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DISSERTATION

Thema: p53 codon 72 polymorphism in HPV-related cervical cancer

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1 INTRODUCTION

1.1 Aim of the study

Cervical cancer is strongly linked to infections with high-risk HPV-types. E6 acts as a viral oncoprotein of HPV and has the ability to associate and neutralize the function of the p53 tumor suppressor gene. P53 is known as a gene with different genotypes-polymorphic alleles of p53 at codon 72 encode for homozygous arginine, homozygous proline or heterozygous arginine and proline. The Arg/Arg genotype of the p53 tumor suppressor gene was found to be more susceptible than p53 Pro/Pro in E6 mediated degradation (Storey A et al. 1998, Saranth D et al., Pegoraro RJ et al., Li et al. 2002, van Duin M et al., Dokianadis DN et al., Agoraskos T et al.). Thus, the Arg/Arg genotype of the p53 antioncogene has recently been identified as a risk factor for HPV-related cervical cancer. However, a number of other studies contradict these findings.

In this study we examined the single nucleotide polymorphism in codon 72 of the p53 tumor suppressor gene by PCR and sequencing in order to analyze the association between codon 72 polymorphism of p53 and infection with high-risk HPV in the development and outcome of cervical cancer. As accurate and robust assays are needed to produce statistically significant and reliable results PCR and cycle sequencing were used for detecting p53 polymorphism. In addition, these methods were applied to detect reliably the HPV type aware of the fact the samples were previously tested by ELISA.

We used PCR and cycle sequencing for DNA analysis from 111 HPV-positive patients with cervical lesions and from 117 healthy pregnant women.

Sequencing detects high-risk HPV ignored by ELISA which distorts results needed for reaching accurate conclusions (8.3 % were added to high-risk HPV group).

68.2 % of homozygous Arg in the high-risk HPV group developed CIN III and invasive carcinoma compared to only 53 % of the heterozygous and 42.9 % homozygous Pro.

These data suggest that women homozygous for Arginine are more susceptible for developing HPV 16/18 -related high–risk cervical lesions. Others who failed proving this association did not take into consideration the high-risk HPV infection, distribution and relation nor did they perform sequencing.

Consequently using cycle sequencing to detect homozygosity of Arg in p53 can serve as an early rapid and cost-effective detection method to identify women of developing higher risk for cervical cancer in HPV-related lesions.

2 CERVICAL LESIONS

2.1 Histopathology

Squamous cell carcinoma accounts for approximately 90 % and adenocarcinoma for approximately 10 % of cervical cancers. Adenosquamous and small cell carcinomas are relatively rare. Primary sarcomas of the cervix have been described occasionally, and malignant lymphomas of the cervix, both primary and secondary, have also been reported.

Stage Information

- Low-grade SIL (squamous intraepithelial lesion) refers to early changes in the size, shape, and number of cells that form the surface of the cervix. Some low-grade lesions spontaneously regress. However, with time, others may grow larger or become more abnormal, forming a high-grade lesion. Precancerous low-grade lesions also may be called mild dysplasia or cervical intraepithelial neoplasia 1 (CIN 1). Such early changes in the cervix most often occur in women between the ages of 25 and 35 but can appear in other age groups as well.
- High-grade SIL defines more significant changes. The cells frequently become cancerous and invade deeper layers of the cervix for many months, perhaps years. High-grade lesions also may be called moderate or severe dysplasia, CIN 2 or 3, or carcinoma in situ. They develop most often in women between the ages of 30 and 40 but can occur at other ages as well.

If abnormal cells spread deeper into the cervix or to other tissues or organs, the disease is then defined as cervical cancer, or invasive cervical cancer. It occurs most often in women over the age of 40 (ACS, American Cancer Society).

Cervical carcinoma origins at the squamous-columnar junction either in the endocervical canal or on the portio of the cervix. The precursor lesion is dysplasia or carcinoma in situ (cervical intraepithelial neoplasia (CIN), which can subsequently become invasive cancer. This process can be quite slow. Longitudinal studies have shown that in untreated patients with in situ cervical cancer, 30 % to 70 % will develop invasive carcinoma over a period of 10 to 12 years. However, in about 10 % of patients, lesions can progress from in situ to invasive in a period of less than 1 year. As it becomes invasive, the tumor breaks through the basement membrane and invades the cervical stroma. Extension of the

tumor in the cervix may ultimately manifest as ulceration, exophytic tumor, or extensive infiltration of underlying tissue including bladder or rectum.

In addition to local invasion, carcinoma of the cervix can spread via the regional lymphatics or bloodstream. Tumor dissemination is generally a function of the extent and invasiveness of the local lesion. While cancer of the cervix generally progresses with size, occasionally a small tumor with distant metastasis is seen. For this reason, patients must be carefully evaluated for metastatic disease.

Stages are defined by the Federation Internationale de Gynecologie et d'Obstetrique (FIGO) and the American Joint Committee on Cancer's (AJCC) TNM classification (Shepherd JH, 1996; Creasman WT, 1995; American Joint Committee on Cancer, 1997).

2.2 FIGO Staging

Stage I

Stage I is carcinoma strictly confined to the cervix without extension to the uterine corpus.

- Stage IA: Invasive cancer identified only microscopically. All gross lesions even with superficial invasion are stage Ib cancers. Invasion is limited to measured stromal invasion with a maximum depth of 5 mm* and no wider than 7 mm. [Note: *The depth of invasion should not be more than 5 mm taken from the base of the epithelium, either surface or glandular, from which it originates. Vascular space involvement, either venous or lymphatic, should not alter the staging.]
    - Stage IA1: Measured invasion of the stroma no greater than 3 mm in depth and no wider than 7 mm diameter.
    - Stage IA2: Measured invasion of stroma greater than 3 mm but no greater than 5 mm in depth and no wider than 7 mm in diameter.
- Stage IB: Clinical lesions confined to the cervix or preclinical lesions greater than stage IA.
    - Stage IB1: Clinical lesions no greater than 4 cm in size.
    - Stage IB2: Clinical lesions greater than 4 cm in size.

Stage II

Stage II is carcinoma that extends beyond the cervix but has not extended onto the pelvic wall. The carcinoma involves the vagina, but not as far as the lower third.

- Stage IIA: No obvious parametrial involvement. Involvement of up to the upper two thirds of the vagina.
- Stage IIB: Obvious parametrial involvement, but not onto the pelvic sidewall.

Stage III

Stage III refers to a carcinoma that extending into the pelvic sidewall. On rectal examination, there is no cancer-free space between the tumor and the pelvic sidewall. The tumor involves the lower third of the vagina. All cases with a hydronephrosis or non-functioning kidney should be included, unless they are known to be due to other causes.

- Stage IIIA: No extension onto the pelvic sidewall but involvement of the lower third of the vagina.
- Stage IIIB: Extension onto the pelvic sidewall or hydronephrosis or non-functioning kidney.

Stage IV

Stage IV is carcinoma that has extended beyond the true pelvis or has clinically involved the mucosa of the bladder and/or rectum.

- Stage IVA: Spread of the tumor onto adjacent pelvic organs.
- Stage IVB: Spread to distant organs.

2.3 Incidence and Prevalence

Cancer of the cervix is the second most common cancer in women worldwide and is a leading cause of cancer-related death in women in underdeveloped countries. Worldwide, approximately 500,000 cases of cervical cancer are diagnosed each year (NCCC, National Cervical Cancer Coalition).

Routine screening has decreased the incidence of invasive cervical cancer in the United States, where approximately 13,000 cases of invasive cervical cancer and 50,000 cases of cervical carcinoma in situ (i.e., localized cancer) are diagnosed yearly (NCCC, National Cervical Cancer Coalition). Cervical cancer rates are higher in Germany than in comparable European countries due to regional differences in screening participation (Rückinger et al. 2008).

It is estimated that more than 6 million women in the United States have HPV infection and proper interpretation of these data is important. Epidemiologic studies convincingly demonstrate that the major risk factor for development of preinvasive or invasive carcinoma of the cervix is HPV infection, which far outweighs other known risk factors such as high parity, increasing number of sexual partners, young age at first intercourse, low socioeconomic status and positive smoking history (Schiffman et al.1993; Brisson et al.1994).

There is also a higher rate of incidence among African American, Hispanic, and Native American women.

Some patients with HPV infection appear to be at minimally increased risk for development of cervical preinvasive and invasive malignancies while others appear to be at significant risk and candidates for intensive screening programs and/or early intervention (Wang and Hildesheim 2003).

While the incidence of cervical cancer in Germany has decreased by more than 60 % (Becker N et al., 1997; Gustafsson L et al.,1997; Black RJ et al., 1997). Since the use of Pap-smear in 1971 from 35.8: 100.000 to 12: 100.000 the incidence of intraepithelial lesions has increased significantly (Anderson GH et al., 1988).

Like in other countries incidence and mortality seem to stagnate in Germany as well (Fisher U. et al).

The most important reason for the persistence of cervical cancer in all countries is the fact that in over 50 % of the cases patients miss to participate in screening programmes (Plaxe

SC et al., 1999; Anderson GH et al., 1992; Janerich DT et al., 1995; Kinney W et al., 1998; Sawaya GF et al., 1999; Massad LS et al., 2000).

More than 50 % of 12,560 cases of cervical cancer in the U.S. in 1997 were not screened at all and 10 % were screened insufficiently; this shows clearly that every strategy that is limited to additional procedures only or new laboratory techniques in early detection of cervical cancer can improve incidence and mortality just marginally.

Shortcomings, lack of quality and efficiency in early cancer detection via Pap smear are observed in several national and international studies; costs and treatment patterns in women with abnormal cervical smears differ among countries due to the different type of screening programme and, consequently due to the histological type. (Mc Crony et al 1999, Schneider et al 2000, Rash B et al 2008).

2.4 Early Detection

If all women had pelvic examinations and Pap tests regularly, most precancerous conditions would be detected and treated before invasive cancer develops. Thereby, most invasive cancers could be prevented. Any invasive cancer that does occur would likely be found at an early, curable stage.

The Pap test is a simple, painless test to detect abnormal cells of the cervix; the best time is between 10 and 20 days after the first day of menstrual period (National Cancer Program U.S.).

The way of describing Pap test results is changing. The newest method is the Bethesda System. Changes are described as low-grade or high-grade SIL. It is believed that the Bethesda System provides more useful information than an older system, which uses numbers ranging from class 1 to class 5. (In class 1, the cells in the sample are normal, while class 5 refers to invasive cancer).

Currently, there are discussions to integrate HPV-tests into the primary screening program due to its relationship with cervical cancer (Schneider A et al.; Iftner T et al., 2000) and the reference to the higher sensitivity (- 32 %) compared to a Pap test.

Sensitivity of the HPV-test is 90 % and significantly higher than the Pap test (Arbyn M. et al 2004, Nieminem et al 2004, Cuzcik J, Szarewski A. et al 2003, Cuzcik J et al 2006, Clavel C. et al 2001, Sherman ME et al 2003).

2.4.1 Diagnosis

Colposcopy and Pap test allow the gynecologist to detect abnormal changes in the cervix. If these examination show that an infection is present, the doctor treats the infection and then repeats the Pap test at a later time. If the exam or Pap test suggests something other than an infection, the Pap test is repeated and further tests are performed to find out the cause of cytological changes.

Colposcopy is a widely used method to check the cervix for abnormal epithelia. The gynecologist applies a vinegar-like solution to the cervix and then uses an instrument much like a microscope (called a colposcope) to look closely at the cervix. The doctor may then coat the cervix with an iodine solution (Schiller test). Healthy cells turn brown; abnormal cells turn white or yellow (Acta Obstet.Gynecol.Scand. 1960; 39: 540-56).

In addition,the gynecologist may remove a biopsy for examination by a pathologist.

Another method used to retrieve a biopsy is called loop electrosurgical excision procedure (LEEP). In this procedure, the doctor uses an electric wire loop to slice off a thin, round piece of tissue.

One may also check inside the opening of the cervix, an area that cannot be seen during colposcopy. Then a procedure is performed called endocervical curettage (ECC).

These tests may not show for sure whether the abnormal cells are present only on the surface of the cervix. In that case, the doctor will then remove a larger, cone-shaped sample of tissue. This procedure, called conization or cone biopsy, allows the pathologist to see whether the abnormal cells have invaded tissue beneath the surface of the cervix. Conization also may be used as treatment for a precancerous lesion if the entire abnormal area can be removed.

In a few cases, it may not be clear whether an abnormal Pap test or a woman's symptoms are caused by problems in the cervix or in the endometrium (the lining of the uterus). In this situation, the doctor may perform dilation and curettage (D and C).

However, in pregnant women CLC (cervical laser conization) or LEEP (loop electrosurgical excision procedure) increase the risk of preterm delivery, low birth weight and pROM (preterm rupture of membrane) Sjeborg et al 2007.

2.5 Stage Dependent Therapy

Stage 0 Cervical Cancer

Therapy of CIN I or CIN II :

If the lesion is limited to the ectocervix (secured by colposcopy), control after 3 months is recommended.

In case of persistence and ectocervical localization, biopsy and CO-2 (DGGG guidelines 2006) is performed.

Properly treated, tumor control of in-situ cervical carcinoma should be nearly 100 %. Either expert colposcopy-directed biopsy or cone biopsy is required to rule out invasive disease before therapy is undertaken. A correlation between cytology and colposcopy-directed biopsy is also necessary before local ablative therapy is done. Even so, unrecognized invasive disease treated with inadequate ablative therapy may be the most common cause of failure (Shumsky AG et al., 1994). Failure to identify the disease, lack of correlation between the Pap smear and colposcopic findings, adenocarcinoma in situ, or extension of disease into the endocervical canal makes a laser, loop, or cold-knife conization mandatory. The choice of treatment will also depend on several patient factors including age, desire to preserve fertility, medical condition and HPV type.

