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„Wir alle leben geistig von dem, was uns Menschen in bedeutungsvollen

Stunden unseres Lebens gegeben haben."

- Albert Schweitzer

KURZFASSUNG

Hintergrund: Der Gebärmutterhalskrebs ist nach wie vor eine der häufigsten Krebserkrankungen bei Frauen weltweit. Es zeigt sich, dass der mTOR-Signalweg ein attraktives therapeutisches Ziel darstellt. Die randomisierte Phase-III-Studie BOLERO-2 hat gezeigt, dass Veränderungen im mTOR-Signalweg mit der Resistenz gegen endokrine Therapien bei Brustkrebs assoziiert sind. Diese Ergebnisse könnten auch für eine effiziente Therapie beim Zervixkarzinom interessant und wichtig sein.

Methoden: Es wurde eine immunhistochemische Analyse durchgeführt um signifikante Unterschiede in den Expressionsniveaus der verwendeten Biomarker in Tumorgewebe im Vergleich zu gesundem Gewebe aufzudecken. Gewebe-Microarrays von 126 Patientinnen wurden präpariert, angefärbt und die Expression von tumorspezifischen Markern (mTOR, ER, PR, PTEN, EGFR) untersucht.

Ergebnisse: Die vorliegende Stichprobe umfasste 126 Patientinnen im Alter zwischen 29 und 68 Jahren mit Gebärmutterhalskrebs. Bei 96 (76.2%) Patientinnen wurde ein Plattenepithelkarzinom diagnostiziert und nur 3 (2.4%) Patientinnen hatten ein Adenokarzinom. Die immunhistochemische Auswertung der Gewebearrays zeigte, dass sowohl mTOR ($p<0.006$, (95% CI -47.8365 - -8.6635)) als auch der Hormonrezeptor ER ($p<0.000$, (95% CI -105.9131 - -53.5991)) im Tumorgewebe im Vergleich zum Kontrollgewebe signifikant höher exprimiert waren. Es konnte eine signifikante Korrelation zwischen mTOR- und ER-Expression im Tumorgewebe gezeigt werden (p= 0,226; sign. 0,014).

Fazit: Die Ergebnisse zeigen eine signifikante Korrelation zwischen den Biomarkern mTOR und ER im Zervixkarzinom im Vergleich zum Kontrollgewebe. Daher wäre es hoch relevant, den Einsatz von Medikamenten in Zukunft in Betracht zu ziehen, wie es zuletzt auch die BOLERO-Studie in Bezug auf Brustkrebs gezeigt hat.

Schlagwörter: Zervixkarzinom, mTOR, ER, PR

ABSTRACT

Background: Cervical cancer is still one of the most common cancers in women worldwide. It turns out that the mTOR signaling pathway represents an attractive therapeutic target. The randomized phase III BOLERO-2 trial has shown that alterations in the mTOR pathway are associated with resistance to endocrine therapies in breast cancer. These results may also be of interest and importance for efficient therapy in cervical carcinoma.

Methods: Immunohistochemical analysis was performed to reveal significant differences in the expression levels of the biomarkers used in tumor tissue compared to healthy tissue. Tissue microarrays from 126 patients were prepared, stained, and the expression of tumor-specific markers (mTOR, ER, PR, PTEN, EGFR) was examined.

Results: The present sample included 126 patients aged 29 to 68 years with cervical cancer. 96 (76.2%) patients were diagnosed with squamous cell carcinoma and only 3 (2.4%) patients had adenocarcinoma. Immunohistochemical evaluation of tissue arrays showed that both mTOR ($p<0.006$, (95% CI -47.8365 - -8.6635)) and hormone receptor ER ($p<0.000$, (95% CI -105.9131 - -53.5991)) were significantly higher expressed in tumor tissue compared to control tissue. A significant correlation between mTOR and ER expression in tumor tissue could be shown ($p= 0.226$; sign. 0.014).

Conclusion: The results show a significant correlation between the biomarkers mTOR and ER in cervical carcinoma compared to control tissue. Therefore, it would be highly relevant to consider the use of drugs in the future, as most recently demonstrated by the BOLERO study in relation to breast cancer.

Keywords: Cervical carcinoma, mTOR, ER, PR

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1. Introduction

Definition

The cervix is a cylindrical shaped structure composed of stroma and epithelium. Cervical carcinoma is a malignant degeneration of the cervix uteri. This cancer forms in tissues of the cervix, the organ connecting the uterus and vagina. It is usually a slow-growing cancer. Alteration of the epithelium may result from maturation disorders of the cells or proliferation of atypical cells. If a deviation of the tissue structure occurs, it is called dysplasia. The degenerate cervical cells may disappear without treatment, remain the same, or develop into cancer cells over many years. In 80% of cases, these are squamous cell carcinomas and in 20% adenocarcinomas or mixed forms. (1,2)

Localization

The cervical carcinoma and its pre-stages are usually located at the transition between squamous epithelium and cylindrical epithelium, the so-called transformation zone. Carcinomatous changes often begin in this area or in higher cervical sections. Cervical carcinomas can either grow out of the surface (exophytic), or be with stromal infiltration with minimal surface growth (endophytic). The localization is also dependent on the female sexual hormones estrogen and gestagen, as this boundary can shift under their influence. (1,3,4)

1.1. Epidemiology: Incidence and Mortality

Cervical cancer is the fourth most frequently diagnosed cancer and the fourth leading cause of cancer death in women with an estimated 570.000 (569.847) new cases and 311.000 (311.365) death in 2018 worldwide. The incidence was 6.6% and the mortality rate was 7.5% in 2018. Nearly 90% of death from cervical cancer occurred in low- and middle-income countries where no screening programs exist and the highest regional incidence and mortality rates are seen in Africa. In contrast, the incidence of cervical cancer in industrialized countries has declined sharply in recent decades due to cervical cancer screening. (5–8) Until the 70s of the last century, cervical carcinoma was the most common malignant tumor in women. Since then, the age standardized incidence has decreased from about 20/100.000 in 1980 to 9.3 in 2010. (9,10)

Since the late 90s, the disease rates have been stable. In the other "old" member states of the European Union, too, the age-standardised morbidity rates have fallen significantly since the 1970s. The reduction in incidence and cancer-specific mortality in the EU, North America and other parts of the world correlates with the establishment of national and regional screening programmes based on cytological (Papanicolaou, Pap) screening. (11,12) Globally, one women dies of cervical cancer every two minutes and nearly 0.6 percent of women will be diagnosed with cervical cancer at some point during their lifetime (based on 2013-2015 data). It is most frequently diagnosed among women aged 35-44 and the median Age at diagnosis is 50. (13,14)

An important point is the fact that in about 30% of death from uterine cancer, it is not more precisely defined which part of the uterus was affected by the carcinoma. That’s the reason why it is more difficult to evaluate the actual numbers. (15)

1.2. Risk factors

The carcinogenesis is multifactorial with different significance and interactions of the influencing factors. (16) Groups of risk factors for the development of investigative cervical carcinoma are identified:

- HPV-Infection

Human Papillomavirus (HPV) infection is the main etiological factor for the development of cervical cancer. (17) Recently it seems certain that an infection with the human papillomavirus does not necessarily lead to cancer, but that it does provide the essential building block for a dysplastic alteration of the epithelium, which over the years can develop into a precursor or an invasive squamous cell carcinoma of the cervix. (18)
Cervical cancer is almost always caused by human papillomavirus (HPV) infection that is spread through sexual contact. (19) A landmark study has shown that HPV DNA can be found in 99.7% of cervical cancer specimens. (20)

There are more than 80 types of human papillomavirus and about 30 of these can infect the cervix. (19) Seven out of 10 of all cervical cancer cases reported throughout the world are caused by only two types of HPV: HPV 16 and 18. Another four high risk HPV types- 31, 33, 45 and 58- are less commonly found to be associated with cervical cancer, with particular types being more prevalent than others in certain geographical areas.(21)
HPV 16 and 18 have two transcriptional units, E6 and E7, that encode proteins essential for viral replication. The E6 oncoprotein exerts its effect by binding to and inactivating the tumor-suppressor gene P53 through ubiquitin degradation, which disrupts an inherent cell-cycle checkpoint. The E7 oncoprotein binds to and inactivates products of the retinoblastoma gene, pRb, which ultimately allows unchecked cell-cycle progression in cells infected with HPV16 or 18. (2)

Representatives of this group are detectable in almost all high-grade dysplasias and investive carcinomas of the lower genital tract and are also associated with a higher progression tendency. Types 6 and 11 belong to the low risk group (low risk HPV, LR-HPV), which are mostly found in condylomatous changes and light-grade dysplasias with only low progression potential. Also double or multiple infections with up to 6 different HPV types are common with about 25%. (15)

The HPV shows a pronounced localization specificity. It produces characteristic proliferative and degenerative changes only in certain areas of the epidermis and mucosa. In the cytological smear, abnormalities can be observed which have been called coilocytes and dyskeratocytes since Papanicolaou (PAP test).(15)
The HPV virus is able to suppress the expression of MHC class-I molecules on the surface of tumour cells. The function of these MHC class-I molecules is to present foreign substances to the immune system. Since the tumor cells prevent this, no peptides of the virus can be presented and therefore no cytotoxic T cell response takes place at the level of the cellular immune system in the body. For this reason, the tumor cells are not recognized by the body as foreign and are not fought against. (22)

The human papilloma virus is detected in over 99% of all cervical carcinomas and thus represents the greatest risk factor for both cervical precancerous and invasive carcinomas. (20)

- Hormonal contraception

An increase of the cervical carcinoma risk is discussed in case of HPV infection and simultaneous oral contraception. The use of mainly combined oral contraception (with estrogen and gestagen) over a longer period (5 years or more) is associated with an increased risk of cervical cancer. (23)

In an analysis of 24 epidemiological studies, it was shown that long-term oral contraception is associated with a higher risk of disease. On the other hand, a risk reduction could be observed after discontinuation of oral contraception regardless of the preceding duration of use. (24)

- Co-Factors

All other known co-factors, such as the woman's age for regular sexual intercourse, number of sexual partners, promiscuity, smoking, other sexually transmitted diseases (HIV), number of births and abortions, and socio-economic status, are related to this factor. Cervical carcinoma can thus be described as a sexually transmitted disease (STD). (15)

Having a weakened immune system, caused by immunosuppression, increases the risk of HPV infection and cervical cancer. Immunosuppression can be caused by HIV virus or medicine given to prevent organ rejection after transplant. These factors weakens the body‘s ability to fight infection. (19)

Most of the time, the body’s immune system can fight the HPV infection before cancer forms. (19) It takes 15 to 20 years for cervical cancer to develop in women with normal immune systems and it can take only 5 to 10 years in women with weakened immune systems, such as those with untreated HIV infection. (25)

