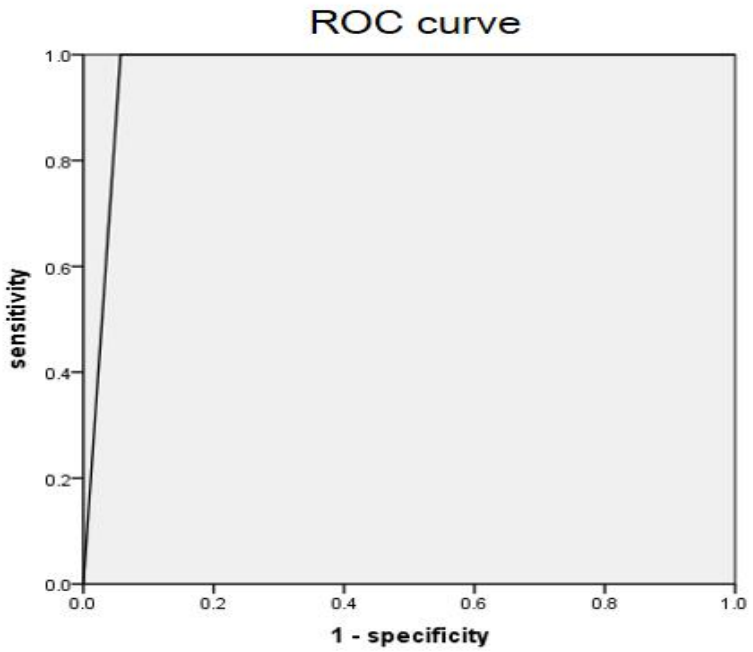

Figure 5: Receiver operating characteristic curves for HPV- mRNA identify lymph node metastasis status of patients. AUC=0.962. [95% confidence interval (CI) 0.929-0.995]

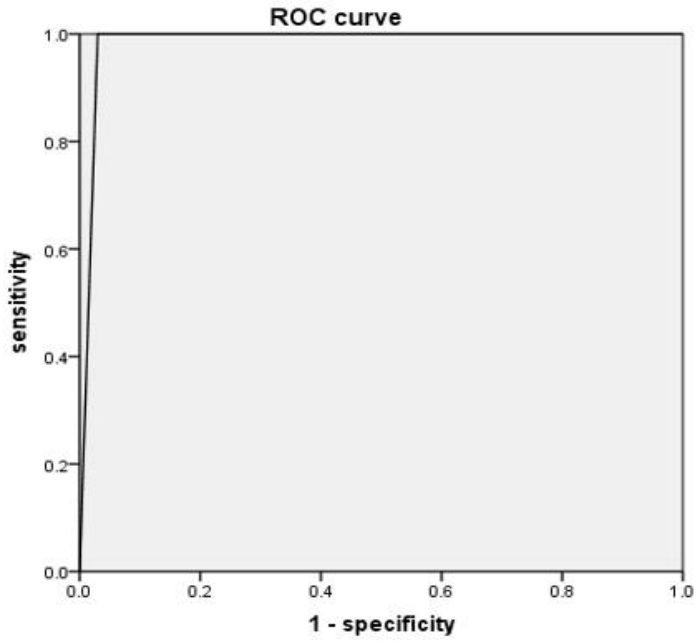

Figure 6: Receiver operating characteristic curves for HPV- mRNA identify metastasis in the lymph node. AUC=0.985 [95% confidence interval (CI) 0.972-0.998]

9 Discussion

9.1 Comparison between histology and molecular HPV status:

Although the incidence of cervical cancer in the developed countries is declining, the epidemiological situation in several developing nations is still critical. Currently, 80% of newly diagnosed cervical cancers occur in developing countries, and this percentage is expected to increase over the next decade[18]. This is probably due to the lack of health care infrastructure and limited financial resources that lead to ineffective prevention and control programs. In these parts of the world, cervical cancer is the most commonly diagnosed cancer and is an important cause of mortality among women. By 2030, it is expected that cervical cancer will be responsible for the death of more than 474,000 women annually. Over 95% of these deaths will occur in low- and middle-income countries[53]. There are large variations in incidence and mortality related to cervical cancer between the member states of the European Union [54]. An almost straight line can be drawn between Western and Eastern Europe. Both incidence and mortality rates are generally higher in Central and Eastern Europe than in Western Europe[55].