Most importanly, the extent of disease must be known.

In selected cases, the outpatient loop electrosurgical excision procedure (LEEP) may be an acceptable alternative to cold-knife conization. This quickly performed in-office procedure requires only local anesthesia and obviates the risks associated with general anesthesia for cold-knife conization (Wright TC Jr et al., 1992; Naumann RW, 1994). However, controversy exists as to the adequacy of LEEP as an alternative to conization (Widrich T et al., 1996).

A trial comparing LEEP with cold-knife cone biopsy showed no difference in the likelihood of complete excision of dysplasia (Girardi F., 1994). However, two case reports suggested that the use of LEEP in patients with occult invasive cancer led to an inability to accurately determine the depth of invasion when a focus of the cancer was transected (Eddy GL et al.; 1994).

Standard treatment options:

Methods to treat ectocervical lesions include:

1. Loop electrosurgical excision procedure (LEEP) (Wright VC, 1992; Bloss JD, 1993).

2. Laser therapy (Tsukamoto N, 1985)

3. Conization (DGGG guidelines 2006)

4. Cryotherapy (Benedet JL et al., 1987).

When the endocervical canal is involved, laser or cold-knife conization may be used for selected patients to preserve the uterus and avoid radiation therapy and/or more extensive surgery.

2.5.1 Treating Precancerous Conditions

Treatment for a precancerous lesion of the cervix depends on different factors. These factors include whether the lesion is low or high grade, whether the woman wants to have children in the future, the woman's age and general health, and the preference of the woman and her doctor.

A woman with a low-grade lesion may not need further treatment, especially if the abnormal area was completely removed during biopsy, but she should have a Pap test and pelvic exam regularly. When a precancerous lesion requires treatment, the doctor may use cryosurgery (freezing), cauterization (burning, also called diathermy), or laser surgery to destroy the abnormal area without harming nearby healthy tissue. The doctor also can remove the abnormal tissue by LEEP or conization. Treatment for precancerous lesions may cause cramping or other pain, bleeding, or a watery discharge.

In some cases, a woman may have a hysterectomy, particularly if abnormal cells are found inside the opening of the cervix. This surgery is more likely to be done when the woman does not want to have children in the future.

2.5.2 Staging

The choice of treatment for cervical cancer depends on different factors like location and size of the tumor, the stage (extent) of the disease, the woman's age and general health, and other factors.

Staging is a careful attempt to find out whether the cancer has spread and, if so, which parts of the body are affected.

Stage IA Cervical Cancer

Equivalent treatment options:

1. Total hysterectomy (Sevin BU et al., 1992): If the depth of invasion is less than 3 millimeters proven by cone biopsy with clear margins (Jones WB et al., 1993) and no vascular or lymphatic channel invasion is noted, the frequency of lymph node involvement is sufficiently low that lymph node dissection is not required. Oophorectomy is optional and should be deferred for younger women.
2. Conization: If the depth of invasion is less than 3 millimeters, no vascular or lymphatic channel invasion is noted, and the margins of the cone are negative, conization alone may be appropriate in patients wishing to preserve fertility (Sevin BU et al., 1992).
3. Radical hysterectomy: For patients with tumor invasion between 3 and 5 millimeters, radical hysterectomy with pelvic node dissection has been recommended because of a reported risk of lymph node metastasis of up to 10 % (Jones WB et al., 1993). However, a study suggests that the rate of lymph node involvement in this group of patients may be much lower and questions whether conservative therapy might be adequate for patients believed to have no residual disease following conization Radical hysterectomy with node dissection may also be considered for patients where the depth of tumor invasion was uncertain due to invasive tumor at the cone margins.
4. Intracavitary radiation alone: If the depth of invasion is less than 3 millimeters and no capillary lymphatic space invasion is noted, the frequency of lymph node involvement is sufficiently low that external beam radiation is not required. One or two insertions with tandem and ovoids for 6,500 to 8,000 milligram hours (10,000 - 12,500 cGy vaginal surface dose) are recommended (Grigsby PW et al., 1991). Radiation should be reserved for women who are not surgical candidates. Creasman WT et al.

Trachelectomy

Surgical removal of the cervix :

Trachelectomy is done in younger women with early cancer of the cervix (with a tumor not larger than 2-3 centimeters). In this surgery, the cervix and the upper part of the vagina are removed but the rest of the uterus is left in place.

The pelvic lymph nodes are also removed, usually by keyhole laparoscopic surgery, to see if the cancer has spread.

After trachelectomy it is sometimes possible for the woman to have children. A stitch is made at the bottom of the uterus like a drawstring and this takes the place of the cervix during pregnancy. There is a higher chance of miscarriage for women who have this procedure, and the baby needs to be delivered by CS (Dargent D et al. 2000).

Stage IB Cervical Cancer

Either radiation therapy or radical hysterectomy and bilateral lymph node dissection, by an experienced professional, results in cure rates of 85 % to 90 % for patients with small volume disease. The choice of either depends on patient factors and availability of local expertise. A randomized trial reported identical 5-year overall and disease-free survival rates when comparing radiation therapy to radical hysterectomy (Landoni F et al., 1997). The size of the primary tumor is an important prognostic factor and should be carefully evaluated in choosing optimal therapy (Perez CA et al., 1992). For adenocarcinomas that expand the cervix greater by more centimeters, the primary treatment should be radiation therapy (Eifel PJ et al., 1991). After surgical staging, patients found to have small volume para-aortic nodal disease and controllable pelvic disease may be cured with pelvic and para-aortic irradiation (Cunningham MJ et al., 1991). The resection of macroscopically involved pelvic nodes may improve rates of local control with postoperative radiation therapy (Downey GO et al., 1989. Hacker NF et al. 1995).

Treatment of unresected periaortic nodes with extended field radiation leads to long-term disease control in those patients with low volume (<2 cm) nodal disease below L3 (Vigliotti AP et al., 1992). A single study showed a survival advantage in patients with tumors larger than 4 centimeters who received radiation to para-aortic nodes without histologic evidence of disease (Rotman M et al., 1995). Toxic effects of para-aortic radiation are greater than pelvic radiation alone, but they are mostly confined to patients with prior abdominopelvic surgery (Rotman M et al., 1995).

Patients with “close” vaginal margins (<0.5 cm) may also benefit from pelvic irradiation (Whitney CW et al., 1999).

Five randomized phase III trials have shown an overall survival advantage for cisplatin-based therapy given concurrently with radiation therapy, (Morris M et al., 1999; Rose PG et al., 1999; Keys HM et al., 1999; Peters WA 3rd et al., 2000; Thomas GM, 1999; Pearcey R et al., 2002) while one trial examining this regimen demonstrated no benefit (Rose PG, et al., 2002). The patient populations in these studies included women with FIGO stages IB2 to IVA cervical cancer treated with primary radiation therapy and women with FIGO stages I to IIA disease found to have poor prognostic factors (metastatic disease in pelvic lymph nodes, parametrial disease, or positive surgical margins) at the time of primary surgery. Although the positive trials vary somewhat in terms of stage of disease, dose of radiation, and schedule of cisplatin and radiation, they all demonstrate significant survival benefit for this combined approach. The risk of death from cervical cancer was decreased by 30 % to 50 % by concurrent chemoradiation. Based on these results, strong consideration should be given to the incorporation of concurrent cisplatin-based

chemotherapy with radiation therapy in women who require radiation therapy for treatment of cervical cancer (Sehouli J et al 2001, Morris M et al., 1999; Rose PG et al., 1999; Keys HM et al., 1999; Peters WA 3rd et al., 2000; Thomas GM, 1999; Pearcey R et al., 2002; Rose PG, et al., 2002; Stitt JA et al., 1992).

Standard treatment options:

1. Radiation therapy: External-beam pelvic irradiation combined with two or more intracavitary applications, based on reports indicating improved outcome with two intracavitary implants rather than one. The use of high-dose-rate brachytherapy for the intracavitary portion of treatment is under clinical evaluation (Thomadsen BR et al., 1992; Eifel PJ, 1992).
2. Radical hysterectomy and bilateral pelvic lymphadenectomy.
3. Postoperative total pelvic irradiation plus chemotherapy following radical hysterectomy and bilateral pelvic lymphadenectomy: Radiation in the range of 5,000 cGy over 5 weeks plus chemotherapy with cisplatin with or without fluorouracil (5-FU) should be considered in patients with positive pelvic nodes, positive surgical margins, and residual parametrial disease (Morris M et al., 1999; Rose PG et al., 1999; Keys HM et al., 1999; Peters WA 3rd et al., 2000; Thomas GM, 1999; Pearcey R et al., 2002).
4. Radiation therapy plus chemotherapy with cisplatin or cisplatin/5-FU for patients with bulky tumors (Morris M et al., 1999; Rose PG et al., 1999; Keys HM et al., 1999; Peters WA 3rd et al., 2000; Thomas GM, 1999; Pearcey R et al., 2002).

Stage IIA Cervical Cancer

Either radiation therapy or radical hysterectomy, by an experienced professional, results in cure rates of 75 % to 80 %. The selection of either option depends on patient factors and local expertise. A randomized trial reported identical 5-year overall and disease-free survival rates when comparing radiation therapy to radical hysterectomy (Landoni F et al., 1997). The size of the primary tumor is an important prognostic factor and should be carefully evaluated in choosing optimal therapy (Perez CA et al.,1992). For bulky (>6 cm) endocervical squamous cell carcinomas or adenocarcinomas, treatment with high-dose radiation therapy will achieve local control and survival rates comparable to treatment with radiation therapy plus hysterectomy. Surgery after radiation therapy may be indicated for some patients with tumors confined to the cervix which respond incompletely to radiation therapy or in whom vaginal anatomy precludes optimal brachytherapy (Thoms WW Jr et al., 1992). After surgical staging, patients found to have small volume para-aortic nodal disease and controllable pelvic disease may be cured with pelvic and para-aortic irradiation (Cunningham MJ et al., 1991). The resection of macroscopically involved pelvic nodes may improve rates of local control with postoperative radiation therapy (Downey GO et al., 1989). Treatment of unresected periaortic nodes with extended field radiation leads to long-term disease control in patients with low volume (<2 cm) nodal disease below L3 (Vigliotti AP et al., 1992). A single study showed a survival advantage in patients who received radiation to para-aortic nodes without histologic evidence of disease (Rotman M et al., 1995). Toxic effects of para-aortic radiation were greater than pelvic radiation alone, but they were mostly confined to patients with prior abdominopelvic surgery (Rotman M et al., 1995). Patients who underwent extraperitoneal lymph node sampling had fewer bowel complications than those who had transperitoneal lymph node sampling (Vigliotti AP et al., 1992; Weiser EB et al., 1989; Fine BA et al., 1995). Patients with “close” vaginal margins (<0.5 cm) after radical surgery may also benefit from pelvic irradiation (Estape RE et al., 1998).

Five randomized phase III trials have shown an overall survival advantage for cisplatin-based therapy given concurrently with radiation therapy, (Whitney CW et al., 1999; Morris M et al., 1999; Rose PG et al., 1999; Keys HM et al., 1999; Peters WA 3rd et al., 2000 ; Thomas GM, 1999) while one trial examining this regimen demonstrated no benefit (Pearcey R et al., 2002). The patient populations in these studies included women with FIGO stages IB2 to IVA cervical cancer treated with primary radiation therapy and women with FIGO stages I to IIA disease found to have poor prognostic factors (metastatic disease in pelvic lymph nodes, parametrial disease, or positive surgical margins) at the time of primary surgery. Although the positive trials vary somewhat in terms of stage of disease, dose of radiation, and schedule of cisplatin and radiation, they all demonstrate

significant survival benefit for this combined approach. The risk of death from cervical cancer was decreased by 30% to 50% by concurrent chemoradiation. Based on these results, strong consideration should be given to the incorporation of concurrent cisplatin-based chemotherapy with radiation therapy in women who require radiation therapy for treatment of cervical cancer (Whitney CW et al., 1999; Morris M et al., 1999; Rose PG et al., 1999; Keys HM et al., 1999; Peters WA 3rd et al., 2000; Thomas GM, 1999; Pearcey R et al., 2002; Rose PG et al., 2002).

Application of new cytostatics, platinous combination chemotherapies, sequential instead of simultaneous proceedings and adequate supportive therapies should be considered. Many studies are currently being conducted to evaluate new therapies on life expectancy and quality of life (e.g. Cervix- NOGGO- AGO- Uterus 7- study Sehouli et al. 2006).

Standard treatment options:

1. Radiation therapy: Intracavitary radiation combined with external-beam pelvic irradiation. Radiation to para-aortic nodes may be indicated in primary tumors 4 centimeters or larger. The use of high-dose-rate brachytherapy for the intracavitary portion of treatment is under clinical evaluation (Stitt JA et al., 1992; Thomadsen BR et al., 1992; Eifel PJ, 1992).
2. Radical hysterectomy and pelvic lymphadenectomy.
3. Postoperative total pelvic irradiation plus chemotherapy following radical hysterectomy and bilateral pelvic lymphadenectomy: Radiation in the range of 5,000 cGy over 5 weeks plus chemotherapy with cisplatin with or without fluorouracil (5-FU) should be considered in patients with positive pelvic nodes, positive surgical margins, and residual parametrial disease (Whitney CW et al., 1999; Morris M et al., 1999; Rose PG et al., 1999; Keys HM et al., 1999; Peters WA 3rd et al., 2000).
4. Radiation therapy plus chemotherapy with cisplatin or cisplatin/5-FU for patients with bulky tumors (Whitney CW et al., 1999; Morris M et al., 1999; Rose PG et al., 1999; Keys HM et al., 1999; Peters WA 3rd et al., 2000).