1.3. Development of pre-cancer

The cervical cancer progression model shows the three steps of cervical carcinogenesis. This includes acute human papillomavirus infection, HPV persistence, which is particularly associated with the development of cervical precancer (CIN 3), and invasion. A sharp increase in HPV prevalence in the years after the average age of first sexual intercourse is observed. Depending on the intensity of screening, the peak in prevalence occurs 5 to 10 years after infection, when women are about 25 to 35 years old. The increase in cervical cancer incidence typically occurs many years later. (26)

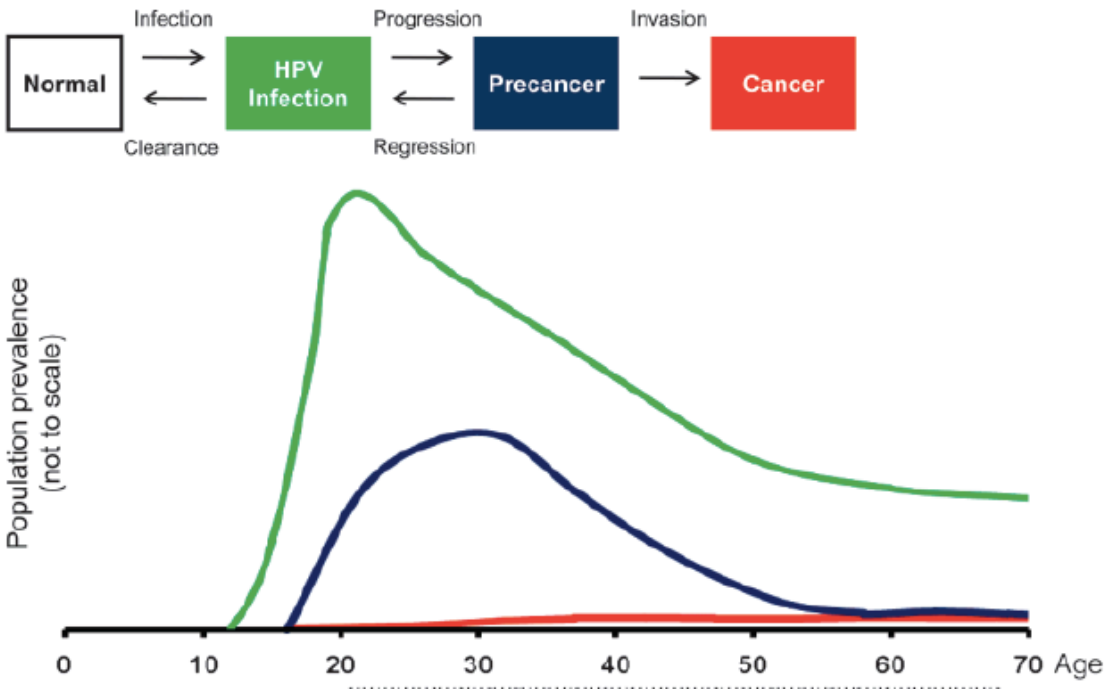

Figure 1. *Timeline of cervical pre-cancer and cancer development. The figure illustrates the age-related prevalence of HPV infection (green), CIN2 and CIN3 (blue), and cervical cancer (red) in the United States. (26–28) (The prevalence curves are not drawn to scale)*

1.4. Pathogenesis

Precancerous lesions and dysplasia resulting from cell maturation defects and proliferation of atypical cells are grouped under the term cervical intraepithelial neoplasia (CIN). This is followed by increased mitosis, loss of polarity and higher cell density. (18) The classification is based on the degree of differentiation into CIN1, CIN2, CIN3 and adenocarcinoma in situ (ACIS). (16) (29) Persistent infection with high-risk (HR) HPV types can lead to cervical intraepithelial neoplasia (CIN), which can develop into cervical cancer over time. (20) More than 70 percent of the virus strains 16 and 18 lead to CIN and carcinomas. (30) CIN1 regressed in up to 60% of cases, CIN2 in 40% and CIN3 in only about 10-15%. (18)

- CIN

The invasive cervical carcinoma is preceded by a long phase of HPV-associated intraepithelial precursor lesions. The most common of these precursor lesions is cervical intraepithelial neoplasia (CIN), which is classified into three degrees of epithelial dysplasia: CIN1 (=LSIL), CIN2 and CIN3 (=HSIL). (29)
Shown in Table 1.

CIN-Classification (Histology)	Degree of Dysplasia	Bethesda-System (Zytology)
CIN 1	Slight dysplasia - Epithelial stratification largely preserved - Polarity disorders and mitoses in the lower third of the epithelium - Possible coilocytes	LSIL
CIN 2	Moderate dysplasia - Removal of the epithelial layer up to the middle third of the epithelium - Possible coilocytes	HSIL
CIN 3	Severe dysplasia or carcinoma in situ (CIS) - Typical epithelial stratification largely eliminated core atypia and mitoses reach into the epithelial surface - The basement membrane is not breached - Possible coilocytes	HSIL

Table 1. *Classification of CIN (31).*

(*LSIL= low grade squamous intraepithelial lesion; HSIL= high grade squamous intraepithelial lesion)*

-Histological subtypes

Squamous cell carcinoma, whether interrogating or not, and adenocarcinoma or adenosquamous carcinoma are the most common histological types. Squamous cell carcinoma is present in about 80% of cases. The percentage of adenocarcinomas has increased from 10% to about 20% in the last 25 years. Other tumor identities such as mixed forms (adenosquamous), neuroendocrine (large or small cell), clear cell or serious papillary carcinomas are rare. (16)

Adenocarcinoma has increased in frequency in recent years, especially in younger women. Studies on outcome in relation to histology show inconsistent results. Some studies state that there is no difference in 5 year-survival. (32)

Others show that in both early and advanced stages, adenocarcinoma has a worse outcome due to its more aggressive behavior. (33)

1.5. Classification

The staging of cervical carcinoma can be done using two classifications, the Federation Internationale de Gynecologie et d'Obstetrique (FIGO) and the Union for International Cancer Control (UICC) TNM staging. The TNM classification is used for all tumor identities of the human body and requires clinical or pathological criteria. The FIGO classification refers specifically to tumors of the female genital tract and is largely based on surgical staging. (34)

Clinically, cervical cancer was classified based on the 2009 FIGO classification until 2018. To allow staging based on imaging and pathologic findings, the FIGO Committee made a revision in 2018. (35) FIGO staging allows both the selection and evaluation of therapy as well as estimation of prognosis and calculation of end results. (1) In describing the anatomic extent of disease, the FIGO and TNM classification are nearly identical. The TNM nomenclature was previously used for documentation of nodal and metastatic disease status. (36)

TNM	FIGO	Description
Tx		Primary tumor cannot be assessed
T0		No evidence of primary tumor
Tis		Preinvasive carcinoma
T1	I	The carcinoma is strictly confined to the cervix (extension to the uterine corpus should be disregarded)
T1a	IA	Invasive carcinoma that can be diagnosed only by microscopy, with maximum depth of invasion < 5 mm[a]
T1a1	IA1	Measured stromal invasion depth of < 3 mm
T1a2	IA2	Measured stromal invasion depth ≥ 3 mm and < 5 mm
T1b	IB	Invasive carcinoma with measured deepest invasion of ≥ 5 mm (greater than Stage IA), lesion limited to the cervix uteri[b]
T1b1	IB1	Invasive carcinoma with measured deepest stromal invasion of ≥ 5 mm, and greatest dimension of < 2 cm
T1b2	IB2	Invasive carcinoma with greatest dimension of ≥ 2 cm and < 4 cm
-	IB3[d]	Invasive carcinoma with greatest dimension of > 4 cm
T2	II	The carcinoma invades beyond the uterus, but has not extended into the lower third of the vagina or to the pelvic wall
T2a	IIA	Involvement limited to the upper two-thirds of the vagina without parametrial invasion
T2a1	IIA1	Invasive carcinoma with greatest dimension of < 4 cm
T2a2	IIA2	Invasive carcinoma with greatest dimension of ≥ 4 cm
T2b	IIB	With parametrial involvement but not up to the pelvic wall
T3	III	The carcinoma involves the lower third of the vagina and/or extends to the pelvic wall and/or causes hydronephrosis or nonfunctioning kidney and/or involves pelvic and/or para-aortic lymph nodes[c]
T3a	IIIA	The carcinoma involves the lower third of the vagina, with no extension to the pelvic wall
T3b	IIIB	Extension to the pelvic wall and/or hydronephrosis or nonfunctioning kidney (unless known to be due to another cause)
N[d]	IIIC[d]	Involvement of pelvic and/or para-aortic lymph nodes, irrespective of tumor size and extent (with r and p notations)[c]
	IIIC1[d]	Pelvic lymph node metastasis only
	IIIC2[d]	Para-aortic lymph nodes metastasis
T4	IV	The carcinoma has extended beyond the true pelvis or has involved (biopsy proven) the mucosa of the bladder or rectum (the presence of bullous edema is not sufficient to classify a case as Stage IV)
	IVA	Spread to adjacent pelvic organs
M1	IVB	Spread to distant organs

When in doubt, the lower staging should be assigned
[a]Imaging and pathology can be used, when available, to supplement clinical findings with respect to tumor size and extent in all stages
[b]The involvement of vascular/lymphatic spaces does not change the staging. The lateral extent of the lesion is no longer considered
[c]The notations of r (imaging) and p (pathology) are added to indicate the findings used to assign a case as Stage IIIC. For example, if imaging indicates pelvic lymph node metastasis, the stage allocation would be Stage IIIC1r, whereas if confirmed by pathologic findings, the stage would be Stage IIIC1p. The type of imaging modality or pathology technique used should always be documented
[d]The revised FIGO classification was recently published (October 2018). TNM (8th Edition) does not include classification for the new FIGO groups IB3, IIIC1, and IIIC2. TNM defines only regional lymph nodes, with N0 (i+) indicating isolated tumor cells in regional lymph node(s) no greater than 0.2 mm, and N1 indicating regional lymph node metastasis

Table 2. *TNM and FIGO Classification of cervical cancer (1,35)*

After surgical removal of the tumor, the specimen obtained can be classified by the pathologist using the TNM classification, which considers the extent of the primary tumor (T) as well as the presence of lymph node metastases (N) and distant metastases (M). (35)

- Grading

Histological and cytological criteria can be used to determine the grades of malignancy of the tumor. The following classification is commonly used: (1,18)

GX	Grade cannot be assessed
G1	Well differentiated
G2	Moderately differentiated
G3	Poorly or undifferentiated