This disease continues to claim large numbers of lives; it is still the leading cause of morbidity and mortality among women worldwide. It remains a serious issue of public health and a burden in terms of morbidity, mortality, and high costs related to diagnosis and therapy. With regard to treatment, women with recurrent and metastatic cervical cancer have limited systemic treatment options, and these tumors are often chemotherapy-resistant. Recurrence compromises the survival and quality of life of patients. However, the rate of recurrence can be potentially better controlled once high-risk factors are recognized and this understanding leads to a more tailored oncological therapy.

The nodal status is considered as the most important prognostic factor in early-stage cervical cancer. Lymph node metastasis has been shown to have a direct impact on mortality[56, 57]. Thus, lymphadenectomy is currently considered as an integral part of surgery for early-stage cervical cancer. However, the incidence of positive nodes in these cases is relatively low (approximately 10%), and there are numerous potential complications associated with the procedure, such as ileus, adhesions, lymphoceles, and debilitating lymphedema.

More recently, sentinel node biopsy has been considered as an alternative to systematic

lymphadenectomy. To incorporate it into the clinical practice, this procedure must have high sensitivity and negative predictive value (NPV). The likelihood of missing metastatic lymph nodes must be negligible, given the major risk of recurrence associated with unrecognized lymph node metastasis.

Different tracers for SLN mapping have already been used. The SLN can be identified either using a cervical injection of a blue dye, which will color the first draining node, or by injection of a radioactive tracer that spreads to the sentinel node and can be detected with a gamma probe[58]. The drawback related to the blue dye method is a relatively low detection rate (60%–90%)[59]. Tests using radioactive tracers are quantitative and highly sensitive, but are more expensive and require nuclear medicine. Thus, currently, there is no consensus about the optimal technique. The method used depends on the experience of the surgeon and the standards of the institution. In our study, we have identified at least one SLN in almost 100% of the patients in whom the mapping was performed with patent blue dye. This detection rate is higher than reported in the literature[60, 61].

The identified and surgically removed SLN(s) is then histologically examined. There is currently no standardized protocol for the pathological processing of the SLN, but serial sectioning and immunohistochemistry are usually performed in most centers to detect the presence of micrometastases and isolated tumor cells[62]. The lymph nodes are conventionally processed and cut into 2 to 3 mm slices, but the SLN must be sectioned into slices not larger than 150–200 μm to guarantee the reliable detection of small metastases between 1 and 3mm (micrometastases)[48]. The presence of macrometastases (MAC), micrometastases (MIC), and isolated tumor cells (ITC) was recorded and classified according to the classification of malignant tumors (TNM) system. MAC was defined as a metastasis > 2mm in diameter, MIC as a metastasis between 0.2 and 2mm, and ITC as individual tumor cells or small clusters of cells < 0.2 mm in diameter, including the presence of single non-cohesive cytokeratin-positive tumor cells[63]. By using more intensive pathological analysis (ultrastaging) on the SLN, MIC can be detected, which would be overlooked by routine pathological processing[50]. Following this rationale, we can identify the high-risk patients more adequately, and consequently, tailor the use of adjuvant therapy that contributes to increased survival of the patient. In addition, SLN biopsy is a diagnostic method used to determine the local and regional lymph node status of solid tumors by taking a targeted

sample rather than by performing a complete lymphadenectomy which makes it possible to decrease short-term and long-term morbidity while preserving the same level of oncological safety. Furthermore, intraoperative determination of sentinel lymph node status may have significant implications in terms of clinical management. If the node is positive for tumor metastasis, the patient will have to receive complementary treatment.