Stage IIB Cervical Cancer

The size of the primary tumor is always an important prognostic factor and should be carefully evaluated in choosing optimal therapy (Perez CA et al., 1992). Survival and local control are better with unilateral than bilateral parametrial involvement (Lanciano RM, et al., 1992). Patients who are surgically staged as part of a clinical trial and are found to have small volume para-aortic nodal disease and controllable pelvic disease may be cured with pelvic and para-aortic irradiation (Cunningham MJ et al., 1991). If postoperative external-beam therapy is planned following surgery, extraperitoneal lymph node sampling is associated with fewer radiation-induced complications than a transperitoneal approach (Weiser EB et al., 1989). The resection of macroscopically involved pelvic nodes may improve rates of local control with postoperative radiation therapy (Downey GO et al., 1989). Treatment of unresected periaortic nodes with extended field radiation leads to long-term disease control in patients with low volume (<2 cm) nodal disease below L3 (Vigliotti AP et al., 1992). A single study showed a survival advantage in patients who received radiation to para-aortic nodes without histologic evidence of disease (Rotman M et al., 1995). Toxic effects of para-aortic radiation is greater than pelvic radiation alone, but was mostly confined to patients with prior abdominopelvic surgery (Rotman M et al., 1995). Patients who underwent extraperitoneal lymph node sampling had fewer bowel complications than those who had transperitoneal lymph node sampling (Weiser EB et al., 1989; Vigliotti AP et al., 1992; Fine BA et al., 1995).

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Standard treatment options:

- Radiation therapy plus chemotherapy: Intracavitary radiation and external-beam pelvic irradiation combined with cisplatin or cisplatin/fluorouracil (Whitney CW et al., 1999; Morris M et al., 1999; Rose PG et al., 1999; Keys HM et al., 1999; Peters WA 3rd et al., 2000; Thomas GM, 1999).

Stage III Cervical Cancer

These studies also reveal a progressive increase in local control and survival paralleling a progressive increase in paracentral (point A) dose and use of intracavitary treatment. The highest rate of central control was seen with paracentral (point A) doses of greater than 8,500 cGy (Lanciano RM et al., 1991). Patients who are surgically staged as part of a clinical trial and are found to have small volume para-aortic nodal disease and controllable pelvic disease may be cured with pelvic and para-aortic irradiation. If postoperative external-beam therapy is planned following surgery, extraperitoneal lymph node sampling is associated with fewer radiation-induced complications than a transperitoneal approach (Weiser EB et al., 1989). The resection of macroscopically involved pelvic nodes may improve rates of local control with postoperative radiation therapy (Downey GO et al., 1989). Treatment of unresected periaortic nodes with extended field radiation leads to long-term disease control in patients with low volume (<2 cm) nodal disease below L3. Patients who underwent extraperitoneal lymph node sampling had fewer bowel complications than those who had transperitoneal lymph node sampling (Vigliotti AP et al., 1992).

There are five randomized phase III trials which show an overall survival advantage for cisplatin-based therapy given concurrently with radiation therapy (Whitney CW et al., 1999; Morris M et al., 1999; Rose PG et al., 1999; Keys HM et al., 1999; Peters WA 3rd et al., 2000; Thomas GM, 1999), while one trial examining this regimen demonstrated no benefit (Pearcey R et al., 2002). The patient populations in these studies included women with FIGO stages IB2 to IVA cervical cancer treated with primary radiation therapy and women with FIGO stages I to IIA disease found to have poor prognostic factors (metastatic disease in pelvic lymph nodes, parametrial disease, or positive surgical margins) at the time of primary surgery. Although the positive trials vary somewhat in terms of stage of disease, dose of radiation, and schedule of cisplatin and radiation, they all demonstrate significant survival benefit for this combined approach. The risk of death from cervical cancer was decreased by 30 % to 50 % by concurrent chemoradiation. Based on these results, strong consideration should be given to the incorporation of concurrent cisplatin-based chemotherapy with radiation therapy in women who require radiation therapy for

treatment of cervical cancer (Whitney CW et al., 1999; Morris M et al., 1999; Rose PG et al., 1999; Keys HM et al., 1999; Peters WA 3rd et al., 2000; Thomas GM, 1999; Pearcey R et al., 2002; Rose PG et al., 2002).

Standard treatment options:

- Radiation therapy plus chemotherapy: Intracavitary radiation and external-beam pelvic irradiation combined with cisplatin or cisplatin/ fluorouracil (Whitney CW et al., 1999; Morris M et al., 1999; Rose PG et al., 1999; Keys HM et al., 1999; Peters WA 3rd et al., 2000; Thomas GM, 1999).

Stage IVA Cervical Cancer

An important prognostic factor is the size of the primary tumor and should be carefully evaluated in choosing optimal therapy (Perez CA et al., 1992). After surgical staging, patients found to have small volume para-aortic nodal disease and controllable pelvic disease may be cured with pelvic and para-aortic irradiation.

Five randomized phase III trials have shown an overall survival advantage for cisplatin-based therapy given concurrently with radiation therapy (Whitney CW et al., 1999; Morris M et al., 1999; Rose PG et al., 1999; Keys HM et al., 1999; Peters WA 3rd et al., 2000; Thomas GM, 1999), while 1 trial examining this regimen demonstrated no benefit (Pearcey R et al., 2002). The patient populations in these studies included women with FIGO stages IB2 to IVA cervical cancer treated with primary radiation therapy and women with FIGO stages I to IIA disease found to have poor prognostic factors (metastatic disease in pelvic lymph nodes, parametrial disease, or positive surgical margins) at the time of primary surgery. Although the positive trials vary somewhat in terms of stage of disease, dose of radiation, and schedule of cisplatin and radiation, they all demonstrate significant survival benefit for this combined approach. The risk of death from cervical cancer was decreased by 30% to 50% by concurrent chemoradiation. Based on these results, strong consideration should be given to the incorporation of concurrent cisplatin-based chemotherapy with radiation therapy in women who require radiation therapy for treatment of cervical cancer (Whitney CW et al., 1999; Morris M et al., 1999; Rose PG et al., 1999; Keys HM et al., 1999; Peters WA 3rd et al., 2000; Thomas GM, 1999; Pearcey R et al., 2002; Rose PG et al., 2002).

Standard treatment options:

- Radiation therapy plus chemotherapy: Intracavitary radiation and external-beam pelvic irradiation combined with cisplatin or cisplatin/fluorouracil Whitney CW et al., 1999; Morris M et al., 1999; Rose PG et al., 1999; Keys HM et al., 1999; Peters WA 3rd et al., 2000; Thomas GM, 1999).

Stage IVB Cervical Cancer

There is no standard chemotherapy treatment for patients with stage IVB cervical cancer that provides substantial palliation. All such patients are appropriate candidates for clinical trials testing single agents or combination chemotherapy employing agents listed below or new anticancer treatments in phase I and II clinical trials (Alberts DS et al., 1987).

Standard treatment options:

1. Irradiation therapy may be used to palliate central disease or distant metastases.
2. Chemotherapy. Tested drugs include:
    - Cisplatin (15 %-25 % response rate) (Alberts DS et al., 1987; Thigpen JT et al., 1989).
    - Ifosfamide (31 % response rate) (Coleman RE et al., 1986).
    - Paclitaxel (17 % response rate) (Kudelka AP et al., 1996; Thigpen T et al., 1995; McGuire WP et al., 1996).
    - Ifosfamide-cisplatin (Buxton EJ et al., 1989; Omura GA et al., 1997).
    - Irinotecan (21 % response rate in patients previously treated with chemotherapy) (Verschraegen CF et al., 1997).
    - Paclitaxel/cisplatin (46 % response rate) (Rose PG et al., 1999).
    - Cisplatin/gemcitabine (41 % response rate) (Burnett AF et al., 2000).

2.5.3 Recurrent Cervical Cancer

There is no standard treatment for recurrent cervical cancer that has spread beyond the confines of a radiation or surgical field. All such patients are appropriate candidates for clinical trials testing drug combinations or new anticancer agents. For locally recurrent disease, pelvic exenteration can lead to a 5-year survival rate of 32 % to 62 % in selected patients (Alberts DS et al., 1987; Morrow CP et al., 1998).

Standard treatment options:

1. For recurrence in the pelvis following radical surgery, radiation in combination with chemotherapy (fluorouracil with or without mitomycin) may cure 40 % to 50 % of patients (Thomas GM et al., 1987).
2. Chemotherapy can be used for palliation. Tested drugs include:
    - Ifosfamide (15 % - 30 % response rate) (Coleman RE et al., 1986; Sutton GP et al., 1993).
    - Ifosfamide-cisplatin (Buxton EJ et al., 1989; Omura GA et al., 1997).
    - Paclitaxel (17 % response rate) (McGuire WP et al., 1996).
    - Irinotecan (21 % response rate in patients previously treated with chemotherapy) (Verschraegen CF et al., 1997).
    - Paclitaxel/cisplatin (46 % response rate) (Rose PG et al., 1999).
    - Cisplatin/gemcitabine (41 % response rate) (Burnett AF et al., 2000)
    - Topotecan / Cisplatin versus Cisplatin mono (Monk BJ et al 2005 GOG 179). This was the first study to show a statistically significant impact on the overall survival rate, median progress free survival rate and median survival, with all outcome measures favoring the two-drug regimens.

2.6 Human papillomavirus infection and cervical cancer

It is estimated that more than 6 million women in the United States have HPV infection and proper interpretation of these data is important. Epidemiologic studies convincingly demonstrate that the major risk factor for development of preinvasive or invasive carcinoma of the cervix is HPV infection, which far outweighs other known risk factors such as high parity, increasing number of sexual partners, young age at first intercourse, low socioeconomic status, and positive smoking history (Schiffman MH et al., 1993; Brisson J et al., 1994). Some patients with HPV infection appear to be at minimally increased risk for development of cervical preinvasive and invasive malignancies while others appear to be at significant risk and candidates for intensive screening programs and early intervention.

HPV DNA tests are unlikely to separate patients with low-grade squamous intraepithelial lesions into those who do and those who do not need further evaluation. A study of 642 such women found that 83 % had one or more tumorigenic HPV types when cervical cytologic specimens were assayed by a sensitive (hybrid capture) technique. Whether HPV DNA testing will prove useful in patients with atypical squamous cells of undetermined significance is being studied by the same group (The Atypical Squamous Cells of Undetermined Significance/Low-Grade Squamous Intraepithelial Lesions Triage Study (ALTS) Group, 2000). Patients with an abnormal cytology of a high-risk type (Bethesda classification) should be thoroughly evaluated with colposcopy and biopsy.

Other studies show patients with low-risk cytology and high-risk HPV infection with types 16, 18, and 31 are more likely to have cervical intraepithelial neoplasia (CIN) or microinvasive histopathology on biopsy (Brisson J et al., 1994; Tabbara S et al., 1992; Cuzick J et al., 1992; Richart RM et al., 1993). One method has also shown that integration of HPV types 16 and 18 into the genome, leading to transcription of both viral and cellular messages, may predict patients who are at greater risk for high-grade dysplasia and invasive cancer (Klaes R et al., 1999). Studies (Brisson J. et al., 1994; Koutsky LA et al., 1992) suggest that acute infection with HPV types 16 and 18 conferred an 11- to 16.9-fold risk of rapid development of high-grade CIN, but there are conflicting data requiring further evaluation before any recommendations may be made. Patients with low-risk cytology and low-risk HPV types have not been followed long enough to ascertain their risk. At present, studies are ongoing to determine how HPV typing can be used to help stratify women into follow-up and treatment groups. HPV typing may prove useful, particularly in patients with low-grade cytology or cytology of unclear abnormality. At

present, has not been finally defined how therapy and follow-up should be altered with low- versus high-risk HPV type.

The frequency of HPV infection is highest among females aged 20 - 25 years.

5 – 30 % of HPV-positive females develop histological changes (Biermann et al., 1999). Prevalence of demonstrable HPV infection decreases with increasing age.

Persistence or progression is observed in only 20 % of the cases. In case of persisting HPV infection of the lower genital tract over years,dysplasia or intraepithelial neoplasia can develop.

However, only a few of those infected by human papilloma virus develop cervical cancer as there are other co-factors which are of importance: circumcision of the man e.g. is identified to be a protective factor (Castellsague et al., 2002).

Laboratory findings suggest that the foreskin is enriched with virus target cells (Drain et al 2006).

Next to immunsuppression, HIV-infection, smoking, use of anticonceptives over years,Chlamydia infections, high parity over all genetic factors seem to be fundamentally in eliminating HPV-infection by the immune system or not as we will describe in the following chapters.

Two prophylactic vaccines against HPV types 6, 11, 16 and 18 have shown great promise in clinical trials, with recent results demonstrating 100 % efficacy against persistent HPV infection and development of CIN up to five years of follow-up. One of these (Gardasil, recently licensed) contains all four HPV types, offering protection against genital warts (types 6 and 11) as well as cervical cancer. The other (Cervarix) contains types 16 and 18, targeting cervical cancer alone. Recent data suggest a degree of cross-protection against types 31 and 45; this could significantly increase the level of protection afforded by the vaccines. It is envisaged that girls between 11 and 12 will be the target, and this is what has been recommended in the United States. In theory, an HPV vaccine could prevent almost all cervical cancer, eventually removing the need for cervical smears (Szarewski A. et al 2007).The HPV- 16/ 18 adjuvanted vaccine induces a persistent immune response (Schneider A et al. 2009).