1.6. Diagnostic

- Anamnesis and Symptoms

In cervical carcinomas at an early stage of the tumor, localized to the cervix, no symptoms occur in many cases. In these cases, the diagnosis is often made as part of cervical carcinoma screening. In symptomatic patients, unspecific symptoms such as hypermenorrhea, postcoital bleeding or vaginal discharge are typically observed. Symptoms of advanced tumors are caused by organ transgression into the parametries or the pelvic wall and adjacent organs (urinary bladder, intestine, bone). In advanced tumor stages, lumbalgia, pelvic pain or lymph congestion may occur in the lower extremities. Urogenital symptoms such as urinary complaints, hematuria or hydronephrosis up to renal insufficiency can be observed with infiltration of the urinary bladder or compression of the ureters. Vesico-vaginal or recto-vaginal fistulas can also develop as a result of tumour infiltration. (30)

- Metastasis

Cervical carcinoma spreads across the cervical wall, into the parametrium and onto the vagina. More advanced tumors invade the urinary bladder and rectum. Lymphogenic metastasis usually occurs to the pelvic and paranormal lymph nodes. The most important prognostic factors are the lymph node status and the number of involved lymph nodes. The 5-year survival rates in stage IB-IIA are 88-95% without lymph node metastasis and 51-78% with lymph node metastasis. Distant metastases usually develop very late affecting mainly the lungs, less frequently the bones or liver. (37,38)

- Clinical examinations

The vaginal and rectal palpation as well as the speculum setting represent essential basics within the framework of the clinical examination. The portio is examined primarily for changes such as erythroplakia, leukoplakia, ulcers and erosions. Furthermore, attention is paid to whether the external cervix is sharply defined. Rectal palpation can be used to feel whether the parametrium is shortened or nodular, which would indicate infiltration of the parametrium. (39)

- Cytology

There are currently 3 different approaches to cervical cancer screening. Cervical cytology, based on the Pap smear, is still the most commonly used screening test. The different variants of cytology include the conventional smear and liquid-based methods, which can be evaluated either completely manually or computer-assisted. (40)

The Pap smear, named by the Greek pathologist George Nicholas Papanicolaou, after its invention more than fifty years ago, is still today one of the most important screening test, which in its history has led to a drastic reduction in the incidence (60-90%) and mortality (90%) of cervical carcinoma in the Western world. For example, cytological screening was introduced in the USA in the sixties and reduced the incidence rate of invasive cervical cancer by 75%. (41,42)

Since the need for screening programs in particularly high in developing countries due to the high incidence of the tumor, and very often resources are scarce, the low cost of the test procedure is a major argument for its use. However, a closer look at the procedure also reveals considerable weakness that call into question the continued use of PAP cytology as a screening method. In particular, the low sensitivity (50%) of PAP cytology is criticized. The probability of a single PAP test to detect a CIN 2,CIN 3 or a cervical carcinoma is 19% to 77%. (41,43) The primary cause of this low number are believed to be errors in the collection, preparation and evaluation of PAP specimens. (44,45)

PAP- Smear method:

In PAP testing, cell smears are taken from the transformation zone of the cervix and the cervical canal. The assessment criteria are cytoplasmic and nuclear changes in the cells. (39) The collected cells are then spread on a microscope slide, fixed with alcohol, stained according to Papanicolaou , covered and examined under a light microscope. (18,22,39)

The assessability depends on the examiner, the obtaining of the tent material, the smear on the slide and the fixation.(18)

For good cell collection, the portio should not be touched before the smear, so no intervention should be made. Two separate smears are performed. For cell collection, smear instruments are used to take the first smear from the surface of the portio with wiping movements, paying attention to the border area of squamous and cylindrical epithelium. The second smear is taken from the cervical canal with a rotating movement of the smear instrument. These two different fractions can then be applied to a microscope slide. (22,39)

The Papanicolaou classification is divided into five groups (Pap I-V) (22) :

Class	Description
I	Absence of atypical or abnormal cells
II	Atypical cytology, but no evidence for malignancy
III	Cytology suggestive of, but not conclusive for, malignancy
IV	Cytology strongly suggestive of malignancy
V	Cytology conclusive for malignancy

Table 3. *The original Papanicolaou classification ; From Papanicolaou, 1954*

It is important that a higher grade pathological Pap smear must be clarified histologically, as only histology can confirm or exclude CIN, especially CIN 3. (22)

Thin-layer cytology:

Better results can be obtained using thin-layer cytology, which has both high sensitivity and high specificity. In this method, the obtained cell material is placed in a container filled with special liquid. This allows more cells to be collected, which increases the detection rate and the cell material can subsequently also be used for further diagnostic procedures (e.g. HPV diagnostics). (39,46)

Colposcopy:

In colposcopy, a binocular magnifying glass, the colposcope, with 10-40x magnification is used. With the help of this magnifying glass, both the cervical surface, the external cervix, the walls of the vagina and the vulva can be assessed. If necessary, the cervical canal can also be spread and the distal section of the examination can be used to view changes in the epithelium. To better visualize the epithelium and any changes, the colposcopic examination is enhanced by acetic acid and iodine sampling. (39)

Acetic acid sample:
2-3% acetic acid is applied to the surface of the portio. Abnormal areas on the epithelium can be better assessed in this way, as the cylindrical epithelium swells due to the acetic acid and abnormal areas appear whitish. (39)

Schiller iodine sample:
4% Lugol's iodine solution is applied to the portio and vaginal tissue. The iodine solution reacts with the glycogen of the tissue. During the reaction, which only takes place in healthy tissue, as there is no glycogen in an abnormal epithelium, the tissue turns red-brown. (39)

HPV diagnostic:

Since it has been shown that human papillomavirus DNA can be found in almost all cases of cervical carcinoma and also precursor lesions, HPV testing occupies a significant position in that it can serve as an additional investigative measure in the identification of high-risk patients in case of unclear findings. The HPV test is also under discussion as a primary screening tool due to its good sensitivity (96% vs. 50% with cytology), which has been proven in several studies. (20,43)

The combination of liquid-based cytology and HPV-DNA detection (DNA and PAP) could further improve the sensitivity to detect precancerous lesions. This would increase the cost of a single test, but because of the safety of the test, the screening interval could be consistently extended if the test is negative. (21,47)

- Histological clarification

Histological clarification can be performed by biopsy, cervical curettage or conization. Biopsy allows a tissue sample to be taken specifically from a suspicious area under colposcopic view. Since the cervical canal cannot be viewed colposcopically, cervical canal curettage is used to obtain a tissue sample from the cervical canal. In the case of suspicious lesions without clinical suspicion of carcinoma, conization can be performed. In this procedure, a scalpel is used to remove a cone containing portions of both the portio and the cervical canal. Conization is a more invasive procedure than biopsy and is associated with a higher complication rate. Postoperative bleeding is most common, but cervical insufficiency may result. (39,48)

Once a diagnosis of cervical carcinoma has been made, further diagnostic measures are useful before initiating the appropriate therapy. To assess the extent of the tumor, a renal sonography and an excretory urogram can be performed. If infiltration of the rectum and/or bladder is suspected, a rectoscopy or cystoscopy is performed. Chest x-ray helps to rule out pulmonary metastases. (37,48)

1.7. Therapy of cervical carcinoma

Treatment of cervical cancer is based on factors such as size, stage, histologic characteristics of the tumor, and lymph node involvement. Primarily, treatment of cervical cancer is by surgery or radiotherapy, with chemotherapy and endocrine therapy being a valuable adjunct. (1) Depending on the FIGO stage detected, the general condition of the patient and the desire for fertility preservation, there are different therapeutic approaches.

- Surgical therapy

In early stages, radiotherapy and surgery are equally effective. As long as the tumor has not reached the pelvic wall (FIGO stage IIb), surgical therapy is preferred. In principle, extended abdominal radical surgery according to Wertheim Meigs is performed. The uterus, the parametria, a vaginal cuff as well as the pelvic and possibly also the paraaortic lymph nodes are removed via a lower median laparotomy. Especially in premenopausal patients, the ovaries are often left in place. The risk of ovarian metastases in stage Ib squamous cell carcinoma is 0.5% and in stage Ib adenocarcinoma 1.7%. (37) For FIGO stage Ia1, conization is usually sufficient due to the low risk of lymph node metastases (<1%) and very rare parametranal involvement. (48) Stegeman et al described parametranous involvement in stage Ia-Ib1 $\leq$2cm in only 1.9% of cases. (49) Conization can also be attempted for stage Ia2, especially in the case of an existing desire to have a child and the absence of high-risk factors. If lymphatic vessel invasion is described in the histological clarification, the pelvic lymph nodes should also be removed. Although adenocarcinoma is considered by many to be more aggressive than squamous cell carcinoma, it has been shown that in stages Ia-Ib $\leq$2cm, parametranous involvement and positive lymph nodes are very rare. Thus, it seems possible to perform a less radical therapy in the form of local excision even in adenocarcinoma. Thus, this may preserve fertility and reduce morbidity due to radical surgery. (49)

Parametrial section and lymph node status:

Resection of the parametrium represents a difficult step in the treatment of cervical carcinoma. Extensive parametrial resection is the main cause of postoperative morbidity. Limited resection could decrease the complications that occur, such as bladder dysfunction, severe blood loss, and rectal dysfunction. Steed et al. reported parametranous involvement in 5% of cases in stages Ia-Ib1. It has been shown that the risk of spread to the parametrium was associated with larger tumor size, deep invasion, and pelvic lymph node metastases. (50)

It appears that parametranous involvement is rare in small cervical carcinoma, possibly limiting parametranous resection. However, Benedetti-Panici et al. illustrates that subclinical spread of tumor into the parametrium occurs in 31% of cases in stage Ib1, 63% in stage Ib2, and 58% in stage IIa. (51)

Thus, lymph node status in combination with tumor size, could be used to perform appropriate parametrial resection. (50)

Fertility preserving therapy:

Cervical cancer is often diagnosed in young women who have not yet completed their family planning and wish to preserve their fertility. Many studies have looked at the extent to which the radicality of the operation can be safely reduced oncologically in the case of cervical carcinoma and an existing desire to have children.

Radical trachelectomy can be performed abdominally, vaginally, laparoscopically, and robotically. Cervix uteri, the median parts of the parametrium, and an upper vaginal cuff are removed, while the corpus uteri is left in place. Thus, it is possible to potentially become pregnant after trachelectomy. According to Beiner et al, the pregnancy rate after radical trachelectomy ranges from 41-79%. Radical trachelectomy is considered an oncologically safe alternative to radical hysterectomy, especially for early cervical cancer, in patients of childbearing age.