A significant proportion of women (around 15%) treated for early-stage cervical cancer and without nodal metastasis will develop local or distant relapse after successful treatment for the primary malignancy[64]. One possible explanation would be recurrence due to histologically undetectable micrometastases or single tumor cells in the lymphatic system. Micrometastases are not a rare finding in early-stage cervical cancer; they can be found in 10%–15% of the cases[65]. The presence of micrometastatic disease represents an independent prognostic factor and may affect adjuvant therapy[66]. Extensive pathologic examination with RT-PCR of pelvic lymph nodes as shown by Van Trappen et al.[65] reported that in up to 50% of patients with early-stage cervical cancer, tumor cells could be detected in their pelvic lymph nodes, and this presence of tumor cells in the lymph nodes was associated with an adverse prognosis. The clinical implication of this finding is yet unclear, but such a high node-positive rate could explain why systematic lymphadenectomy prevents recurrences even in patients without radiological signs of nodal metastasis. MIC and isolated cancer cells are an earlier biological event than macrometastasis and are difficult to detect by routine pathology examination[67]. A second possibility is the occurrence of skip metastasis, i.e., paraaortic lymph node involvement without pelvic lymph node metastasis; however, this phenomenon is rare, accounting for only 1% of cases.

Cervical cancer commonly occurs in young women who are of childbearing age and who desire fertility preservation. These patients have been increasingly treated with conservative therapies. However, the balance between oncological safety and reproductive results must be considered. Currently, the nodal status is a key factor for further fertility-sparing surgery. In addition, nodal status is also crucial for the treatment of cervical cancer during pregnancy. In this case, before the start of the oncological therapy, a pelvic SLN biopsy is usually suggested. Although there is no clear conclusion, based on previous experience doctors should recommend their patients to terminate their pregnancy and further therapy must be given immediately if nodal metastasis is histologically confirmed. The optimal oncological therapy, as well as the preservation of the

health of the fetus, have to be taken into consideration. An accurate preoperative radiological evaluation of lymph nodes is extremely difficult; this is why the surgical nodal triage is considered as the method of choice in this scenario.

Currently, intraoperative pathology analysis of the lymph nodes using frozen sections is frequently utilized to rule out metastasis and, consequently, define the best therapy option. However, the accuracy of frozen section analysis for SLNs in current published research varies from 33.3 to 100%, probably due to the difficulties in detecting ITC and MIC[68]. Moreover, although intraoperative pathology has advanced in developed countries, in other parts of the world performing this examination is still very difficult. Given the large global disparities in cervical cancer burden, it seems unlikely that the use of frozen sections will meet the needs of all populations. In lower-resource settings, there are often technical and financial barriers, including the absence of significant financial investment, basic laboratory procedures, and quality-control measures. Additionally, there is a lack of experienced pathologists to perform effective and reliable frozen section examinations. Hence, innovative strategies for intraoperative pathology analysis are urgently needed mainly in underprivileged areas of the globe.

Although the performance of various cross-sections of dissected lymph nodes and consequent performance of immunohistochemical staining may reduce the false negative rates of conventional histology, these methods are time-consuming and cannot be incorporated into the clinical practice of frozen section biopsy. Therefore, the technique of intraoperative analysis of the SLN needs to be improved. Potentially, the use of novel biological markers may provide accurate information on the nodal status of patients with early-stage cervical carcinoma.

Since molecular technology can be used in the diagnosis of metastasis in the lymph node, many steps have already been taken to overcome the limitations of pathology alone such as p53 protein[69], Cytokeratin 19 mRNA [70]. However, currently, no test can be utilized in clinical practice. In addition to clinical performance, the choice of tests may also be influenced by costs and accuracy. The choice of the assay should be based on an optimal balance of sensitivity and specificity. The appreciation of causal links between HPV infection and cervical cancer as well as of complex interactions between host and HPV genome has opened new possibilities for molecular diagnostics.

Regional and systemic cervical cancer metastatic cells express the transformed genotype induced