2.7 Tumor Suppressor Gene

Using their protein products some genes are able to suppress tumor formation by inhibiting mitosis. Tumor suppression continues as long as the cell contains one normal allele. In contrast to oncogenes can predispose the cell to tumor formation by only one defect allele.Consequently oncogenes behave as dominant genes compared to tumor suppressor genes (Levine AJ et al 1990)

Example of p53

Located in the short arm of the human chromosome 17 (17p13.1) the p53 tumor suppressor gene encodes a protein of 393 amino acids and the product is a protein of 53 kilodaltons (Levine AJ et al 1992).

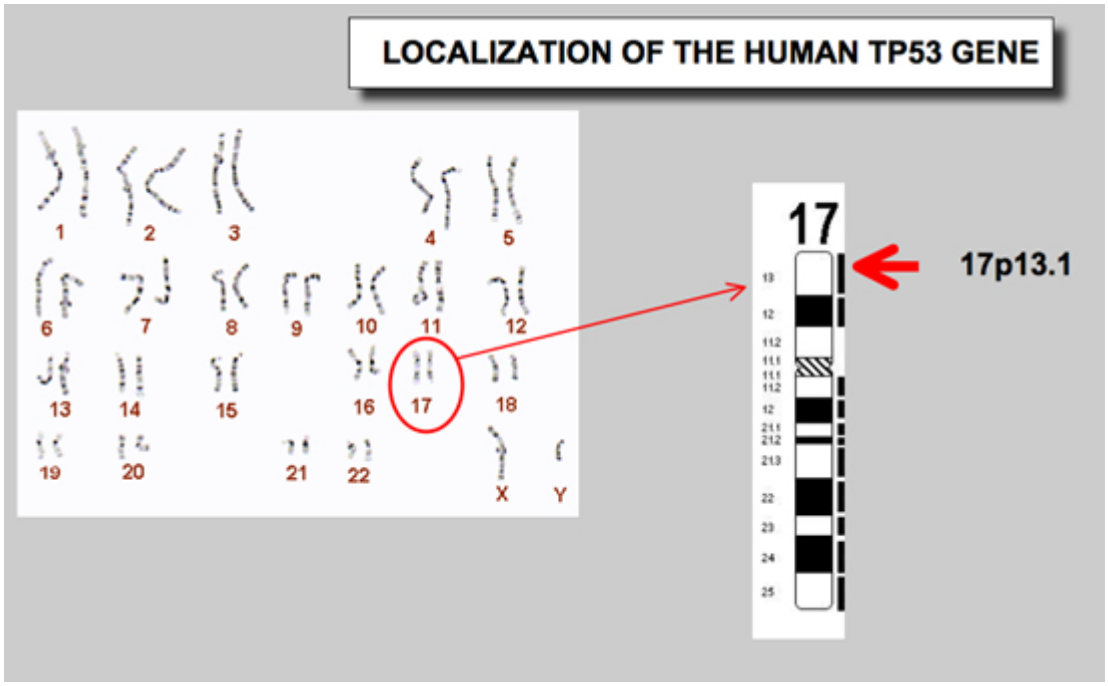


Figure 2.1: The TP53 gene is localized on chromosome 17 (short arm, 17p13), a region that is frequently deleted in human cancer.

p53 is one of the most important cellular proteins in guarding repair processes and maintaining chromosomal stability.
It prevents a cell from completing the cell cycle if its DNA is not correctly replicated in the S-phase.

It binds to a transcription factor called E2F; this prevents E2F from binding to the promoters of such proto oncogenes as c-myc and c-fos. Transcription of c-myc and c-fos is needed for mitosis, blocking the transcription factor is needed to turn on these genes prevents cell division. So p53 tumor suppressor gene is a transcription factor that controls the expression of many different genes involved in various cellular pathways

(Levine AJ et al 1993, Shai et al 2007).

2.7.1 Interaction between E6 / E7 Oncoproteins on Tumor Suppressor Genes

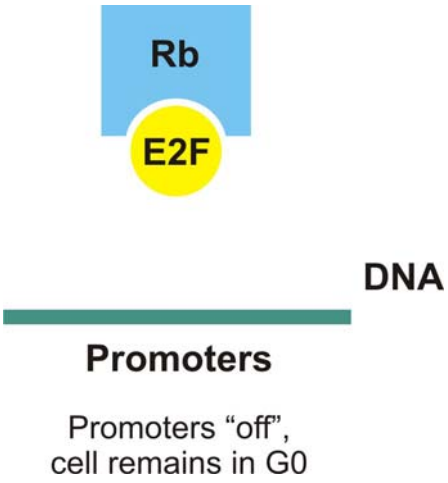


Figure 2.2: Interaction between Rb and transcription factor E2F

Genes like **RB** and **p53** are also called anti-oncogenes or tumor suppressor genes. They were first given this name because they reverse, at least in cell culture, the action of known oncogenes.

Mechanism: The Rb protein prevents cells from entering the S phase of the cell cycle. It does this by binding to a transcription factor called E2 F.

E2F stands for family of transcription factor (TF) in higher eucaryotes. Three of them are activators: E2F1, 2 and E2F3a.

This prevents E2F from binding to the promoters of such proto-oncogenes as c-myc and c-fos. Transcription of c-myc and c-fos is needed for mitosis,so blocking the transcription factor needed to turn on these genes prevents cell division.

In case of minor damages, p53 halts the cell cycle until the damage is repaired, in case of major damages which cannot be repaired, p53 triggers the cell to commit suicide by apoptosis.

Both the Rb protein and the p53 protein form a complex directly in the cell with an oncogene product of some human papilloma viruses.

Through binding to viral proteins (in our case E6 or E7 is an oncoprotein product of one of the HPV) as a result of mutations in the p53tumor suppressor gene: it is inactivated in most human cancers.

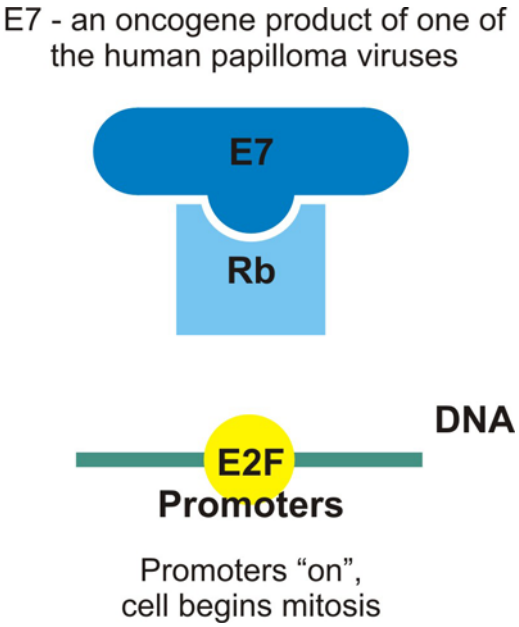


Figure 2.3: Interaction between Oncoprotein E7 on Tumor Suppressor Gene Rb

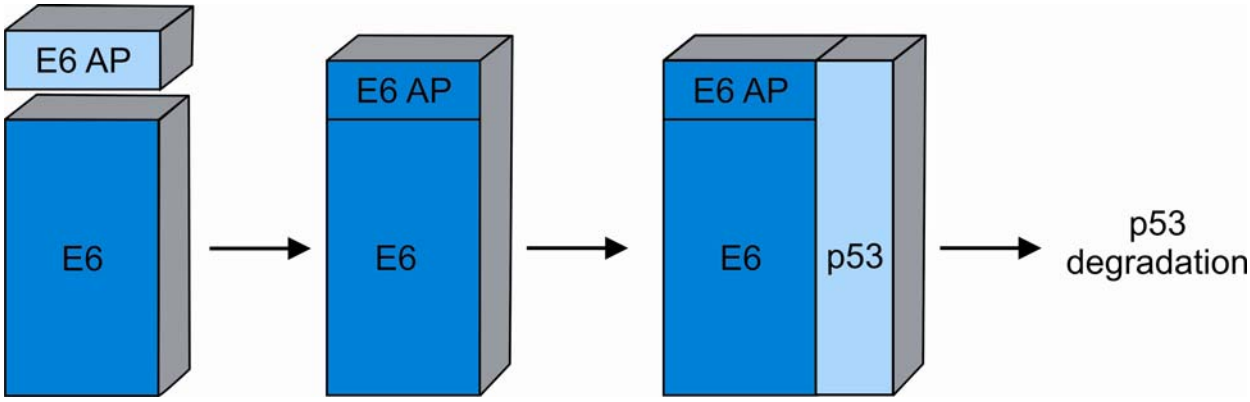


Figure 2.4: E6 protein and the cellular ubiquitin-protein ligase E6 AP (E6 associated protein) form a complex which cause the ubiquitination and degradation of p53.

After the end of the G1- phase Rb normally binds to E2F to inhibit it. Also E7 (HPV-oncoprotein) can bind to Rb so Rb loses its function as inhibitor.

Result: The cell begins with DNA –synthesis in the S-phase.

P53 senses DNA damage and can halt progression of the cell cycle in both G1 and G2.

It also is a key player in apoptosis forcing mutated cells to commit suicide. If E6 protein (HPV-oncoprotein) is implicated in cervical cells it binds the p53 protein targeting it for destruction by proteasomes.

As a result, p53 cannot stop progression of cell cycle in both G1 and G2. (Agarwal ML et al,1995).

Once inside the cells of their host, human papilloma viruses synthesize a protein designated E6 and a protein designated E7.

Of the >30 strains of HPV that infect humans, several have been implicated as a risk factor for **cervical cancer**. The **E7** protein of one of these binds to the **Rb** protein preventing it from binding to the host transcription factor **E2F**.

Result: E2F is now free to bind to the promoters of genes (like c-myc) that cause the cell to enter the cell cycle (right). Thus this version of E7 is an **oncogene product**.

The E6 protein of a second human papilloma virus implicated in cervical cancer binds the **p53** protein targeting it for destruction by proteasomes and thus removing the block on the host cell's entering the cell cycle.

Understanding these mechanisms of guarding repair processes and maintaining chromosomal stability makes p53 consequently a key player in protecting human beings against cancer (Hallstein et al 1991).

In 1986, an intragenic polymorphism of p53 was discovered that leads to the expression of two different p53 proteins, with Arginine or Proline in codon 72 in a region rich in proline residues (Harris et al., 1986). This region could be involved in the apoptotic activity of p53 (Walker and Levine, 1996). The distribution of this polymorphism in the general population is heterogeneous with a frequency of the Pro/Pro haplotype of 16 % in Scandinavian populations and 63 % in Nigerian populations (Beckman et al., 1994). The reason for this North/South gradient is unknown at the present time. Many studies have investigated whether one of the haplotypes could be associated with a higher susceptibility to developing cancers. The results of these studies are very contradictory and have not demonstrated any highly significant findings. In 1998, Storey et al. showed that p53Arg

was very sensitive to the degradation activity induced by papillomavirus protein E6, while p53Pro was more resistant. This observation, that has not been contested, was associated with an epidemiological study on cervical cancer, which showed an over-representation of women with cervical cancer presenting the p53Arg allele (Makni et al., 2000; Storey et al., 1998).

2.7.2 Human Papilloma Virus Tests

Methods to prove HPV- infection vary in their sensitivity. For reliable results experience of the laboratory is of highest importance, especially when performing PCR-tests is required. Classical methods to diagnose viral infections like electron-microscopy, cell culture or other immunological methods are not suitable to detect HPV infection.

An established method to prove Human Papilloma Virus is hybridization of viral nucleic acid by:

- Hybrid Capture® HPV DNA Test 2 (hc2)
  hc2 connected with the Pap-test is certified by the FDA
- PCR Polymerase Chain Reaction (Villa LL et al).

Using Hybrid Capture® HPV DNA Test 2 (hc2) (Fa. Digene, USA) 1 pg HPV DNA /ml can be proven. Advantages of this test consist in its simple use and its good reproducibility of results. Sensitivity and specificity are thought to be comparably similar to those of the PCR-based test, but the results of our work will clarify this point.

Identification of the exact HPV-type is not possible with the Hybrid Capture Test, only the distinction between "low-risk" (6, 11, 42, 43, 44) and "high-risk" (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68) HPV-genotype-groups is possible.

Amplification of DNA during PCR tests in specialized laboratories permits a high sensitivity which exceeds that of the Hybrid Test. However, result deviation between different laboratories is substantial.

For scientific research the HPV- test by PCR performed in highly specialized laboratories is accepted to be method of choice, our result findings however will illustrate this aspect critically (JU Cope, A Hildesheim, MH Schiffman et al, 1997).

3 PATIENTS AND METHODS

Blood and tissue specimens:

Genomic DNA was extracted from paraffin-embedded cervical tissues from 157 patients who were all previously HPV-tested by ELISA. All tissue specimens were taken from the archives of the Department of Pathology RWTH Aachen.

I also extracted DNA from peripheral blood specimens taken from 117 pregnant females (healthy control group) who were tested all PAP 1 with normal cells picture in PAP-smears. Blood samples were taken from Moenchengladbach Labor Dr. Stein and partners using Sarstedt monovettes.