There is no significant difference in survival, recurrence rate, and complications between radical trachelectomy and radical hysterectomy. However, radical trachelectomy results in less blood loss and the postoperative hospital stay is shorter. Fertility may be preserved. (52)

Since this form of surgery should only be performed under certain conditions, there are some criteria for performing a trachelectomy: (53)

-Desire for fertility preservation
-Proven diagnosis
-Squamous cell carcinoma, adenocarcinoma or adenosquamous carcinoma
-Tumor size < 2cm
-Tumor limited to the cervix according to MRI or PET-CT
-Stage Ia1 with lymphatic vessel invasion, stage Ia2 or Ib1
-No existing infertility

Although fertility is preserved by trachelectomy, the main concern in a subsequent pregnancy appears to be the increased rate of preterm birth and miscarriage. One study showed that in the 1st trimester 17% of miscarriages, in the 2nd trimester 10% of miscarriages and 22% of preterm births resulted.
Some authors described that 65% of patients with early cervical carcinoma do not have tumor in the trachelectomy specimen after diagnostic conization. Therefore, conization in combination with lymphadenectomy is suggested as definitive fertility-preserving therapy, since the outcome of pregnancy after conization is significantly better than after trachelectomy. (54,55)

Chemotherapy and Radiotherapy

Classical radiotherapy is composed of a combination of percutaneous high-voltage irradiation and brachytherapy. Especially in the advanced stages (from FIGO IIb), primary radiochemotherapy is the standard therapy. Studies have shown that the combination of radiotherapy and chemotherapy in the advanced stages significantly improves outcome. (56)

In young patients with good general condition in the early stages, surgery is preferred to primary radiotherapy, despite simultaneous outcomes. However, a major disadvantage of radiotherapy is the loss of ovarian function.

Adjuvant radiotherapy is given for large tumors and additional risk factors such as lymphatic vessel invasion and pelvic metastasis. Adjuvant radiochemotherapy is preferred for extensive lymph node involvement and/or parametranous involvement. If recurrence and metastasis occur, palliative radiotherapy may help improve quality of life. (37,48) Cervical cancer patients with intermediate risk factors such as large tumor size and deep stromal invasion do not need further adjuvant therapy, whereas adjuvant CRT is recommended for high-risk patients with one or more negative prognostic factors such as positive lymph nodes or microscopic parametrial involvement. (4)

Chemotherapy can be used for both squamous cell carcinoma and adenocarcinoma. If an initially inoperable tumor is diagnosed, neoadjuvant chemotherapy (e.g., cisplatin) can be used to try to shrink the tumor. Although a meta-analysis also reported significant benefits with non-platinum agents, the most commonly used regimen is weekly cisplatin 40mg/m2. In about 75% of cases, these tumors can subsequently be submitted to surgery. (37,48,57) The use of neoadjuvant chemotherapy mainly involves the reduction of the size of the primary tumor, which allows operability, and also the reduction of the number of hypoxic cells. (58–60) A meta-analysis has shown that NACT followed by radical surgery results in a highly significant 35% reduction in the risk of death compared to RT alone (HR=0.65; p= 0.0004). An absolute improvement in 5-year survival from 50% to 64% was also shown. (61)

Furthermore, another meta-analysis confirmed that patients treated with NACT followed by surgery had better local control compared with patients treated with surgery alone. (62) These results suggest that NACT may offer an advantage over surgery alone in cervical cancer patients.

Preference is most often given to surgery followed by chemotherapy or combined CRT for limited, potentially respectable disease or definitive CRT for locally advanced, nonrespectable but nonmetastatic disease. Palliative chemotherapy is preferred for metastatic disease using chemotherapy regimens typically used for small cell lung cancer. (4)

Follow-up care and prognosis

Follow-up care is composed of a focused medical history, gynecological examination, and assessment of the kidneys by ultrasound. Recurrences and complications occur especially in the first post-therapeutic years. Therefore, follow-up is performed four times a year for the first two years and then every 6 months until the 5th year. (37,48)

The 5-year survival rate for all FIGO stages is approximately 70%. (37)
Important prognostic factors include tumor volume, parametrial involvement, lymph node involvement, lymphatic vessel invasion, depth of invasion, and grading. In particular, parametrial involvement is associated with a significantly increased recurrence rate. (50)

HPV vaccination

For primary prevention, there are two vaccines against HPV16/HPV18 (Cervarix) and against HPV 6,11,16 and 18 (Gardasil). The optimal age for vaccination is between 9 and 12 years, most favorably before the first sexual contact. Since in about 70% of cervical carcinomas are caused by HPV16 and 18, a decrease in cervical carcinomas and cervical precursors is expected in the future due to vaccination. (37)

1.8. Biomarker

In HPV-associated tumors and tumor precursors, the tumor suppressor protein p16 is upregulated as a consequence of transcription of the viral oncogene E7 and therefore immunhistochemical evidence of p16 overexpression can be considered an effective marker of transcriptionally active HPV infection. In this regard, a strong and diffuse block-like staining reaction for p16 is considered positive and a patchy and incomplete p16 immunoreaction is considered negative. (63)
However, there are other interesting biomarkers that may be helpful in diagnosis.

- mTOR

mTOR, a serine/threonine protein kinase, consists of two distinct protein complexes known as mTOR complex 1 and mTOR complex 2. The signaling cascade of mTOR (mechanistic Target of Rapamycin) has an important role in many fundamental aspects of cellular biology. Multiple cellular stimuli can activate the PI3K/AKT/mTOR signaling pathway to regulate various physiological functions such as cell survival, proliferation, migration, energy metabolism and growth in cancer. Dysregulation of mTOR can causes progression of various cancers. (64,65)

Figure 2 shows that mTORC1 regulates cell growth and metabolism through growth factors and amino acids, mTORC2 instead controls cell survival and proliferation through growth factors primarily by phosphorylating various members of the protein kinase family (PKA,PKG,PKC). (66)

In recent years, extensive research has led to a better understanding of the mechanism of this complex signaling pathway, centered on the serine/threonine kinase AKT, which comprises three different isoforms (AKT 1-3). An important role of mTORC2 is the phosphorylation and activation of AKT thereby promoting cell survival, proliferation and growth. (67)

Constitutive activation of the PI3K/AKT pathway has been demonstrated in cervical cancer samples. (68)

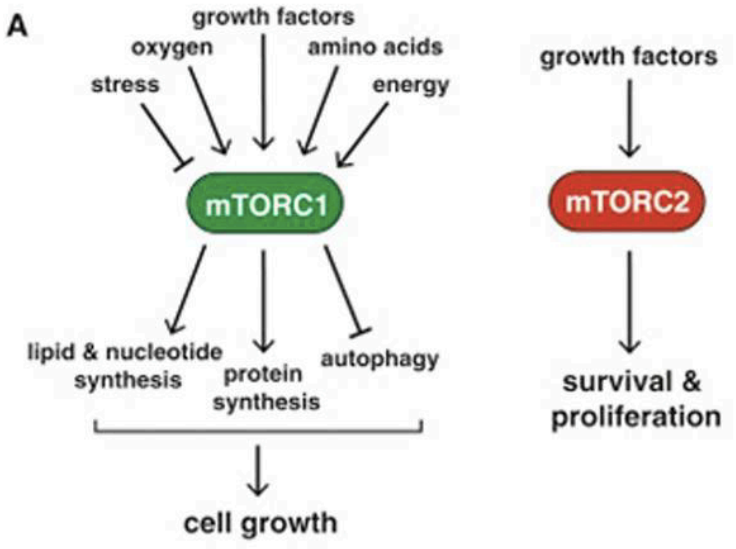

Figure 2. *The mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) signaling pathways. (66)*

For a long time, mTOR has been studied as a target in various cancers. Patients with adenocarcinoma of the biliary tract showed significantly shorter overall survival with phosphorylated-mTOR (p-mTOR) positive tumors than those with p-mTOR negative tumors. (69)

The BOLERO-2 breast cancer trial impressively demonstrated the benefit of a combination of mTOR inhibitor (everolimus) and an aromatase inhibitor (exemestane). Everolimus and Exemestane were shown to significantly prolong progression-free survival compared to placebo plus Exemestane alone in the patient population.

However, this is still limited to breast cancer patients. (70) These results lead to the question of whether this combination could possibly be used in cervical cancer.
There are few published studies on mTOR expression and radiation resistance in patients with cervical carcinoma. However, it has been observed that patients with advanced cervical cancer who express the serine/threonine kinase AKT as well as mTOR and were treated with cisplatin-based neoadjuvant chemotherapy have a good prognosis. This suggests that mTOR may be a potential new target for therapeutic intervention in patients with cervical cancer. (71)
The mTOR signaling pathway has been reported to be activated in cervical carcinomas, and thus inhibition of this pathway may be a potential therapeutic strategy. (72)

- PTEN

PTEN is one of the most important tumor-suppressor gene located on chromosome 10q23.3. It has important functions in controlling cell growth, promoting apoptosis, downregulating adhesion and suppressing cell migration. (73–75) Its mutation or loss of expression is often associated with various tumor identities, including cervical carcinoma. Furthermore, PTEN functions as a key regulator in angiogenesis. (76)
A number of other human cancers, including brain, prostate, breast, thyroid, lung and endometrium have been found to have a deletion and/or mutation of the PTEN gene. (77–82) Loss of PTEN function can occur via various genetic and epigenetic mechanism such as deletion, mutation and methylation. (83)
In a study by T.-H.Cheung et al. , PTEN methylation and loss of PTEN expression occurred in a subgroup of CIN and cervical cancer. They found that tumors with PTEN methylation are likely to be more aggressive and have worse prognosis. (83)

The tumor-suppressor gene PTEN encodes a lipid and protein phosphatase enzyme with dual specifity. It dephosphorylates and reduces the level of phosphatidylinositol-3,4,5-triphosphate, which is required for activation of serine-threonine kinase (AKT). The function of AKT is to promote cell survival and proliferation. (73)

When growth factors bind to cell membrane receptors, PIP-3 is increased, which activates the AKT complex and thereby promotes cell proliferation. On the other hand, PIP3 levels and phosphorylated AKT are reduced by high PTEN levels and thus apoptosis is induced. (74) Therefore, PTEN plays an important role in modulating cell cycle progression and/or apoptosis and thus loss of PTEN may contribute to the development of metastasis. (73,75)

- uPAR

The urokinase plasminogen activator system (uPA system) is a proteolytic enzyme system that plays a central role in metastasis and growth of diverse solid tumors. The uPA system consists of the urokinase-type plasminogen activator, physiological inhibitors (PAI-1 and PAI-2), and the uPA receptor (uPAR, CD87), which is expressed on the surface of many tumor cells. Through this system, tumor cells are enabled to degrade the extracellular matrix (ECM) surrounding them to form metastases. Binding of urokinase to the urokinase receptor activates plasminogen to plasmin on the surface of tumor cells and subsequently induces enzymatic degradation of the ECM, thereby promoting tumor cell spreading.