by HPV in 99% of the cases[71]. Thus, detection of actively transcribed nucleic acids of HPV oncogenes in the lymph nodes indicates the presence of metastatic cancer cells. The analysis of HPV DNA, generally integrated within the genome of the host cancer cells, could represent a sensitive marker for micrometastases and ITC in pathologically-negative lymph nodes and could lead to the hypothesis that HPV-DNA detection in pelvic lymph nodes could be a risk factor for recurrence and poor prognosis. Since the first report by Lancaster et al.[72] on the utility of HPV DNA detection in lymph nodes, which was regarded as a surrogate marker of cervical cancer metastasis, several retrospective and prospective studies were conducted to validate the clinical usefulness of this approach. Noventa et al. showed that HPV DNA was present in 75% of cases with at least one lymph node metastasis (488 women), and in 39% of cases without metastatic involvement (913 women)[73]. Higher rates of HPV positivity have been reported by Slama et al., who found viral genomes in 66.6% of histologically negative lymph nodes from patients without histological metastatic involvement[74]. One may conclude that HPV-DNA is not a good biomarker for identifying metastases in the lymph nodes. The reason is that in some cases, the HPV positivity is due to the ability of immune-competent phagocytes to transport the HPV-positive cells or viral particles from the primary tumor to the lymph nodes. HPV DNA could not only remain in the invasive squamous cells but also in nuclei and cytoplasm of lymphocytes from germinal centers or cortical areas, in endothelial cells, in macrophages, and stromal cells. Indeed, this fact limits the utilization of the HPV-DNA assay as a reliable method for detecting metastases in the lymph nodes. Detection of metastasis in the lymph node needs methods of higher analytical sensitivity and specificity.

More recently, the HPV E6/E7 mRNA test was evaluated in screening studies that revealed a more accurate profile in diagnosis and prevention of cervical cancer[75, 76, 77].

The mRNA-based assays are potentially more reliable than HPV-DNA tests for the following reasons:

(1) Detection of HPV-DNA only indicates the presence of HPV and cannot distinguish transient infections from persistent ones. Since the mRNA is a temporal template for HPV synthesis, it spontaneously disappears after the termination of HPV DNA synthesis. A positive mRNA HPV test, therefore, reflects the activity of HPV infection. Thus, detection of HPV E6/E7 mRNA allows for the monitoring of the oncogenic activity of the virus by detection of active

transcription of viral DNA. Previous studies have shown that the level of HR-HPV E6/E7 transcripts correlates with the severity of the histological abnormality. Negative mRNA results in an HPV DNA-positive patient reflect the fact that not all HR HPV infections express E6 and E7, which is expected in transient infections. Thus, HPV E6/E7 mRNA testing may serve as a specific discriminator between transient cervical dysplasia and potentially progressive lesion. Identification of the transcripts of the viral oncogenes E6/E7 implicated in the oncogenic process through mRNA techniques is widely accepted as the present gold-standard test to elucidate the oncogenic role of HPV in the tumor.

(2) E6/E7 is highly expressed when HPV DNA is integrated into the host's genome, but this expression remains undetectable by HPV DNA tests.

(3) The smaller volume needed for mRNA analysis than for DNA analysis is more suitable for collecting samples before histology examination.

(4) Compared with mRNA tests, DNA tests lead to further unnecessary examinations for gynecologists and laboratories.

(5) The use of high-risk HPV E6/E7 mRNA may reduce the overdiagnosis and overtreatment associated with HPV-DNA testing. DNA from HPV was detected more frequently than the E6 and E7 mRNA. Several studies have reported that tests using HPV E6/E7 mRNA have good diagnostic and prognostic values that allow the follow-up intensity in women with HPV DNA-positive and negative colposcopy or histology to be reduced. For these reasons, current evidence suggests that the utilization of mRNA-based tests can improve the accuracy of the diagnosis of cervical dysplastic lesions in different settings. The mRNA-based tests represent a valuable improvement in terms of specificity and, consequently, positive predictive value (PPV) in detecting high-grade cervical lesions. The use of this technology will certainly reduce overdiagnosis and overtreatment.

The utility of HPV mRNA testing for predicting CIN has been well-documented in some published studies[78,79,80], with data suggesting HPV mRNA testing is more specific and less sensitive than HPV DNA testing and cytology. Recently, trials are being conducted to determine whether HPV mRNA could be used as a better diagnostic tool in the field of lymph node metastasis.

Durst et al.[81] evaluated the expression of HPV E6/E7 mRNA as a molecular marker for the

detection of tumor cells in biopsies of histologically negative sentinel lymph nodes and showed that recurrence-free survival was significantly longer for patients with HPV mRNA negative sentinel lymph nodes (log rank p = 0.002). However, the methods used in these studies were also technically complex as well as time-consuming, not appropriate for clinical practice. The availability of commercially standardized assays for the detection of HPV E6/E7 mRNA has opened new prospects for its use in the diagnostics and therapy of cervical cancer. However, some recent studies have reported considerable rates of false negative results for DNA-based HPV tests in histologically confirmed cervical carcinoma[82], possibly due to the inability to detect some rare carcinogenic types of HPV.