3.1 DNA Isolation

3.1.1 DNA- isolation from peripheral blood

DNA extraction was performed using the following protocol:

1. 20 µl of Protein K was pipetted into the bottom of a 1.5 ml microcentrifuge tube.
2. 200 µl of the blood was added into the microcentrifuge tube.
3. 200 µl buffer AL was added to the sample and mixed by pulse-vortexing for 15 seconds.
   Buffer AL contained chaotropic salts which have two functions:
4. it lyses the cells together with Proteinase K and it is responsible for binding conditions. Proteinase K degrades haemoglobin and makes the lysate more fluid.
5. The mixture was incubated at 56°C for 10 minutes.
6. 200 µl of ethanol (96 %-100 %) was added to the sample mixed briefly and centrifuged.
   Ethanol together with buffer AL is important for efficient binding of nucleic acid to the silicae membrane.
7. The mixture was applied to the spin column and centrifuged at 8000 rpm for 1 min.
8. 500 µl buffer AW 1 was added and centrifuged again at 8000 rpm for 1 min. Buffer AW1 also contains chaotropic salts and removes remaining carbohydrates, fatty acids and proteins.

9. 500 µl buffer AW 2 was added and the spin column were cetrifuged at 14,000 rpm for 3 min. AW 2 removes remaining chaotropic salts from the membrane but retains binding conditions.

3.1.2 DNA-isolation from paraffin-embedded cervical tissue

To extract DNA from paraffin-embedded tissue blocks, appropriate specimens were selected by conventional light microscopy and then subjected to the following protocol:

Day 1:

1. 3-5 sections with a thickness of 10 µm were vortexed with 1 ml xylol for 30 min and centrifugated at 14,000 rpm for 5 min.
2. After xylol extraction, 1 ml ethanol was added , vortexed and centrifugated 5 min. with a speed of 14,000 rpm. This step was repeated again and ethanol was discarded.
3. After 15 min. transpiration of ethanol 180 µl ATL-buffer warmed up to 55°C was added and vortexed together with 20 µl proteinase K (20 µg/µl) .
4. The mixture was incubated over night at 55°C.

Day 2:

1. 200 µl AL-buffer was added, vortexed and incubated at 70°C for 10 min.
2. 210 µl ethanol was added, vortexed and prepared into a spin column, centrifuged at 8000 rpm for 1 min, the filtrate was discarded.
3. 500 µl AW-buffer was added and centrifugated at 8000 rpm for 1 min. This step was repeated and centrifugated further 2 min. at 14.000 rpm.
4. Qiamp spin coloumns were filled with 200 µl tris buffer and incubated for 10 min. at 70°C and centrifugated 1 min. at 8000 rpm.
5. 10 µl of the extracted DNA was mixed with 2 µl loading buffer for gel-electrophoresis (1 % agarose) at 80 Volts for 45 min.

3.2 PCR Amplification

The purpose of a PCR (Polymerase Chain Reaction) is to generate a large number of gene copies from a limited number of such copies, this is necessary to have enough starting template for sequencing.

There are three major steps in a PCR, which are repeated for 30 or 40 cycles. This is done on an automated cycler, which can heat and cool the tubes with the reaction mixture in a very short time.

1. Denaturation at 95°C:
   During the denaturation, the double strand melts to single stranded DNA, all enzymatic reactions stop (for example: the extension from a previous cycle).
2. Annealing at 55°C
   Ionic bonds are constantly formed and broken between the single stranded primer and the single stranded template. The more stable bonds last longer (primers that fit exactly) and on that little piece of double stranded DNA (template and primer), the polymerase can attach and start copying the template. Once a few bases are synthesized in, the ionic bond is so strong between the template and the primer that it does not melt anymore.
3. Extension at 72°C:
   This is the ideal working temperature for the polymerase. The primers, where there are a few bases built in, already have a stronger ionic attraction to the template than the forces breaking these attractions. Primers that are on positions with no exact match, dissociate again (because of the higher temperature) and do not result in extension of the fragment. The bases (complementary to the template) are coupled to the primer on the 3' side (the polymerase adds dNTP's from 5' to 3', reading the template from 3' to 5' side, bases are added complementary to the template).

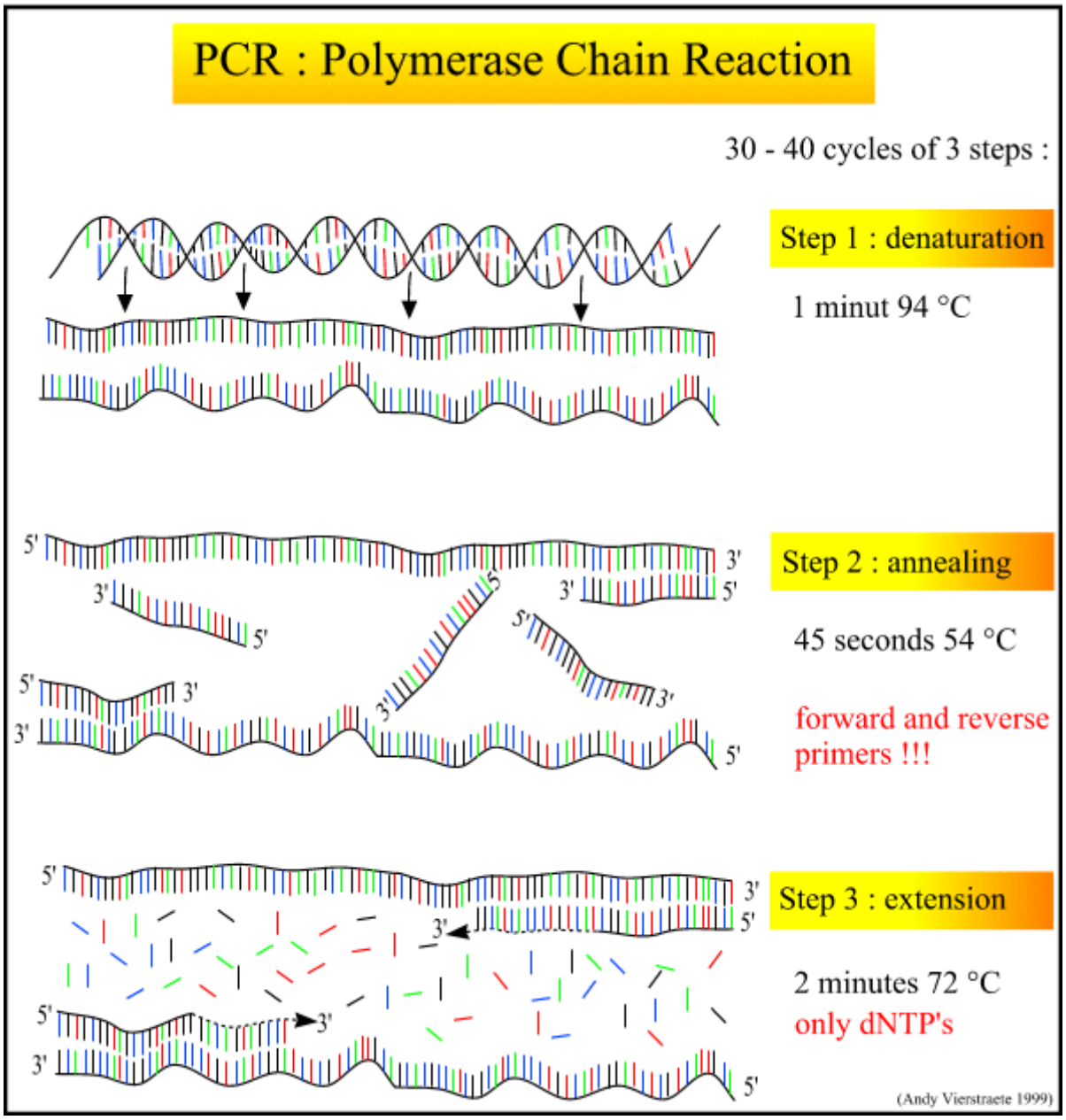


Figure 3.1: PCR steps denaturation, annealing and extension

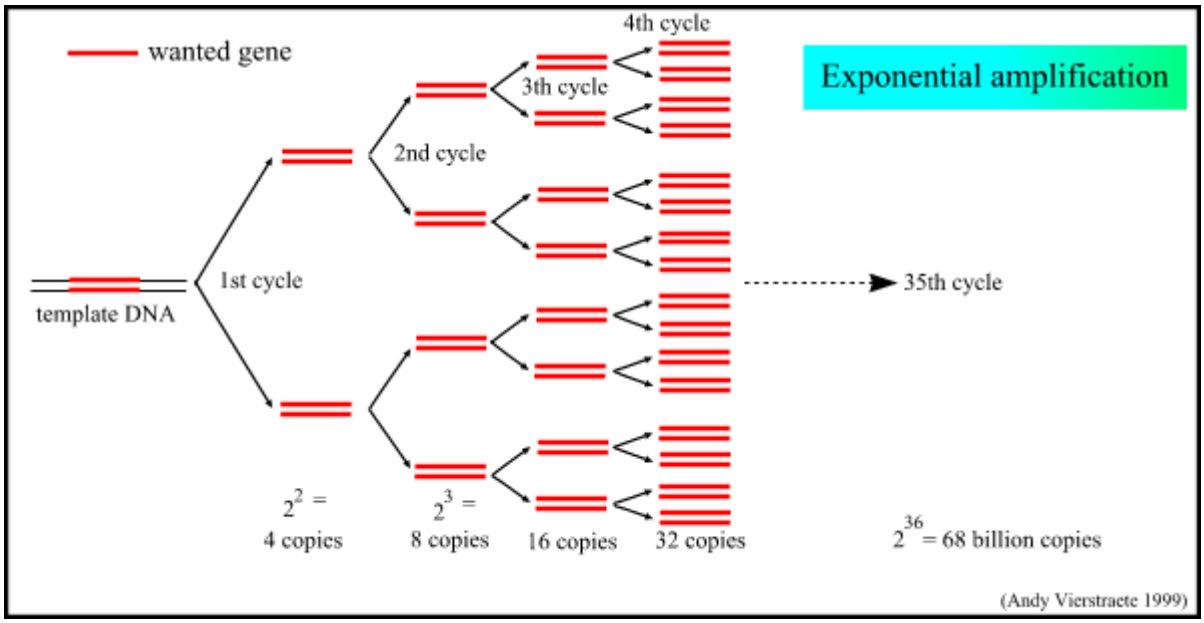


Figure 3.2: Exponential amplification of the gene in PCR

3.2.1 PCR amplification of p53 polymorphic sequences

P53 Pro sequence were detected by PCR using the primer pair p53+/ p53-
Primer pairs (0,5 µl each) were mixed in a total volume of 50 µl.
PCR was performed at 95°C denaturating temperature 5 minutes 40 cycles,
followed by 40 cycles for 30 seconds at 95°C. Annealing temperature was at 55°C for 30 seconds, then 30 seconds at 72°C elongating and the last step was 5 minutes at 72°C.

Each step was repeated 40 cycles:
95°C – 5 min.
95°C – 30 sec.
55°C – 30 sec.
72°C – 30 sec.
72°C – 5 min.

Primer p53+ sequence:

5’-TCC CCC TTG CCG TCC CAA-3’

Primer p53 - sequence:

5’-CGT GCA AGT CAC AGA CTT-3’

The primers were purchased from INTERACTIVA Biotechnology GmbH in 89077 Ulm, Germany.

3.2.2 PCR amplification of HPV-DNA

HPV-positive samples were analyzed by PCR using the primer pair GP5/GP6 and HPV1/HPV2.

Sequence of HPV1: CGT-CCM-ARR-GGA-WAC-TGA-TC

Sequence of HPV2: GCM-CAR-GGW-CAT-AAYY-AAT-GG.

Primer pairs (0,5 µl each) in a total volume of 50 µl. This resulted in a concentration 10 pmol/reaction.

The PCR was performed at 53°C annealing temperature for 30 seconds and at 72°C for 7 min. The cycler we used was a Gene AMP 2400 by Perkin Elmer.

(Perkin Elmer Headquarter, Wellesley, USA)

95°C – 3 min.

95°C – 30 sec.

53°C – 30 sec.

72°C – 45 sec.

72°C – 7 min.

Each cycle was repeated 40 times.

3.3 Cycle Sequencing

We used 4 µl Terminator Ready Mix, 1 µl primer and 20 µl aqua dest with an adequate amount of template. The Cycle Sequencing PCR was performed with Gene Amp 9600 by Perkin Elmer PE annealing temperature 40°C. The protocol of cycle sequencing begins with denaturating temperature 95°C for 5 minutes and 30 cycles, followed by 30 seconds at 95°C and then 30 cycles extension.

Annealing temperature is 50°C for 30 seconds. The last step of sequencing is at 72°C for 1 minute.

3.4 Detection of p53 codon 72 polymorphism

10 µl of p53 PCR-product were mixed with with 1.1 µl Bsh 1236 I restriction enzyme (10 u/µl) by MBI Fermentas (Fermentas GmbH, St. Leon-Rot, Germany).