Since most cancer deaths are not caused by the growth of the primary tumor but by the metastasis of the tumor, agents that specifically inhibit metastatic processes would be indicated. Although the exact biochemical processes of metastasis have not been fully investigated, it has already been demonstrated that the uPA system plays a critical role in tumor cell invasion, metastasis and primary tumor growth. It has been shown that high expression of uPA and PAI-1 in tumor tissue is associated with unfavorable disease progression and poor prognosis. (84)

Numerous studies found out that uPAR is a key regulator of several steps in angiogenesis and also affects the regulation of PTEN. (76,85–89)

The functional role of the urokinase receptor (uPAR, CD87) in growth factor-induced endothelial cell activation was previously described by Unseld et al. and others. (85–89)

Upon endothelial cell activation, the protein uPAR is up-regulated and over expressed in many tumor cells. (90,91)

Unseld et al. observed that uPAR, when induced by major growth factors, is essential for effective endothelial cell migration and invasion. (86) Another study by Unseld et al. found that overexpression of uPAR led to its interaction with integrin adhesion receptors to disrupt PTEN transcription in an NFkB-dependent manner. The implication is that when uPAR suppresses PTEN expression, AKT-dependent cell behavior, such as cell motility and survival in vitro and endothelial cell invasion in vivo, is promoted. (76)

- EGFR

EGFR is a transmembrane glycoprotein receptor which is encoded by the HER-1 proto-oncogene located on chromosome 7p12. Docking of a ligand induces dimerization, activating a tyrosine kinase domain to regulate multiple functions including differentiation, cell growth, gene expression and development. The receptor is found in many normal tissues and is also expressed in a variety of solid tumors, including cervical cancer. (92) Normally, EGFR is expressed in normal cervical mucosa in the cytoplasm and membrane of cells within the basal layer. Upon cell differentiation, there is a shift towards the cytoplasm. Due to increased cytoplasmic EGFR expression with increasing grade of intraepithelial neoplasia, EGFR expression is associated with HPV infection. (93)

EGFR can be activated by binding of the E5 protein of HPV type 16 to a subunit of the protein pump ATPase. This leads to reduced degradation of EGFR receptors, an increase in EGFR recycling, and overexpression of EGFR. (94–96) Also, expression of high-risk HPV E6 has been associated with an increase in EGFR levels. Furthermore, changes in the functional level of HPV E6/E7-proteins, may alter the growth rate of cervical cancer cell lines by decreasing the stability of EGFR at the post-transcriptional level. (97–99)

According to Soonthornthum et al. most carcinoma cells express less EGFR than normal epithelial cells. Moreover, EGFR is expressed in 50-70% of cases in squamous cell carcinomas, with lower expression in adenocarcinoma and adenosquamous carcinomas. (100)

HPV-induced genetic instability has been hypothesized to mutate EGFR. Soonthornthum et al. and others have shown that EGFR mutations are uncommon in high-grade cervical cancer lesions and invasive cervical carcinomas. Thus, it can be concluded that high-risk HPV proteins affect EGFR at the protein level but not at the genomic level. (100–103)

- ER and PR

Estrogen receptors consist of 2 isoforms (ERalpha and ERbeta), each encoded by a different gene. Both isoforms form homo- or heterodimers to interact with estradiol and perform different functions. (104)

ER are highly expressed in the endocervical mucosa regardless of the phases of the menstrual cycle. ERs are expressed by basal as well as parabasal cells of the squamous epithelium in the exocervix mainly during the proliferative phase of the menstrual cycle and by metaplastic cells at the squamous-columnar junction. (105,106) The most studied ER is ERalpha, which is also routinely studied in patients with breast and endometrial cancer.

PRs also consist of 2 isoforms (PRB and PRA) encoded by a single gene. The 2 isoforms are actually 2 functionally distinct transcription factors that mediate their own response genes and also physiological effects. (107) Since the expression pattern of PRs in the cervix depends on the transactivation of the estradiol-bound ER at the gene promoter, it is similar to the expression pattern of ERs. The only difference is that PRs are expressed in the luteal phase, whereas ERs are not expressed in this phase. (108)

In cervical intraepithelial neoplasia, expression of both ER and PR is downregulated and progressively decreases with increasing lesion severity. These receptors were found to be less expressed in adenocarcinomas and rarely detected in invasive squamous cell carcinomas. (105,109–111)

ER and PR are expressed in the epithelium of the adult uterine cervix in the basal and parabasal cells, but rarely in squamous cell carcinomas. (105,109)

The expression of ERalpha is progressively downregulated during cervical epithelial transformation, according to Zhai . The loss of ERalpha expression plays an important role in mediating the invasion and progression of cervical carcinoma. (112)

2. Aim of the Thesis

2.1. The primary aim

The aim of this study is to investigate whether there is a significant correlation between the expression rates of PTEN, EGFR, mTOR and hormone receptors (ER and PR) in cervical cancer compared to healthy tissue, with respect to molecular mechanisms and with additional consideration of the role of tumor formation and metastasis. An important focus was to determine whether mTOR and the hormone receptors ER and PR show a significant correlation in cervical cancer, as also shown in the BOLERO-2 study. Furthermore, it will be examined whether the expression rates of the individual biomarkers differ or whether there is a significant correlation between them and if this correlation differs between tumor and healthy tissue.

2.2. The secondary aim

As a secondary goal, the correlation of the expression rate of the respective protein and survival with age, grading stage and TNM will be analyzed. The correlation between survival status, survival time and expression rate will also be evaluated.

Therefore, the aim of the study is to optimize the treatment of patients with cervical carcinoma, which is associated with the biomarkers PTEN, EGFR, mTOR and the hormone receptors ER and PR.

Results should demonstrate the relationship between growth-inhibiting and growth-promoting proteins and tumor tissue in order to identify possible therapeutic options to stop tumor proliferation and progression.

3. Material and Methods

3.1. Study Design

We performed a preclinical, monocentric study including 126 women with histologically proven cervical cancer. These patients underwent surgery between January 2010 and September 2011.

IHC staining with PTEN, EGFR, mTOR, ER and PR was performed on the cervical tissue microarrays. Both the microarrays, as well as the positive controls were deparaffined and the non-specific bindings' blocking were produced by peroxidase. Subsequently, the primary and secondary antibodies were applied and the results of the IHC evaluation were analyzed pathologically and statistically with the Clinical Data.

Using a light microscope, the immunohistochemically stained sections were evaluated pathologically without information about the clinical patient data. The intensity of staining was evaluated by four intensity categories: no staining (0), weak staining (1+), moderate staining (2+), strong staining (3+).
To obtain an immunohistochemical score for each biomarker in each tissue, the detected intensity was multiplied by the percentage of positive cells and summed up. Furthermore, the presence (+1) and absence (0) of the biomarkers in stromal cells was also assessed by immunohistochemical staining.

Immunohistochemistry was performed using anti-Estrogen receptor rabbit monoclonal antibody (source: Ventana, clone: 790-4324 SP1, Tuscon, Arizona, ready to use), anti-Progesteron receptor rabbit monoclonal antibody (source: Ventana, clone: 790-2223 1E2, Tuscon, Arizona, ready to use), anti-mTOR rabbit monoclonal antibody (source: Cell Signaling, clone: 2976 49F9, dilution 1:50), anti-EGFR mouse monoclonal antibody (source: Ventana, clone: 790-2988, Tuscon, Arizona, ready to use) and anti-PTEN rabbit monoclonal antibody (source: Abcam, clone: Ab32199, Cambridge, UK, dilution 1:50).

The present study has been approved by the Ethics Committee of the Medical University of Vienna (EK Nr. 1182/2019) and has been conducted according to the principles expressed in the Declaration of Helsinki.

3.2. Study Population and samples

The tissue microarray was purchased from "BioCat GmbH". The tissue samples were stained and histologic analyzed by IHC. From each patient, samples exist for both tumor tissue and normal tissue. Anonymized data of the patients were already available. Following arrays with provided Clinical Data are concerned.

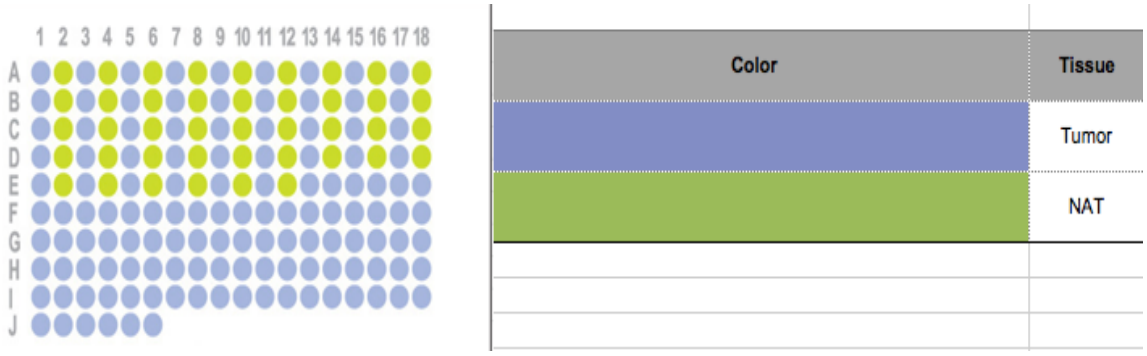

Figure 3. *Microarray panel display*

Array catalogue number: HUteS168Su01-BX

Description: Human cervix tissue

Species: Human (Mongoloid)

Fixative: Formalin

Total Cases: 126

Total Cores: 168

Core Diameter: 1.5mm

Section Thickness: 4 micrometers

Validation: Validated by immunohistochemistry

Tissue summary: Human cervix tissue; 126 cases; 42 cases have 2 cores (tumor and NAT) and 84 cases have tumor only

Storage: Short term RT; Long term 4 °C

3.3. Benefit and Risk Evaluation

The patients included have no direct benefit from this study. As this was a transnational non-therapeutic biomedical research (basic research), no risk was expected at any time during the study. By anonymizing and restricting access, the disclosure of sensitive data was minimized so that no conclusions can be drawn about individual patients from the results. Therefore, data protection and ethical aspects could be considered as fulfilled.
The contents are stored on a restricted PC at the Institute of Internal Medicine I/Oncology of the Medical University of Vienna and are only available to authorized personnel in case of need (Priv. Doz. Dr. Matthias Unseld, PhD).

3.4. Inclusion Criteria / Exclusion Criteria

In this study, women also of childbearing age with a minimum age of 18 years and a maximum age of 90 years with histologically proven cervical cancer were included. The data of the patients included are checked for the presence of cervical cancer. If this is not the case, the patients will be excluded from the study. Minors are also not included in the study.

3.5. Parameter

In order to be able to sufficiently assess the biomarkers examined by us, we have collected several parameters.

The following Parameters were evaluated:

- Age
- Organ
- Pathological diagnosis / Pathological Type
- Grading
- Stage
- TNM-Classification
- Surgery date (J/M/D)
- Survival status (alive/deceased)
- Survival time (months)
- Staining intensity IHC score of PTEN, mTOR, EGFR, ER, PR

3.6. Statistical Analysis

Descriptive statistics were generated for each biomarker using Microsoft Exel and presented in tabular form, including mean values, the respective standard deviation, and the 95% confidence interval of the difference.