In this study, we used Aptima testing to detect the status of HR-HPV mRNA in the sentinel lymph nodes of patients with cervical cancer. The mRNA HPV test detects 14 different types of high-risk HPV, and the analysis runs in a fully automated system. The results from the APTIMA HPV test reveal a sensitivity similar to that reported in DNA detection assays and a higher specificity. Thus, it is used to identify clinically relevant infections with high viral oncoprotein transcript levels, possibly leading to progression. In the current retrospective study, we have identified HPV E6/E7 mRNA in all histologically positive samples. Eight out of 115 (7%) women had at least one HPV-positive lymph node sample without histological evidence of metastatic spread. Our current results are consistent with those in published studies. Until now, there have been only three publications addressing expression of HPV- mRNA in the lymph node of patients with cervical carcinoma, and they all confirmed the association of HPV mRNA detection with the presence of histologically confirmed nodal metastases in 100% of the cases[63, 83, 84]. Rose BR et al. reported that HPV 16 E6/E7 mRNAs were present in a small but significant proportion (6/42, 14%) of the histologically negative lymph node[83]. However, in our research, HPV E6/E7 mRNA is only identified in 3% of histologically negative lymph nodes. The reason for the lower HPV E6/E7 false positive detection rate may lie in a larger sample number, which can decrease the sampling error, as well as in the use of Aptima HR-HPV detection kits, which have fewer procedural steps than the PCR method. All of the reasons mentioned above decrease the possibility of contaminants and human errors. Since there is no further related publication, the exact specificity in other studies cannot be known. The sensitivity and specificity of HPV mRNA tests in our study were better than that of HPV DNA tests reported in previously

published papers[85, 86, 87]. Therefore, it is believed that E6/E7 mRNA detection can not only have a high prognostic value but also improve the specificity and positive predictive value when compared with the HPV-DNA test.

In previous studies, it was observed that the positivity for RNA-based HPV tests is almost three times less frequent compared with the DNA-based assays. There may be several reasons for this significant difference, although the most probable explanation is the fact that mRNAs are not as stable as DNA. Moreover, other technical difficulties involving the standardization of RNA assays in fresh and fixed materials may also be important. Different results have been reported regarding the performance of commercially available molecular assays for HPV DNA and RNA[88, 89, 90], although these generally provide superior specificity for RNA and higher sensitivity for DNA. However, it was found that expression of HPV E6/E7 mRNA was detected in all the samples that gave positive results in pathology examinations, which means that the sensitivity of HPV E6/E7 mRNA in detecting metastasis in the lymph node is 100%. These observations may support the future use of HPV E6/E7 mRNA technology to detect the presence of micrometastasis in the lymph nodes of patients with cervical cancer.

9.2 Issues that need to be discussed and solved

In our current research, we found that the sentinel nodes in 8 patients were histologically-free while HPV-E6/E7-mRNA were positive. Altogether, 9 sentinel lymph nodes that were histologically negative had detectable HPV-E6/E7-mRNA. The possible reasons may be the following.

9.2.1 True-positivity

1) The HPV mRNA detection method is more sensitive than histology detection.

2) For the histological examinations, samples were taken from lymph nodes that had been removed from the patients. Theoretically, one cannot rule out the possibility that micrometastases or single tumor cells have been lost before microscopy.

3) Limitation of taking samples. The number of lymph nodes available for HPV detection is limited; therefore, ruling out errors in sampling is impossible. This means that although results

on HPV and pathology were both negative in the selected lymph nodes, perhaps micrometastases or IHC could be missed by conventional pathology and HPV detected in other lymph nodes.