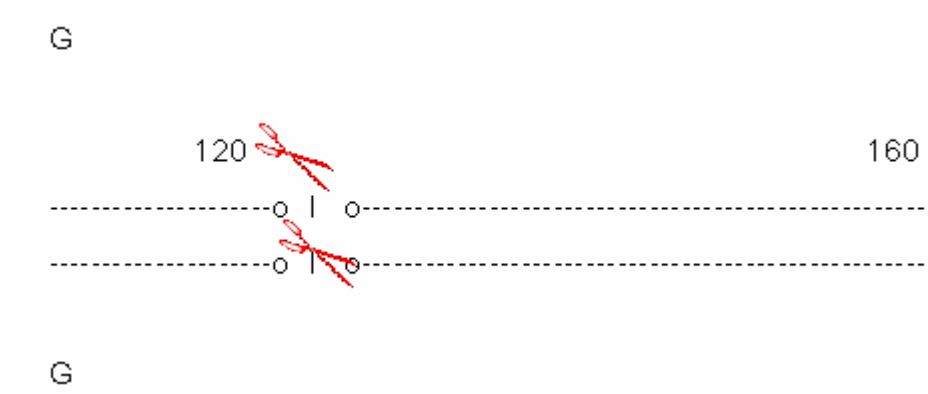
The source of Bsh 1236 I is Bacillus sphaericus RFL 1236. One unit is defined as the amount of enzyme required to digest 1µg of lambda DNA in 1 hour at 37°C in 50 µl of assay buffer. The mixture of Bsh-enzyme and PCR product p53 was incubated at 37°C for 2 hours. *Bsh 1236* I enzyme cuts p53 product at the specific position of the p53 polymorphism I was interested in :

5'…CG CG…3'

3'…GC GC…5'

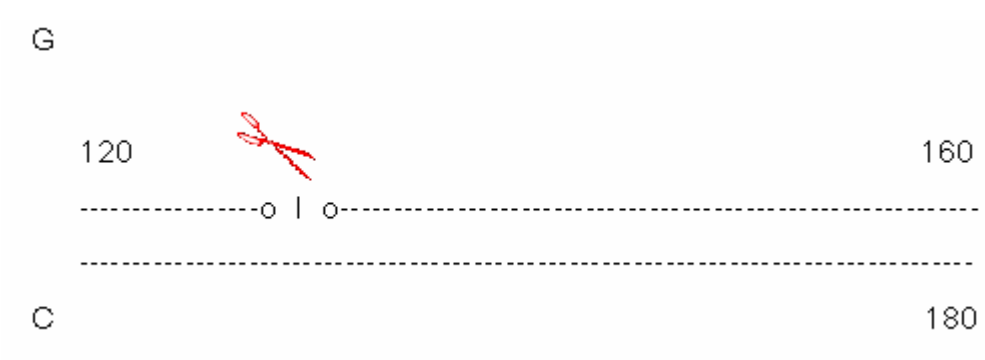
The p53 PCR product is 280 bp long. Bsh 1236 I cuts between the positions 120 and 160. So in case p53 is homozygous (GG) it encodes for Arginine; Bsh 1236 I cuts both DNA-templates into two products of 120 and 160 bp length. In this case one can identify two different bands in the 2 % agarose gel, one at 120 and the other at 160 bp.

3.4.1 HOMOZYGOUS (GG) p53 gene polymorphism for Arginine



When p53 is homozygous for Arginine the entire PCR product will be specifically cut by the enzyme Bsh 1236 I and therefore two different bands result in electrophoresis as explained in the illustration above.

3.4.2 HETEROZYGOUS (GC) p53 gene polymorphism



As illustrated above, the Bsh 1236 I enzyme only cuts one of the p53 PCR products at the specific position and therefore three different bands with the following length result: 120,160 and 180 bp.

3.4.3 HOMOZYGOUS (CC) p53 gene polymorphism

In this case none of the DNA-product is cut by Bsh 1236 I at the specific position. All bands are 280 bp long and therefore only one single band of 280 bp results.

I added 6 µl Loading Dye Solution to 10 µl of the p53 PCR-product and the 1.1 µl Bsh 1236 I to have good results seen in electrophoresis on the agarose gel. Following electrophoretic separation on agarose gels the DNA bands were visualised by ethidium bromide staining.

4 RESULTS

My results described here contain the different genotypes of p53 in the HPV-related cervical lesion and the healthy control group. Furthermore, the results of HPV-PCR and sequencing are also illustrated. The use of various HPV-PCR and its outcome for the same samples are shown.

Finally, I analyzed the distribution of p53 polymorphism with respect to injection by high risk HPV types.

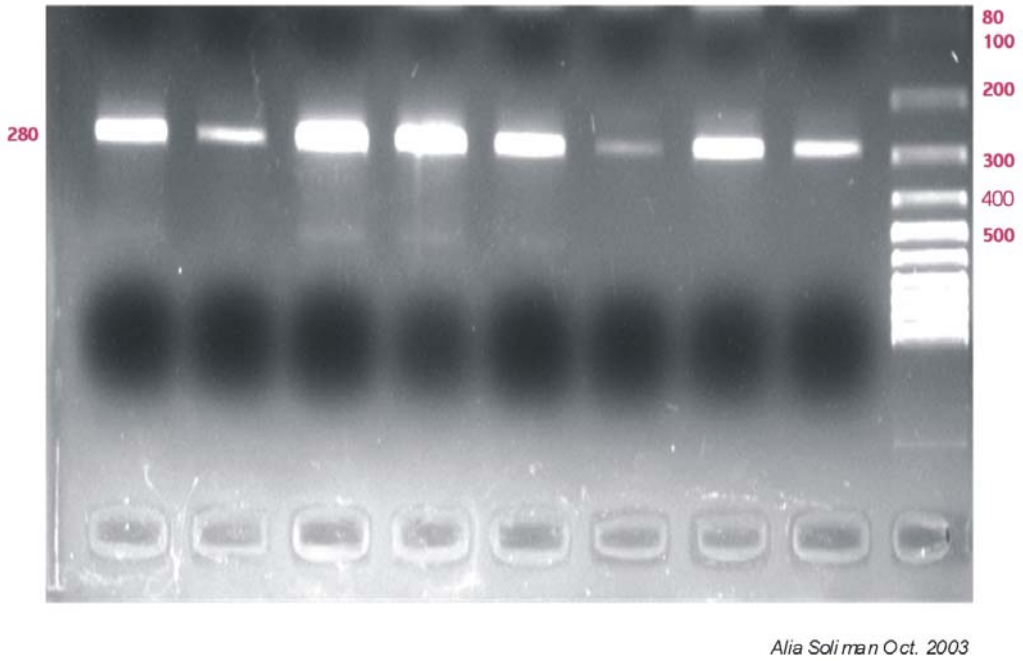


Figure 4.1: PCR Product of *p53* in the absence of Bsh 1236 I digestion

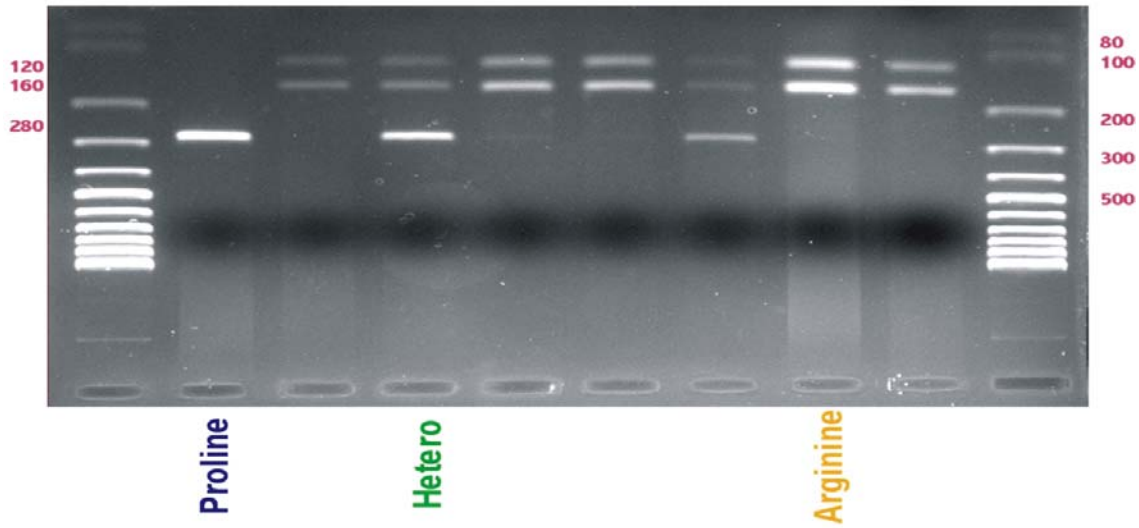


Figure 4.2: PCR products after digestion with the restriction enzyme *BSH1236i.*

4.1 Results of p53 PCR and digestion with Bsh 1236 i

Figure 4.1 shows the PCR product of p53 determined by gel electrophoresis which was expected to be 280 bp long, whereas figure 4.2 illustrates the same samples after digestion with the restriction enzyme Bsh 1236 i.

4.2 Sequencing results of p53 PCR

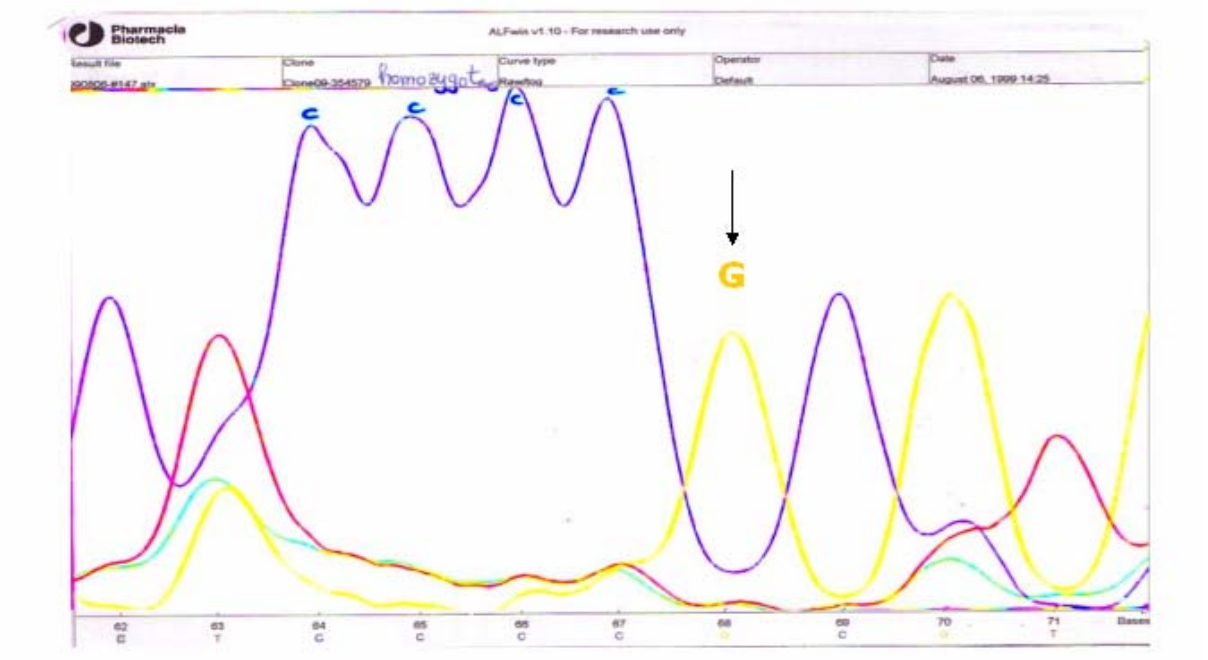


Figure 4.3: Sequencing of the p53 PCR product homozygous for ARGININE G/G

The PCR results were verified by sequencing as shown in figure 4.4. Sequencing shows that the genotype of p53 is homozygous for ARGININE. This proves the correctness of the PCR restriction digest results and indicates that our results obtained by PCR are very accurate and reliable.

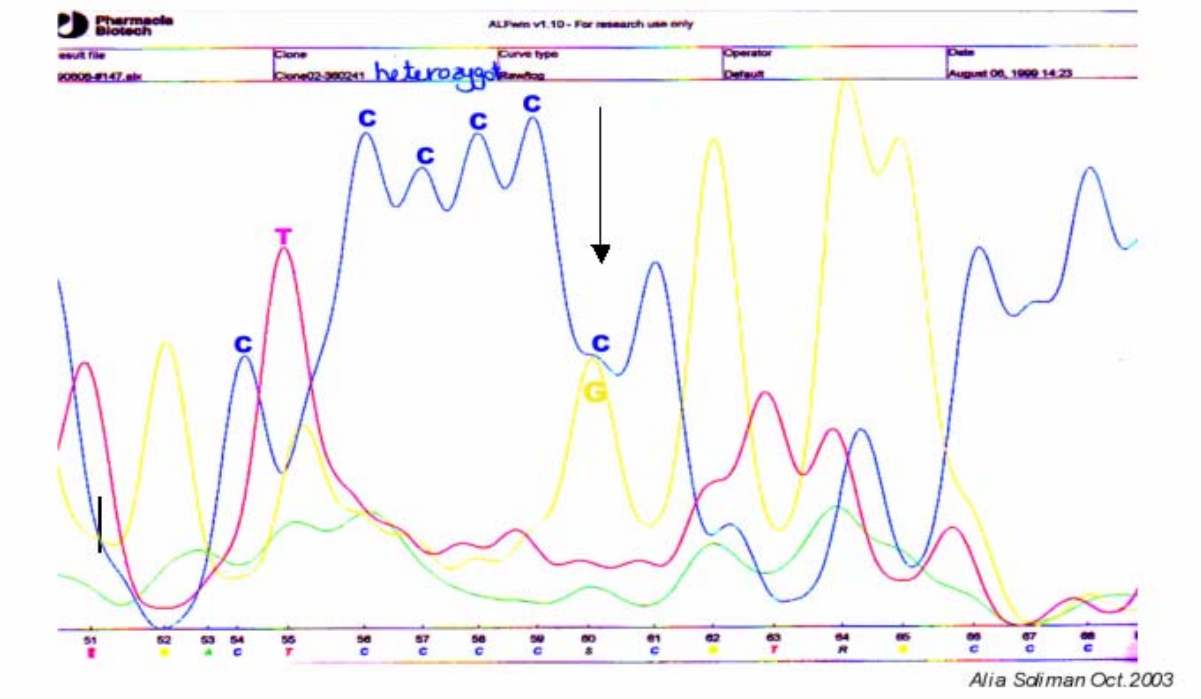


Figure 4.4: Sequencing of the p53 heterozygous PCR product.