The paired Student's t-test was used to examine a significant difference in the expression levels of the biomarkers PTEN, EGFR, mTOR, ER and PR between tumors and controls.

The Kaplan-Meier method with log-rank test for overall survival was performed to assess the correlation between biomarker expression in tumor and normal cervical tissues and biomarker expression in stromal tissues with overall survival and thereby the probability of survival. The Kaplan-Meier curve was performed for graphical representation.

Pearson's correlation coefficient was used to calculate significant correlations between biomarker expressions in each tissue. However, because this is not resistant to outliers, a strong linear correlation can be simulated. The rank correlation coefficient according to Spearman, on the other hand, is resistant to outliers and was also used in this case. This is shown in correlation tables for each tumor and control tissue.

The chi-square test was performed because biomarker expression levels were measured according to a dichotomized (positive or negative staining) scheme.

Statistical analyses were carried out using SPSS 24.0 software for Windows (Chicago, IL). A p-value of < 0.05 was defined as statistically significant.

4. Results

In this section, we first present the patient- and disease-specific characteristics and then present the results to answer the central research question.

4.1. Patient's characteristics

Patients characteristics	Frequency (n)	Percentage (%)
Age		
< 60	105	83,3
≥ 60	21	16,7
mean	47,5	
Pathology diagnosis		
squamous carcinoma	96	76,2
Adenosquamous carcinoma	6	4,8
severe atypical hyperplasia of cervical squamous epithelium, partly squamous carcinoma	20	15,9
small cell neuroendocrine tumor, with adenocarcinoma	1	0,8
Adenocarcinoma	3	2,4
TNM Stage		
I	69	54,8
II	30	23,8
III	24	19,0
IV	3	2,4
Distant metastasis		
No	125	99,2
Yes	1	0,8
Lymph node metastasis		
No	25	19,8
Yes	101	80,2
Grade		
I	6	4,8
II	21	16,7
III	78	61,9
Missing	21	16,7
Survival (in months)		
Mean	63,8	
stdev	20,4	

Table 4. *Patient- and disease specific characteristics/ Sample characteristics (n=126)*

Table 4 shows the baseline characteristics of the patient sample.
The present study included 126 patients with cervical cancer. Out of these, 105 (83.3%) patients were < 60 years old and 21 (16.7%) were ≥60 years old. The patients showed a mean age of 47.5 years. 42 patients showed corresponding adjacent control tissue (NAT) and in 84 patients corresponding adjacent control tissue was not available.

96 patients were diagnosed with squamous carcinoma (76.2%), 6 with adenosquamous carcinoma (4.8%). This table shows that squamous carcinoma with 76.2% (n=96) occurs significantly more frequently than a small cell neuroendocrine tumor with adenocarcinoma with 0.8% (n=1). 69 patients showed a TNM Stage I (54.8%) and TNM Stage IV was found in 3 patients (2.4%). The distribution of the stages is pictured in Table 2. 6 individuals were identified as Grade I (4.8%), 78 patients as Grade III (61.9%). In addition, it can be clearly seen that there is often lymph node metastasis (80.2%, n=101) but rarely distant metastasis (0.8%, n=1). Mean survival was 63.8 months (stdev 20.4).

4.2. Expression rate

The expression rates of the biomarkers were analyzed for all carcinoma types. Due to the fact that tumor tissue wasn't always available the percentage value does not reach 100% in most cases. Only 42 Patients are available with control tissue. The results are represented in Table 5.

Percentage out of tumor tissue (126) control tissue (42) (%)	EGFR Ca	EGFR Ctrl	PTEN Ca	PTEN Ctrl	mTOR Ca	mTOR Ctrl	ER Ca	ER Ctrl	PR Ca	PR Ctrl
	114 (90,5)	41 (97,6)	122 (96,8)	40 (95,2)	124 (98,4)	41 (97,6)	119 (94,4)	41 (97,6)	124 (98,4)	40 (95,2)
Mean	184,1	113,2	106,9	75,6	18,3	52,8	8,1	89,3	0,0	8,0
stdev	97.7	70.1	101.2	58.1	41.3	45.9	35.5	85.8	0.4	37.8

Table 5. *Expression rates of EGFR, PTEN, mTOR, ER and PR in tumor (126) and control tissue (42).*

126 patients were available with tumor tissue and 42 patients with control tissue.

For EGFR 114 samples were successfully analyzed in tumor tissue (90.5%), 41 samples in control tissue (97.6%). The mean expression rate for EGFR was **184.1** (stdev 97.7) in tumors and **113.2** (stdev 70.1) in controls.

For PTEN 122 samples were successfully analyzed in tumor tissue (96.8%), 40 samples in control tissue (95.2%). The mean expression rate for PTEN was **106.9** (stdev 101.2) in tumors and **75.6** (stdev 58.1) in controls.

For mTOR 124 samples were successfully analyzed in tumor tissue (98.4%), 41 samples in control tissue (97.6%). The mean expression rate for mTOR was **18.3** (stdev 41.3) in tumors and **52.8** (stdev 45.9) in controls.

For ER 119 samples were successfully analyzed in tumor tissue (94.4%), 41 samples in control tissue (97.6%). The mean expression rate for ER in tumors was **8.1** (stdev 35.5) and in controls **89.3** (stdev 89.3 (stdev 85.8).

For PR 124 samples were successfully analyzed in tumor tissue (98.4%), 40 samples in control tissue (95.2%). The mean expression rate for PR in tumors was **0.0** (stdev 0.4) and in controls **8.0** (stdev 37.8).

Images of histological sections with IHC staining of mTOR, ER, and EGFR expression in tumor, control, and stromal tissues are shown in Figure 4.

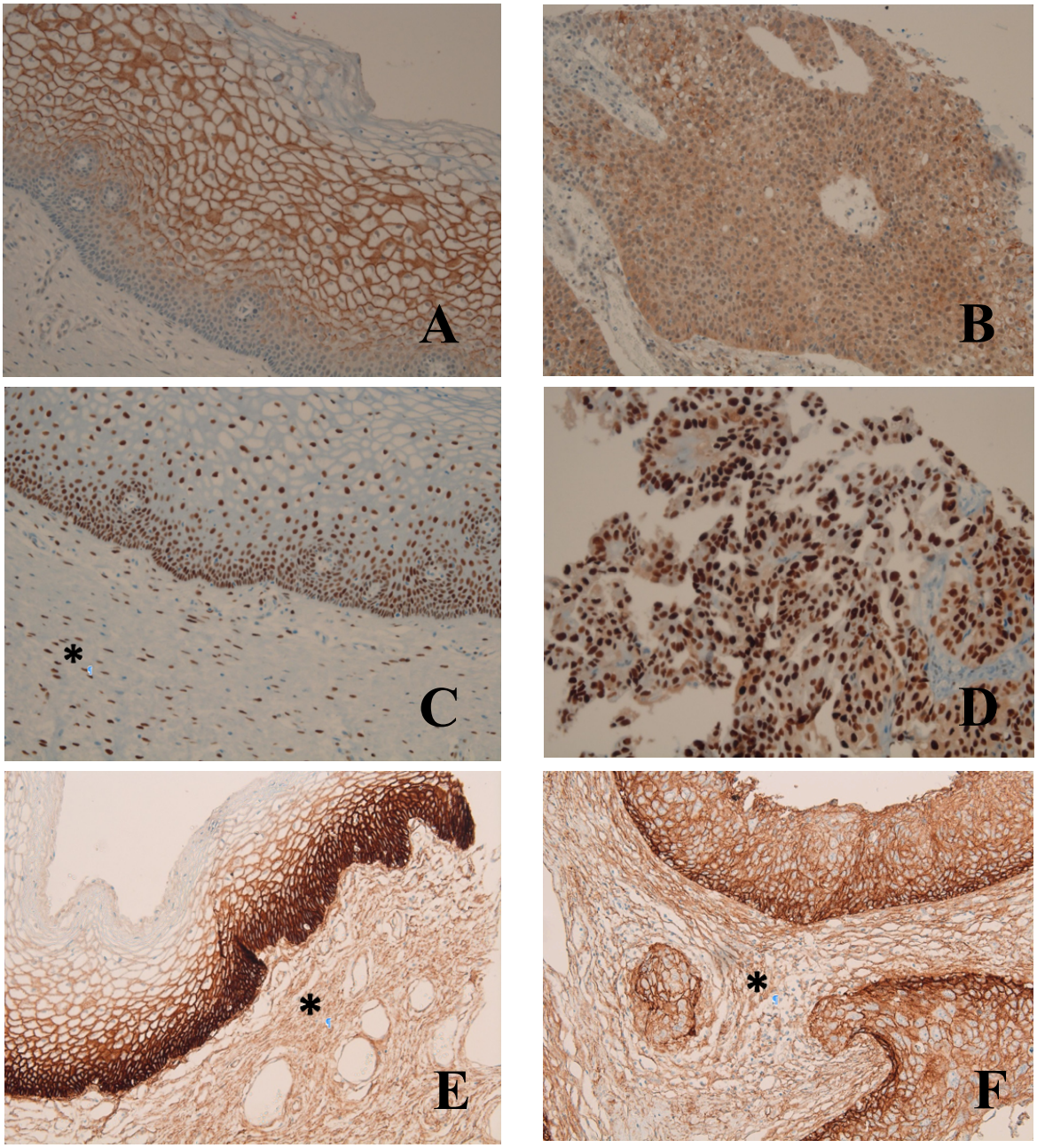

Figure 4. *IHC staining of mTOR, ER and EGFR in cervical cancer, normal cervical mucosa and stromal cells*

(A)Expression of mTOR in cervical mucosa (original magnification 20x)
(B)Expression of mTOR in primary cervical cancer (original magnification 20x)
(C)Expression of estrogen receptor in cervical mucosa and stromal cells (*) (original magnification 20x)
(D)Expression of Estrogen receptor in primary cervical cancer (original magnification 20x)
(E)Expression of EGFR in cervical mucosa and stromal cells (*) (original magnification 20x)
(F) Expression of EGFR in primary cervical cancer and in tumor surrounding stromal cells (*) (original magnification 20x)

Correlation and Difference in Expression

	N	Correlation	Significance
EGFR Ca – EGFR Co	41	0.302	0.055
PTEN Ca – PTEN Co	38	-0.236	0.154
mTOR Ca – mTOR Co	40	0.359	0.023
ER Ca – ER Co	41	0.337	0.031
PR Ca – PR Co	39		

Table 6. *Correlation among the biomarkers in the tumor tissue and control tissue using Pearson's correlation coefficients (Pearson's correlation)*

Results (as pictured in Table 6) show that there was a significant correlation between mTOR tumor tissue and mTOR control tissue (Pearson sig. = 0.023) as well as for ER in tumor and control tissue (Pearson sign. = 0.031).