9.2.2 False-positive

PCR-based molecular techniques are very sensitive when it comes to detecting minimal quantities of genetic material, but their usefulness for predicting cancer is limited by the high rate of false positive results. The problem of false positives could be avoided by using the mRNA assay. According to the instructions for the Aptima HPV detection kit, the specificity of this kit is 99.7%; this could be one explanation for our current results. An additional explanation for the results could be false positivity due to cross-reactivity, which has previously been described for the low-risk types of HPV (26, 67, 70 and 82). Unfortunately, we did not perform the low-risk HPV test in the current research. In the next step of the study, this analysis is planned in order to exclude the impact of these four low-risk HPV types on the experimental results.

9.3 Discussion of the clinical significance of clear HPV status

9.3.1 The significance of HPV PPV

From previous research, it has been found that the reccurrence rate of cervical cancer is higher for patients with histologically clear lymph nodes that show positive results in HPV tests than for those that are negative by both pathology and HPV, which supports the notion that mRNA assays can evaluate the possibility of relapse by testing HPV in lymph nodes. It is known that the presence of HPV in LNs is an independent oncological risk factor, and the presence of HPV-DNA in LNs is probably an early sign of metastases[91]. The reason may be the ability of the HPV E6/E7 viral proteins to transform cells. The active transcription of these two HPV oncogenes and its effects on the cervical cells of the host can be monitored directly through the detection of E6/E7 viral mRNA transcripts. By using E6/E7 mRNA as a biological marker of cervical disease, it has been found that positive samples are associated with malignant transformation. Concerning recurrence in the case of metastasis-free LNs, the most likely explanation is that HR-HPV has the potential intrinsic ability to transform normal looking cervical cells to neoplastic cells that migrated through lymphatic drainage. These “normal” cells can still contain viral genomes, and with the virus life cycle becoming “re-activated”

subsequently following immune suppression or by other mechanisms, these “normal” cells are transformed into neoplastic cells. Thus, the detection of E6 and E7 mRNA expression in some women with “normal” lymph nodes may reflect the oncogenic activity of the above HR-HPV types before initiation of the transformation of the infected cells. This point is especially important in older women (above 35 years of age) who are more likely to harbor persistent HPV infections that drastically increase their risk of normal cells being transformed into neoplastic ones. Although there have been no specific agents for HPV clearance until now, the development of novel, more targeted intervention therapies is progressing and promising possible treatments such as therapeutic vaccines. Clearance of persistent HPV infection is indeed becoming a reality. Thus, knowing the HR-HPV status in metastases not only provides prognostic information but also determines whether a patient is eligible for clinical trials with antiviral drugs. Some authors affirm that the increased sensitivity of HPV testing reflects earlier detection rather than over-diagnosis[92].

Based on these considerations, we conclude that the status of HPV mRNA is of great clinical significance even if the current pathology did not find metastatic lesions. Interestingly, there was one patient whose pelvic lymph node was HPV- and pathology-positive, and whose paraaortic histology was negative but gave a positive result in the HPV mRNA test. Does the HPV-positive imply undetectable tumor cells by histology? Can HPV transform cells showing no pathological changes into a tumor? Also, could this phenomenon explain well why patients with pelvic lymph node metastases are more likely to suffer recurrence events without any metastases in another level lymph node? Obviously, these assumptions have to be confirmed by collecting follow-up information. Moreover, we need to use an additional method to verify the existence of HPV mRNA. This point is important, because, the PPV in our paper is 56.3% which means around half of the patients would be HPV mRNA-positive but without pathological changes observed in histology examinations. Too much emphasis on HPV mRNA positive, especially unconfirmed false-positivity, may have led to more false-positive results, which adversely affected specificity, with the consequence of more women having been referred for frequent follow-ups and even over-diagnosis and treatment, and cause more financial as well as psychological burden.

9.3.2 The significance of HPV NPV

In our project, we found that the HPV NPV are both 100% for predicting pathology metastasis whatever it is based on patients or lymph node. On the one hand, the knowledge that women are negative for HPV infection may provide reassurance to pathologists in reaffirming negative pathology results as well as resolution of differences in pathological section readings obtained by different personnel or due to a decline in diagnosis time. Shorter diagnosis time, which means shortened surgery time, has an important clinical significance, as subsequent surgery depends on these results.