Again, the above results were verified by sequencing as shown in figure 4.4. In this case sequencing shows that the genotype of p53 is heterozygous. Once again, this is evidence of the correctness of the results and indicates that these results obtained by PCR and restriction digest are very accurate and reliable.

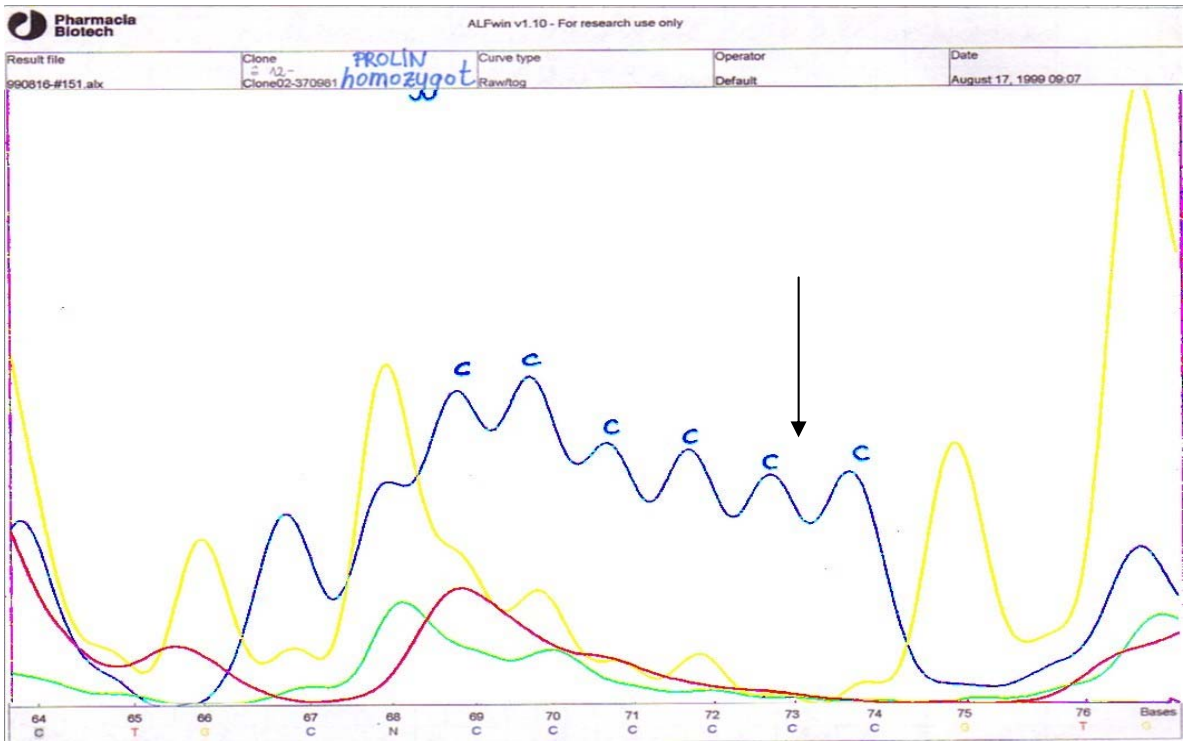


Figure 4.5: Sequencing of p53 homozygous for PROLINE C / C

As for the above mentioned cases, through sequencing of the p53 PCR product the position of the marked nucleotide is cytosin, homozygous C/C encodes for PROLINE.

The distribution of p53 genotypes in the healthy control group (n = 117) was as follows:

Arginine n = 59 (50.4 %),
Heterozygous n = 54 (46.1 %),
Proline n = 4 (3.4 %).

Out of 157 HPV-positive cases (high and low risk) the p53 genotype was distributed as follows:

Arginine 53 %,
Heterozygous 38.8 %,
Proline 8.2 %.

Divided into pathological criteria, 11 out of 111 HPV-positive samples were CIN I and 6 were CIN II (15.31 %).

Herewith 17 cases were pathological low-risk (15.31 %).

Another 35 cases (42.34 %) were benign lesions like ectopic changes, chronic inflammation, parakeratosis, papilloma and condylomata accuminata without dysplasia.

42 cases were CIN III and 5 cases already showed invasive carcinoma.

Thus, 47 out of 111 were carcinomas (in situ or invasive / 42.34 %).

Table 4.1: Distribution concerning pathological criteria

Pathological criteria	Total number	%
Benign lesions	47	42.34
CIN I and CIN II	17	15.31
CIN III and invasive carcinoma	47	42.34
Total	111	100

4.3 Results of HPV PCR with different primer pairs GP5/6 and HPV1/2

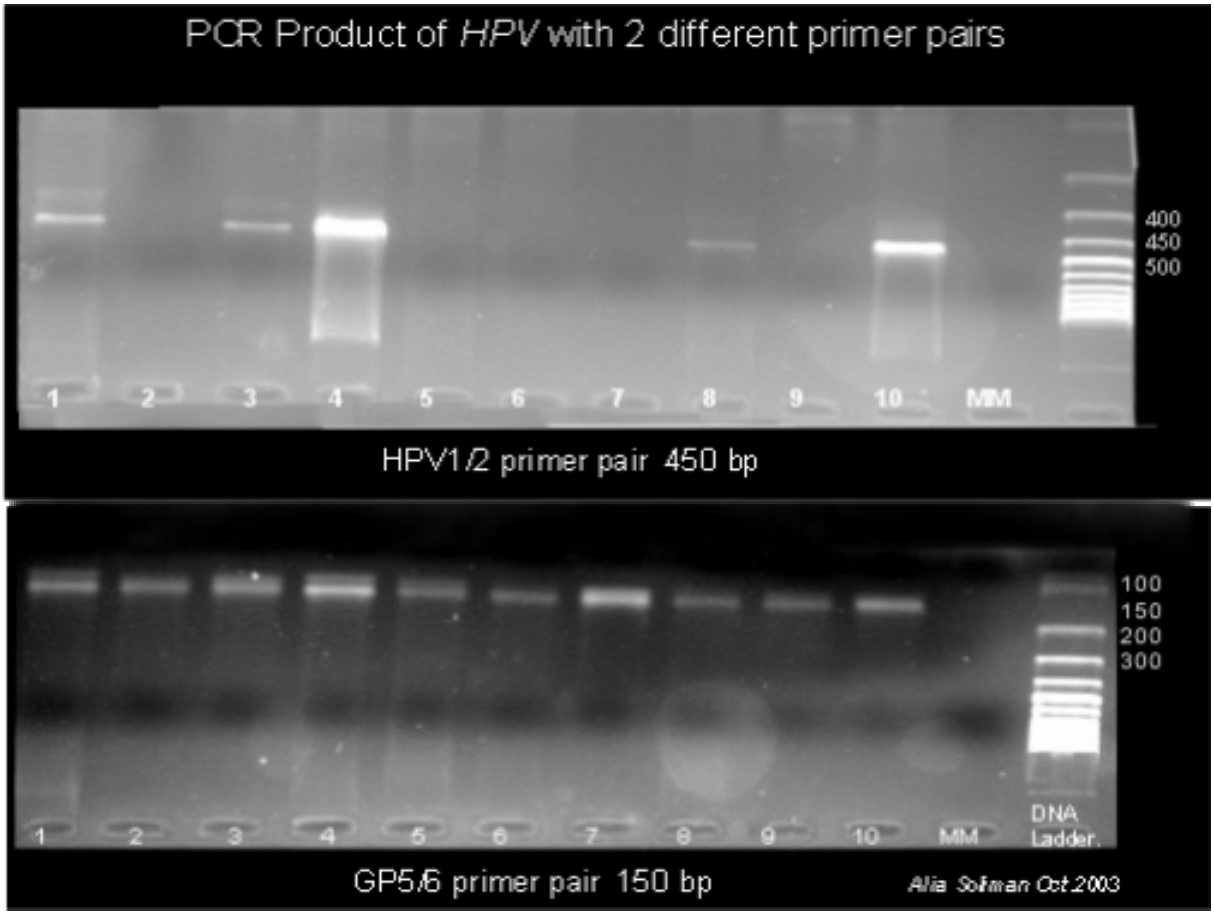


Figure 4.6: PCR of HPV DNA with primer pair GP5/6 and HPV1/2

The figure above shows HPV-PCR products of certain samples, each of them performed once with the primer pair GP5/6 and once with HPV1/2.

Primer pair HPV1/2 detects and amplifies DNA fragments of approximately 450 bp, while GP5/6 detects and amplifies DNA fragments of 150bp.

This explains why samples 2, 5, 6, 7 and 9 are negative when performed by PCR with primer pair HPV1/2 while the same samples are positive when performed with primer pair GP5/6. This illustrates the necessity of reliable PCR primers to detect HPV infection performing PCR as well as the reasonable use of both, PCR-primer GP5/6 and PCR-primer HPV1/2.

Out of 105 HPV-PCR 36 did not show a band – were negative with primer pair HPV1/2 but the same samples were positive when performed with GP5/6.

This means that 34.29 % of the HPV PCR were false negative or undetected by PCR that uses only HPV1/2. My results strongly indicate that HPV detection is much more reliable and sensitive when analyzed by a short range PCR.

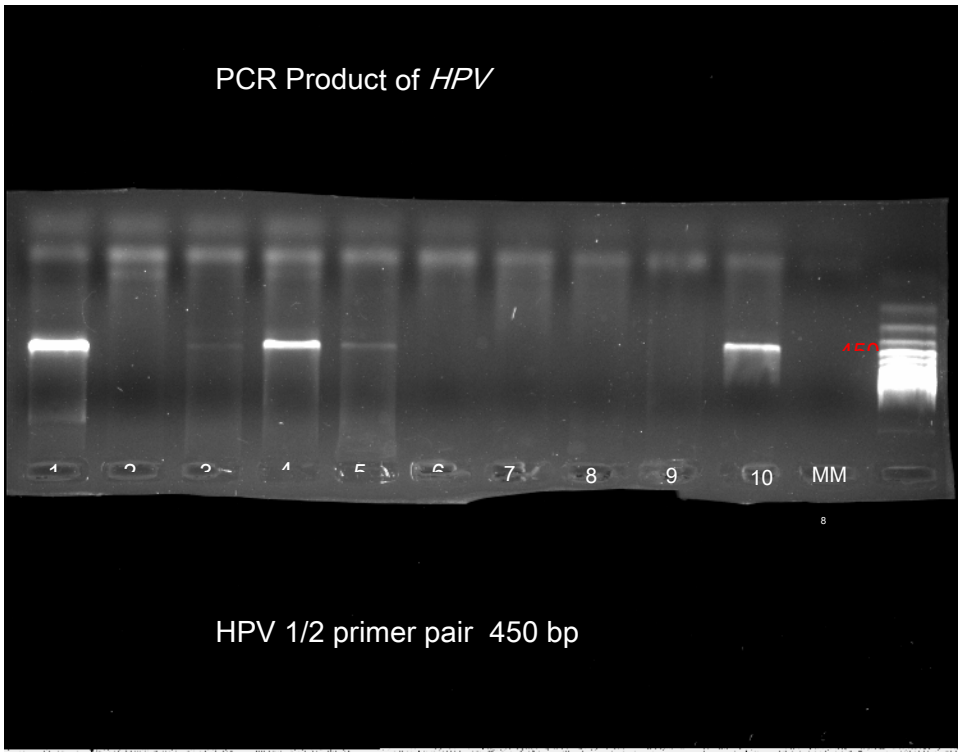


Figure 4.7: PCR of HPV DNA with primer pair HPV1/2

The figure shown above illustrates HPV-PCR performed by primer pair HPV1/2. The bands are 450 bp long. Samples 1, 4 and 10 show strong bands which are 450 bp long- which means they are positive for HPV; while samples 3 and 5 show weak bands and are also positive for HPV.

Samples 2, 6, 7, 8 and 9 show no bands and are negative using HPV1/2 primer. MM is the Master Mix control, no band is detected which means that there is no contamination and assures that all bands seen are not false positive.