The correlation between EGFR tumor tissue and EGFR control tissue did not lead to significant results (Pearson sign. = 0.055).

No significant correlation between PTEN expression in tumor and PTEN expression in control tissue could be demonstrated (Pearson sign. = 0.154).

	Mean	Stdev	Paired differences-Standard error of the mean	95% confidence interval lower	upper	T	df	Sig. (2-tailed)
EGFR Ca – EGFR Co	50.0000	108.5415	16.9513	15.7401	84.2599	2.950	40	0.005
PTEN Ca – PTEN Co	33.5526	125.6492	20.3830	-7.7472	74.8525	1.646	37	0.108
mTOR Ca – mTOR Co	-28.2500	61.2430	9.6834	-47.8365	-8.6635	-2.917	39	0.006
ER Ca – ER Co	-79.7561	82.8700	12.9421	-105.9131	-53.5991	-6.163	40	0.000
PR Ca – PR Co	-8.0769	38.2962	6.1323	-20.4911	4.3373	-1.317	38	0.196

Table 7. *Difference in expression in tumor (126) and control tissue (42) (t-test)*

Based on the examination of IHC staining in the cervical carcinoma samples, EGFR was found to be highly tumor-specific, with an increased mean expression level ($p<0.005$ (95% CI 15.7401 – 84.2599)) within the tumor compartment and very limited expression in normal cervical tissue (mean difference: 50). Results are shown by paired Student's t-test.

The mean expression rate of mTOR shows a significant ($p<0.006$, (95% CI -47.8365 – -8.6635)) difference for mTOR expression in tumor versus control tissue as well as the mean expression rate of ER tumor versus ER control tissue ($p<0.000$, (95% CI -105.9131 - -53.5991)). The mean difference for mTOR was -28.2500 and for ER -79.7561.

The mean expression rate of PTEN shows no significant ($p<0.108$, (95% CI -7.7472 – 74.8525)) difference for PTEN expression in tumor versus control tissue as well as the mean expression rate of PR in tumor versus control tissue ($p<0.196$, (95% CI -20.4911 – 4.3373)). The mean difference for PTEN was 33.5526 and for PR -8.0769.

Correlation between EGFR, PTEN, mTOR, ER and PR

		EGFR Ca	EGFR Ctrl	PTEN Ca	PTEN Ctrl	mTOR Ca	mTOR Ctrl	ER Ca	ER Ctrl	PR Ca	PR Ctrl
EGFR Ca	Spearman	1,000	,311*	**,246****	-,059	-,207*	-,127	,020	,104	,100	-,245
	Sig. (2-tailed)	.	,048	,009	,718	,027	,431	,832	,517	,291	,127
EGFR Ctrl	Spearman	,311*	1,000	,011	,145	-,254	-,198	-,223	,269	.	-,158
	Sig. (2-tailed)	,048	.	,949	,372	,114	,221	,161	,093	.	,330
PTEN Ca	Spearman	**,246****	,011	1,000	-,198	**,187***	-,019	,150	,167	,119	-,236
	Sig. (2-tailed)	,009	,949	.	,234	,040	,907	,106	,310	,195	,154
PTEN Ctrl	Spearman	-,059	,145	-,198	1,000	,230	,373*	-,005	,566**	.	,305
	Sig. (2-tailed)	,718	,372	,234	.	,159	,018	,976	,000	.	,056
mTOR Ca	Spearman	-,207*	-,254	**,187***	,230	1,000	,240	**,226***	,085	-,084	-,121
	Sig. (2-tailed)	,027	,114	,040	,159	.	,136	,014	,604	,358	,465
mTOR Ctrl	Spearman	-,127	-,198	-,019	,373*	,240	1,000	,051	,451**	.	,170
	Sig. (2-tailed)	,431	,221	,907	,018	,136	.	,750	,004	.	,295
ER Ca	Spearman	,020	-,223	,150	-,005	**,226***	,051	1,000	,225	**,278****	-,047
	Sig. (2-tailed)	,832	,161	,106	,976	,014	,750	.	,157	,002	,775
ER Ctrl	Spearman	,104	,269	,167	,566**	,085	,451**	,225	1,000	.	,044
	Sig. (2-tailed)	,517	,093	,310	,000	,604	,004	,157	.	.	,792
PR Ca	Spearman	,100	.	,119	.	-,084	.	**,278****	.	1,000	.
	Sig. (2-tailed)	,291	.	,195	.	,358	.	,002	.	.	.
PR Ctrl	Spearman	-,245	-,158	-,236	,305	-,121	,170	-,047	,044	.	1,000
	Sig. (2-tailed)	,127	,330	,154	,056	,465	,295	,775	,792	.	.

**. The correlation is significant at the 0.01 level (two-tailed).

*. The correlation is significant at the 0.05 level (two-tailed).

Table 8. *Correlation among the biomarkers in tumor tissue and control tissue using Spearmans's rank correlation coefficients*

The correlation between EGFR tumor tissue and PTEN tumor tissue expression was significant (Spearman p=0.246, sign. 0.009). We could also find a significant correlation in cervical tissue between PR tumor tissue and ER tumor tissue expression (Spearman p=0.278, sign. 0.002). Furthermore, Table 8. shows that the correlation between mTOR tumor and PTEN tumor expression was significant (Spearman p=0.187, sign. 0.040) as well as the correlation between mTOR and ER expression in tumor tissue, with a Spearman p-value of 0.226, sign. 0.014.

4.3. Patient Survival

The overall survival rate was observed in 126 patients and was described in months. Of these, 90 patients survived and 36 patients died during the observation period.

The median OS was 63.76 month (stdev 20.356). The longest OS rate was 86 month and the shortest was 8 month.

Biomarker expression in tumor, control tissue, and stroma, as well as clinicopathologic parameters such as tumor size, grade, lymph node status, and metastasis were analyzed for their correlation with survival.

Kaplan-Meier curves are used to display the results. The survival time in months is demonstrated on the abscissa (x-axis) and the survival probability on the ordinate (y-axis).

Overall Survival

EGFR Expression and survival

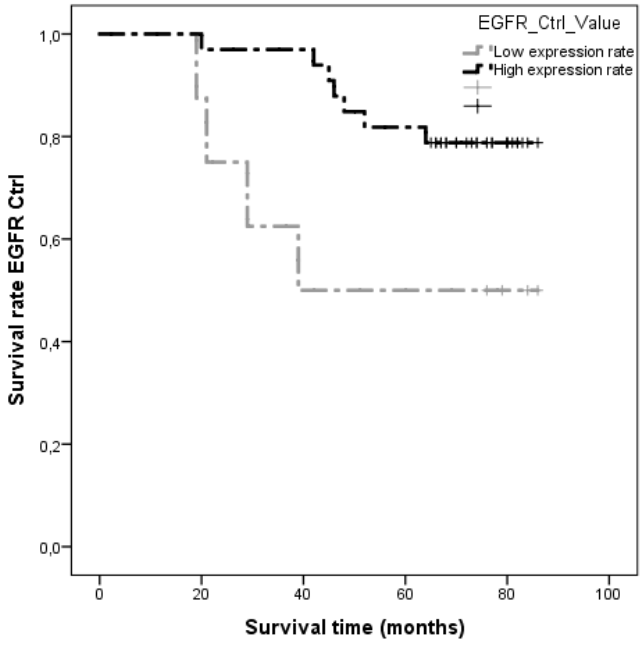

Figure 5. *Kaplan-Meier curve for OS associated with EGFR expression in control tissue*

In the control tissue, the EGFR expression rate has a significant influence on overall survival. The threshold of EGFR expression is at 50 with a p-Value (log rank) at 0.034. (n=41)

In the tumor tissue we found that a threshold of EGFR expression of 80 showed no significant differences.

PTEN Expression and survival

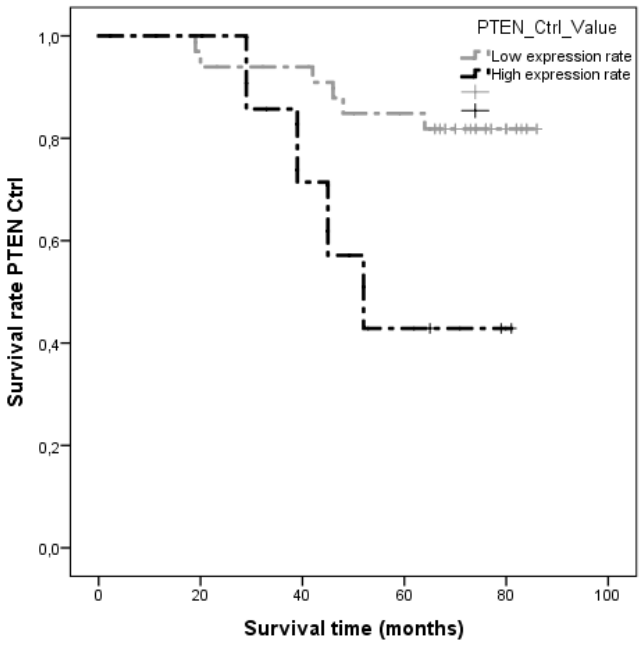

Figure 6. *Kaplan-Meier curve for OS with PTEN expression in control tissue*

In the control tissue, we found that a threshold of PTEN expression rate has significant influence on overall survival. The threshold of PTEN expression is at 125 with a p-Value (log rank) of 0.021. (n=40)

No threshold for PTEN could be found in the tumor tissue and therefore no plot of the results could be generated.

mTOR Expression and survival

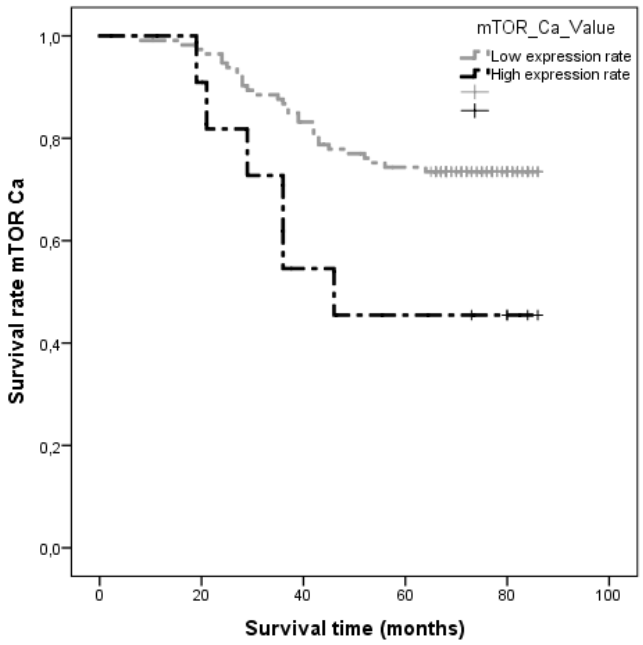

Figure 7. *Kaplan-Meier curve for OS with mTOR expression in tumor tissue*

In determining a threshold for mTOR, we found that mTOR Ca expression rate has a significant influence on overall survival, with a threshold at 85. (p-value= 0.024).