On the other hand, even in women with positive cut margins, if the HPV mRNA results are negative, the rate of development of high-grade lesions is as low as that in women whose results were negative both pathologically and by detection of the virus. Lack of detectable HPV mRNA demonstrated a negative predictive value of 100% for high-grade lesions. As reported in previous studies, the finding of HPV mRNA in the lymph node indicates the existence of tumor cells even before they are visible by histology detection. The status of HPV mRNA in the lymph node is a negative prognostic parameter for patients with cervical cancer. Thus, negative results in HPV testing may provide increased reassurance for women, thereby permitting the safe extension of follow-up intervals. The most important point for clinical practice is that if the expression of HPV mRNA in the lymph node is negative, it can help the patients to avoid unnecessary invasive surgery.

Nowadays, because of delayed pregnancy, fertility-sparing surgery has important implications for young cervical cancer patients as well as their families and society. Confirmation of the pathology status of lymph nodes is important for this fertility-sparing surgery decision. Detection of HPV mRNA has high sensitivity and PPV, and if the result is negative, it means that there is no risk of metastases and recurrence. Thus, the status of HPV mRNA in the lymph node will probably play an important role in surgery decisions. However, although monitoring of the activity of HPV oncogene transcripts seems to be a reasonable strategy to identify clinically relevant HPV infections with high-risk HPV genotypes, HPV mRNA negative doesn’t mean that there is no HPV infection at this pointing time. If HPV-DNA exists, once it is activated, it may transform normal cervical cells into cancer more quickly. A phenomenon can confirm this hypothesis：CIN patients after treatment are more likely to suffer from CIN or cervical cancer

than normal healthy women. Currently, there are few studies that have investigated the association of HPV infection with recurrence at both the HPV DNA level and the HPV oncogene mRNA level. Therefore, we will further detect HPV DNA status in the lymph node of current patients in order to confirm our hypothesis.

10 Outlook and Conclusion

In conclusion, prevention of any condition is described in terms of primary, secondary and tertiary prevention. While primary prevention deals with modification of risk factors to prevent disease occurrence, secondary prevention essentially signifies early diagnosis and treatment, while tertiary prevention seeks to limit the disability caused by the condition. This includes accepting a molecular testing resource for other health care professionals across various medical specialties and ongoing education in molecular diagnostics through participation in professional societies to ensure that best practices and the highest quality of patient care are maintained. The ability to use molecular testing for the diagnosis and treatment of patients enables health care professionals to act with greater precision than ever before. This molecular testing method can help to identify patients with risk factors for recurrence, so that it can perform medical intervention at an early stage, reduce the recurrence rate and improve the survival rate to help to achieve tertiary prevention of cervical cancer.

Since the beginning of the molecular level in the diagnosis of micrometastases in the lymph node and prevention of cervical cancer recurrence, many steps have already been taken to overcome the limitations of pathology alone; HPV mRNA testing actually represents a new challenge, HPV tests are necessary to identify any metastasis in the lymph node of cervical cancer. APTIMA HR-HPV mRNA testing, as a commercial kit, can detect HR-HPV quickly. It shows promise as an effective molecular tool in identifying patients at risk of lymphatic metastasis and avoiding unnecessary invasive surgery for patients without lymphatic metastasis.Consequently, it can help to reduce the recurrence of invasive cervical cancer in women as well as psychological and economic burdens in the near future, which is particularly necessary for countries and regions with limited medical resources.

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12 Affidavit

“I, Xin Le certify under penalty of perjury by my own signature that I have submitted the thesis on the topic „Molekulare Diagnose von Lymphknotenmetastasen in endoskopisch behandeltem Gebärmutterhalskrebs: Genauigkeit des APTIMA Tests zur Detektion von High Risk Humanen Papillomvirus mRNA in Wächterlymphknoten”. I wrote this thesis independently and without assistance from third parties, I used no other aids than the listed sources and resources.

All points based literally or in spirit on publications or presentations of other authors are, as such, in proper citations (see "uniform requirements for manuscripts (URM)" the ICMJE www.icmje.org) indicated. The sections on methodology (in particular practical work, laboratory requirements, statistical processing) and results (in particular images, graphics and tables) correspond to the URM (s.o) and are answered by me. My interest in any publications to this dissertation correspond to those that are specified in the following joint declaration with the responsible person and supervisor. All publications resulting from this thesis and which I am author correspond to the URM (see above) and I am solely responsible.

The importance of this affidavit and the criminal consequences of a false affidavit (section 156,161 of the Criminal Code) are known to me and I understand the rights and responsibilities stated therein.

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Date Signature

13 Declaration of any eventual publications

Köhler C, **Le X**, Dogan NU, Pfiffer T, Schneider A, Marnitz S, Bertolini J, Favero G. Molecular Diagnosis for Nodal Metastasis in Endoscopically Managed Cervical Cancer: The Accuracy of the APTIMA Test to Detect High-risk Human Papillomavirus Messenger RNA in Sentinel Lymph Nodes. J Minim Invasive Gynecol.201; 23(5):748-52.

Contribution in detail: Le Xin contributed to the concept and study protocol of all behavioral experiments. She collected all the samples from patients' lymph node after every surgery. And send the samples medium to perform HPV detection. In addition, she participated in collect clinical information of every patient. She analyzed the respective results obtained from her experiments, performed the statistical tests, and wrote part of the methods and results section. Finally she contributed to the introduction and discussion of the manuscript, revised the final draft and approved the final version of the manuscript.

Signature, date and stamp of the supervising University teacher

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Signature of the doctoral candidate

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14 Curriculum Vitae

My curriculum vitae does not appear in the electronic version of my paper for reasons of data protection.

15 List of publications

1. Favero G, Anton C, Le X, Silva E Silva A, Dogan NU, Pfiffer T, Köhler C, Baracat EC, Carvalho JP. Oncologic Safety of Laparoscopy in the Surgical Treatment of Type II Endometrial Cancer. Int J Gynecol Cancer, 2016. [Epub ahead of print]

2. Köhler C, Foiato T, Marnitz S, Schneider A, Le X, Dogan NU, Pfiffer T, Jacob AE, Mölgg A, Hagemann I, Favero G.Potential Surgical and Oncologic Consequences Related to Skin Tattoos in the Treatment of Cervical Cancer. J Minim Invasive Gynecol, 2016, S1553-4650(16)30:158-3.

3. Köhler C, Le X, Dogan NU, Pfiffer T, Schneider A, Marnitz S, Bertolini J, Favero G.Molecular Diagnosis for Nodal Metastasis in Endoscopically Managed Cervical Cancer: The Accuracy of the APTIMA Test to Detect High-risk Human Papillomavirus Messenger RNA in Sentinel Lymph Nodes. J Minim Invasive Gynecol, 2016, 23(5):748-52.

4. Xin L, Ma X, Xiao Z, Yao H, Liu Z.Coxsackievirus B3 induces autophagy in HeLa cells via the AMPK/MEK/ERK and Ras/Raf/MEK/ERK signaling pathways. Infect Genet Evol, 2015,36:46-54.

5. Ma X, Wu F, Xin L, Su G, He F, Yang Y, Sun J, Liu Z.Differential plasma microRNAs expression in juvenile idiopathic arthritis. Mod Rheumatol, 2016,26(2):224-32.

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10. Xin L, Xiao Z, Ma X, He F, Yao H, Liu Z.Coxsackievirus B3 induces crosstalk between autophagy and apoptosis to benefit its release after replicating in autophagosomes through a mechanism involving caspase cleavage of autophagy-related proteins. Infect Genet Evol, 2014,26:95-102.

16. Acknowledgements

Firstly, I would like to thank Prof. Dr. med. Marnitz for her friendly and kindly commitment, dissertation and workplace;

I would like here to give my special thanks to Prof. Dr. med. Köhler, without whose patience, support, constructive suggestions and cooperation I could not have done this work. I am also very grateful for his great commitment to the support of my doctorate and his constant willingness to help. He is an important mentor father for me and I will remember him for my whole life;

I would like to thank Dr. Giovanni Fevero who gave me many constructive suggestions for my work and taught me a lot of knowledge;

Also Prof. Dr. Schneider, I thank you for with the trials and the technical assistance;

I would like to thank my family, especially my husband and my parents, for their great support during my studies and beyond.

Finally, thanks any person who ever gave me help!