This suggests that higher expression of mTOR in the tumor compartment correlates with shorter survival. (n=124)

No threshold for mTOR and ER could be found in control tissue. Similarly, no threshold for ER was found in the tumor tissue. Therefore, no plot of the results could be generated.

PR Expression and survival

In the control tissue we found that the threshold of 1 for PR expression showed no significant influence on overall survival.

Clinicopathological parameters and survival

By using chi-square test, we found that tumor size and lymph node status have a significant influence on survival. However, the stainability of EGFR and the grading have no significant influence on survival.

	Chi-Square	df	Sign.
Tumor Size	42.203	3	0.000
Lymph node	19.182	1	0.000
EGFR Ca Stroma	0.099	1	0.753
Grade	3.287	1	0.070

Table 9. *Chi-square test*

Tumor size (TNM) and Lymph node status (TNM)

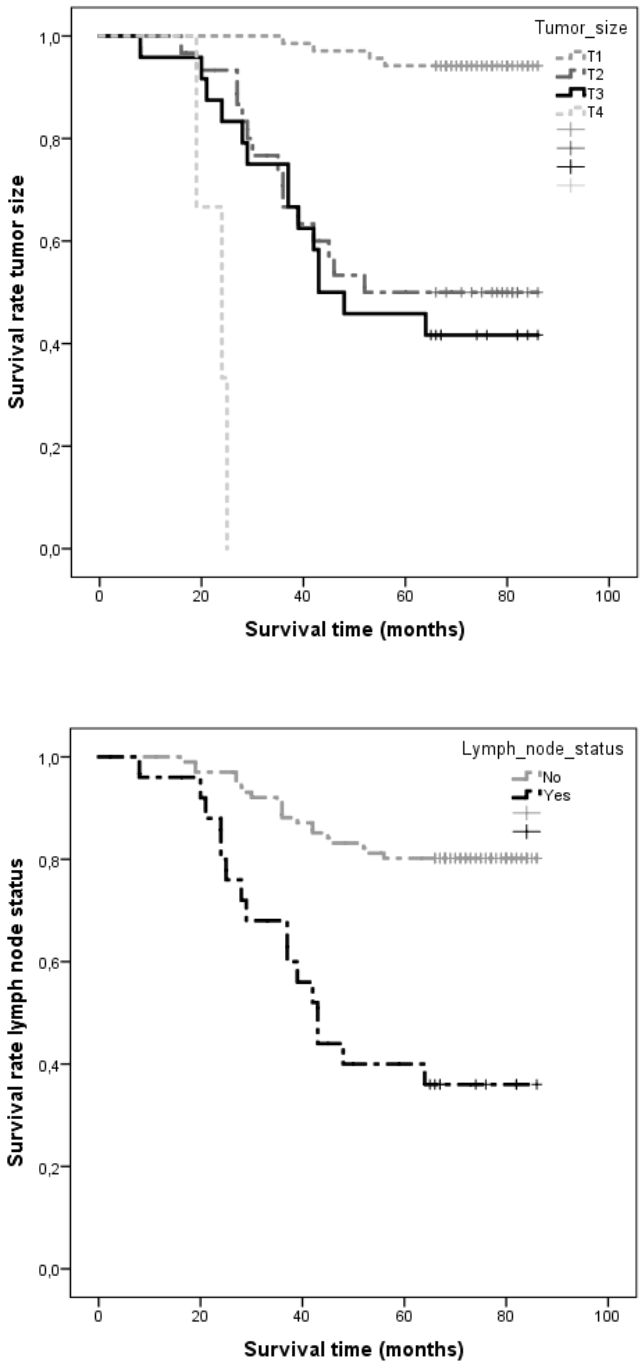

Figure 8. *Kaplan-Meier curves of OS related to tumor size and lymph node status*

Follow-up data showed that tumor size (TNM) with a p-value (log-rank) of $p < 0.000$ and lymph node status (TNM) with a p-value (log-rank) of $p < 0.000$ had a significant influence on overall survival.

EGFR stroma and Grading

Furthermore, the analysis showed that neither stainability of EGFR in the stroma (p= 0.753) nor grading (p= 0.07) had a significant influence on survival.

Multivariate analysis was not performed (Cox regression).

5. Discussion

In the present study, we investigated a possible correlation of mTOR and the hormone receptors ER and PR in 126 patients. By immunohistochemical analysis, we demonstrated that the mTOR pathway significantly correlates with the hormone receptor ER.

We found that mTOR showed a significant difference in expression level between tumor and control tissues, and therefore mTOR is of strong interest in the treatment and therapy of cervical carcinoma. We found the same for ER. The expression level of ER also showed a significant difference in tumor versus control tissue. As expected, the results for EGFR showed an increased expression level within the tumor tissue and very low expression in the control tissue. This concludes that EGFR is highly tumor specific.

Our Kaplan-Meier curves show that a low expression rate of mTOR in the tumor tissue correlates with a longer survival time. A high expression rate of mTOR in tumor tissue correlates with a shorter survival time. Furthermore, we demonstrated by Kaplan-Meier curves that tumor size and lymph node status have a significant impact on overall survival.

The prognosis of cervical cancer patients has been associated with the PI3K/AKT/mTOR signaling pathway, similar to several other cancers. This has been demonstrated by many research groups. High levels of phosphorylated AKT and activated mTOR were found to be associated with worse prognosis and shorter survival of patients. (71,113,114)

An interesting study found that AKT phosphorylation detected in cervical cancer samples indicates constitutive activation of the PI3K/AKT pathway. It was shown that an increased activation state of AKT kinase seems to be present in cervical carciogenesis and can be explained by PIK3CA amplification. (68)
In addition, mTOR inhibitors block phosphorylation and significantly reduce the level of HPV E7 oncoprotein in *in vitro* models. This leads to accumulation of cells in G1 phase and thus apoptosis is induced. (115) These results support the role of mTOR in cervical cancer.

Further results are provided by a morphoproteomic study of the mTOR pathway in HSIL and invasive cervical SCC compared with normal uterine cervical epithelium. This study also supports that alterations leading to activation and dysregulation of the PI3K/AKT/mTOR pathway could serve as potential targets for drug treatment. (116)

There is a large body of evidence that the ER and mTOR pathways have both distinct and overlapping signaling cascades and outputs. (117–122) It is known that both the mTOR and estrogen signaling pathways regulate G1 phase progression. (123,124) These data highlight that the mTOR signaling pathway and its relationship to the estrogen response are of central importance.

Currently, the highly regarded randomized phase III BOLERO-2 study shows that alterations in the mTOR pathway are associated with resistance to endocrine therapies. This was impressively confirmed with respect to progression-free survival in breast cancer patients. (70)

Many research groups are currently focusing on the development of potent and selective PI3K/AKT/mTOR inhibitors and their use in combination to make cervical cancer therapy more efficient for patients. (125,126)

Substances targeting the signaling pathway of mTOR represent promising therapeutic options and may delay or break the development of resistance to endocrine therapy.

In conclusion, it is of great interest to consider this combination of therapy (mTOR inhibition+endocrine therapy) also for the treatment of cervical carcinoma. Overall, these results support the importance of both markers (mTOR+ER) in gynecologic tumors and further investigation in high-quality, randomized clinical trials is required.

6. Conclusion

Finding new prognostic factors is necessary to improve current conventional treatments. Researchers have increased efforts to develop novel biomarkers for early diagnosis and evaluation and monitoring of therapeutic treatments. This approach will help in developing early diagnoses and increasing treatment efficacy with reduced recurrence.
Our study shows that there are clear correlations between mTOR and hormone receptors ER/PR in cervical cancer. Thus, it would be of importance to consider dual blockade of the endocrine and mTOR pathway in cervical carcinoma and also to test it with drugs. Therefore, further studies should be made to investigate this therapeutic strategy in cervical carcinoma. Combination therapy (mTOR+ER/PR) would significantly improve the prognosis of cervical cancer and provide significant clinical benefit.

7. Limitations

This study examined 126 patients, thus a larger number of patients would be beneficial. Since in our case it is only an immunohistochemical study, further clinical investigations should be considered.

8. Declaration

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9. List of Tables and Figures

10. List of abbreviations

ACIS - adenocarcinoma in situ

ATPase - Adenosintriphosphatasen

BOLERO-2 - Breast Cancer Trials of oral Everolimus-2

Ca - Carcinoma

CD87 - Cluster of Differentiation 87

CI - confidence interval

CIN - cervical intraepithelial neoplasia

CIS - carcinoma in situ

CRT - Chemoradiotherapy

CT - Computed tomography

Ctrl - Control

DNA - Deoxyribonucleic acid

ECM - extracellular matrix

EGF - Epidermal Growth Factor

EGFR - Epidermal Growth Factor Receptor

ER - Estrogen Receptor

FIGO - Federation Internationale de Gynecologie et d'Obstetrique

HER - human epidermal growth factor receptor

HIV - Human Immunodeficiency Virus

HPV - Human Papillomavirus

HR - Hazard ratio

HR-HPV - high risk Human Papillomavirus

HSIL - high grade squamous intraepithelial lesion

IHC - Immunohistochemistry

LR-HPV - low risk Human Papillomavirus

LSIL - low grade squamous intraepithelial lesion

MHC - major histocompatibility complex

MRI - Magnetic Resonance Imaging

mTOR - Mammalian target of rapamycin

mTORC1 - Mammalian target of rapamycin complex 1

mTORC2 - Mammalian target of rapamycin complex 2

NACT - neoadjuvant chemosterapy

NAT - normal adjacent tissue

NFkB - Nuclear factor-kappaB

OS - Overall survival

p-mTOR - phosphorylated-mTOR

P53 - tumor protein p53

PAI - plasminogen activator inhibitor

Pap - Papanicolaou

PET - Positron emission tomography

PI3K - Phosphatidylinositol-3-Kinase

PI3KCA - Catalytic phosphoinositide 3-kinase PI3K subunit p110 α

PIP-3 - Phosphatidylinositol-3,4,5-trisphosphat

PKA - Protein kinase A

PKB/AKT - Proteinkinase B

PKC - Protein kinase C

PKG - Protein kinase G

PR - Progesterone Receptor

pRb - retinoblastoma protein

PTEN - Phosphatase and Tensin Homolog Gene

RT - Radiotherapy

STD - sexually transmitted disease

Stdev - standard deviation

TNM - Tumour-Node-Metastasis

UICC - Union Internationale Contre le Cancer

uPA - Urokinase-Type Plasminogen Activator

uPAR - Urokinase-Type Plasminogen Activator Receptor

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