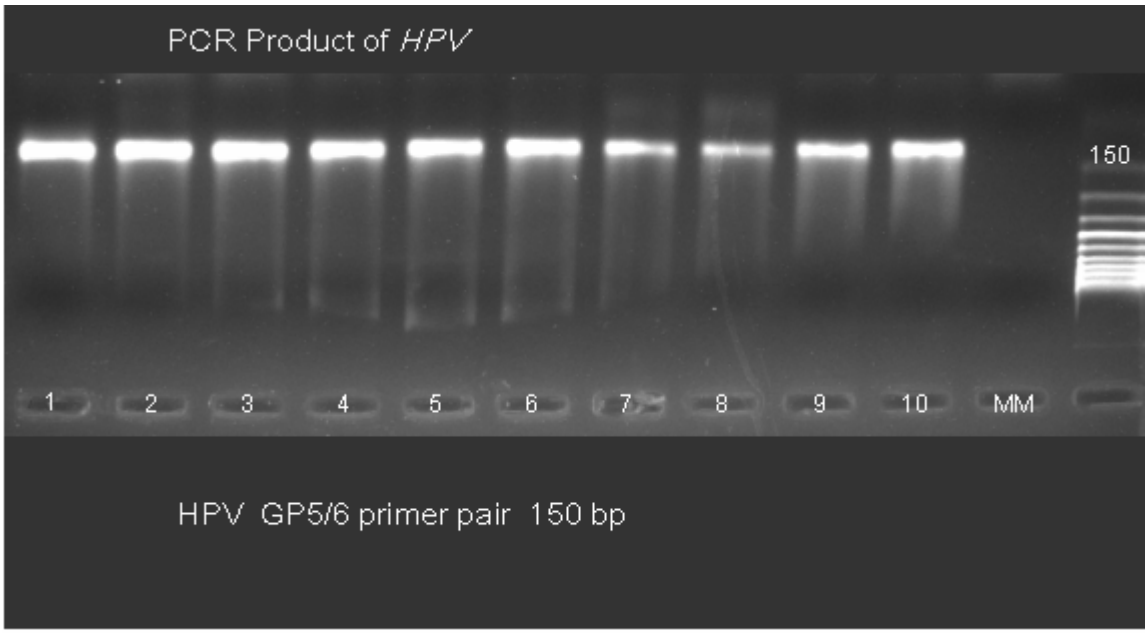


Figure 4.8: PCR Product of HPV DNA with primer pair GP5/6

This figure shows PCR product of HPV performed with primer pair GP5/6. The bands are 150 bp long. All samples show clear bands which means that they are all HPV positive. Master Mix Control is negative which shows that there is no contamination. The samples which are positive in this figure are the same as in figure 4.6. That means that samples 2, 6, 7, 8 and 9 are HPV positive performed with primer pair GP5/6 but undetected with primer pair HPV1/2 as seen in figure 4.6.

Sequencing Results of HPV PCR

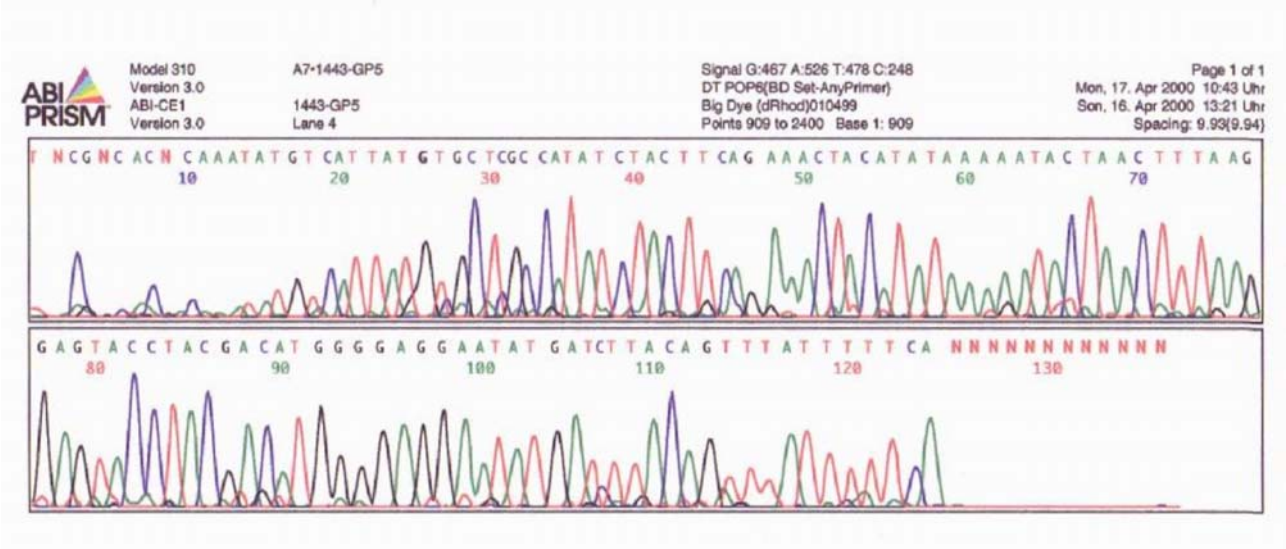


Figure 4.9: Sequencing of HPV PCR GP 5/6

Each HPV-PCR sample was sequenced using ABI PRISM Sequencer Model 310 Version 3.3. The sequence of basepairs was compared with the NCBI databank.

The figure above shows a sequence of a HPV-PCR performed with GP5/6.

After comparing the specific nucleotide sequence with the NCBI databank it became clear that this nucleotide sequence is an HPV 16 sequence and not an HPV 6 sequence as primarily tested by ELISA.

After comparing sequences of 97 HPV-PCR samples it became evident that 8 out of 96 samples were HPV 16 (N=7) or HPV 45/87 (N=1) which all belong to the high-risk HPV group - tested by ELISA all these 8 samples were identified as HPV 6- which belongs to the low risk HPV group.

This means that 8.2 % of the HPV infections were not detected as high-risk HPV 16. This shows the importance of accurate and highly specific detection techniques realized by cycle sequencing which is eminent in analysis and interpretation of the results.

Successful sequencing of 97 HPV PCR samples showed the following distribution:

Table 4.2: distribution of low and high risk HPV

Low-risk HPV	N	frequency
HPV 6	30	30.90 %
HPV 11	4	4.10 %
Total	34	35.00 %

High-risk HPV	N	frequency
HPV 16	56	57.30 %
HPV 18	3	3.10 %
HPV 31	3	3.09 %
HPV 45/87	1	1.03 %
Total sum	63	65.00 %
Total of both groups	**97**	**100 %**

The data illustrated in the table above show that altogether 65 % of the sequenced HPV samples showed high risk infection with HPV 16/18/31/45/87.

gb|U89348.1|HPU89348

Human papillomavirus type 16 variant, complete sequence

Length = 7905

Score = 190 bits (96), Expect = 7e-47

Identities = 96/96 (100 %)

Strand = Plus / Plus

Query: 30 gctgccatatctacttcagaaactacatataaaaatactaactttaaggagtacctacga 89

Sbjct: 6673 gctgccatatctacttcagaaactacatataaaaatactaactttaaggagtacctacga 6732

Query: 90 catggggaggaatatgatttacagtttatttttcaa 125

Sbjct: 6733 catggggaggaatatgatttacagtttatttttcaa 6768

gb|AF001600.1|AF001600

Human papillomavirus type 16 integrated SiHa HPV-16

variant DNA,

capsid protein gene, complete cds, and flanking cellular

DNA

Length = 4550

Score = 190 bits (96), Expect = 7e-47

Identities = 96/96 (100 %)

Strand = Plus / Plus

Figure 4.10: Results of NCBI inquiry of sequenced HPV samples

The figure shown above is a typical result of the NCBI inquiry after alignment of our HPV-samples. Comparison of our sequences with the sequences of the data bank shows that this sequences encodes clearly for the HPV-type 16 with 100 % accuracy.

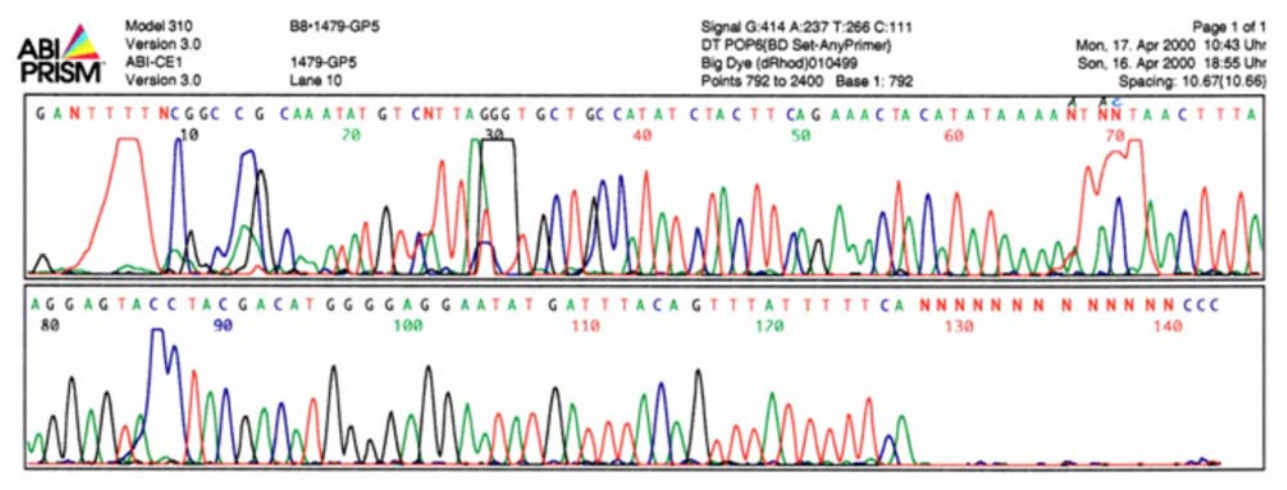


Figure 4.11: Sequencing of HPV PCR

The figure above shows a sequence of HPV-PCR. This sequence was also compared with the NCBI data bank results. The result proved that this sequence encodes for high-risk HPV 16 and not the low-risk HPV 6 as tested by ELISA.

The sequence above is one example for 7 other sequences that were wrongly detected as low-risk HPV by ELISA testing.

Comparison of HPV sequences with the NCBI data bank results revealed that 8 samples were high-risk HPV 16 instead of low-risk HPV 6 – and one sample was high-risk HPV 45/87 instead of low-risk HPV 6.

4.4 Distribution of *p53* genotypes related to cervical lesions

Focusing on the high-risk HPV-infection and high risk lesions of the cervix -
(CIN III and invasive carcinoma) the following distribution was observed:

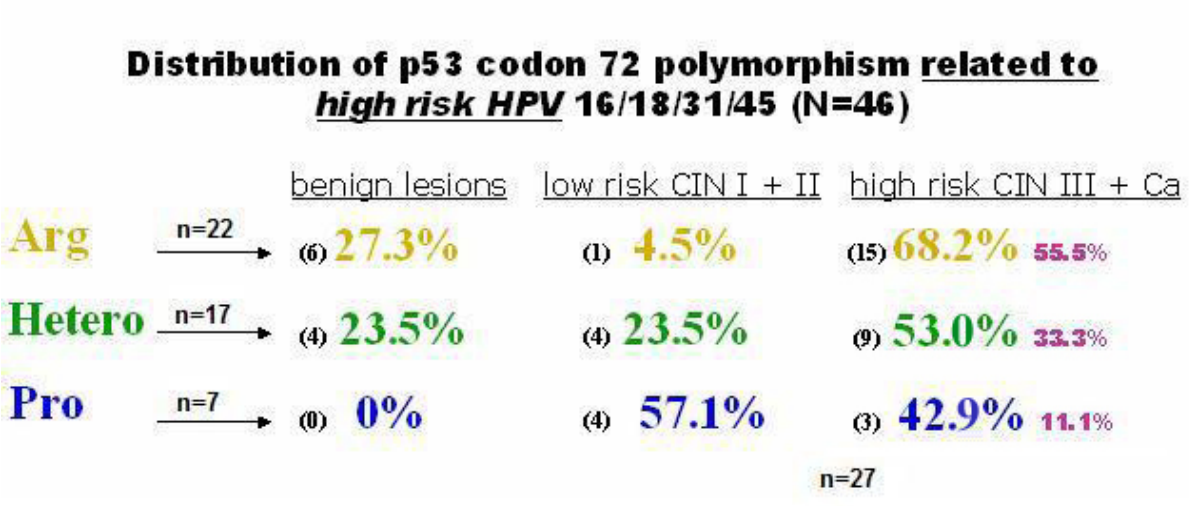


Figure 4.12: Distribution of p53 codon 72 polymorphism related to high-risk HPV types

The data in this figure show the distribution of *p53 genotype* in three different pathological groups:

a) benign lesions,
b) CIN I + CIN II and in
c) CIN III and invasive carcinoma

related to high risk HPV-infection with HPV-types 16,18,31,45 or 87.

Altogether the Arginine group consists of 22 cases: 6 out of the group with the arginine polymorphism showed benign lesion after infection with high-risk HPV, one case a low risk lesion while 15 cases revealed CIN III + invasive carcinoma.

When the group of arginine genotypes was analyzed it became evident that only 4.5 % were at low risk while more than two thirds were CIN III or invasive carcinoma cases.

Altogether more than 68 % of the arginine group showed high risk lesion in the case of high risk HPV- infection.

The group of heterozygous cases comprised a total of 17 cases, four of which were benign,four remained at the low risk stage (CIN I + CIN II) while nine showed CIN III or invasive carcinoma lesions.

Compared to the arginine group (68.2 %) only 53 % of the heterozygous group showed the high risk lesions CIN III and invasive carcinoma.

The group of homozygous proline genotypes comprised seven cases,four were at low-risk CIN I and CIN II, while only three cases of which revealed high risk lesions like CIN III and invasive carcinoma. Thus, only 42.9 % showed high risk lesions compared to 68.2 % .

The total number of cases that revealed high risk lesion like CIN III and invasive carcinoma was 27.

Even if we compare this subgroup with itself Arginine still shows nine cases which revealed high risk lesion, accountin for 55.5 % of the whole group (N = 27), compared with 33.3 % which are heterozygous and 11.1 % which reveal a proline genotype.

Analysing these data it became evident that with 68.2 % the arginine genotype group forms the highest number of all cases with high risk lesions when infected with high-risk HPV 16/ 18/ 31/ 45.

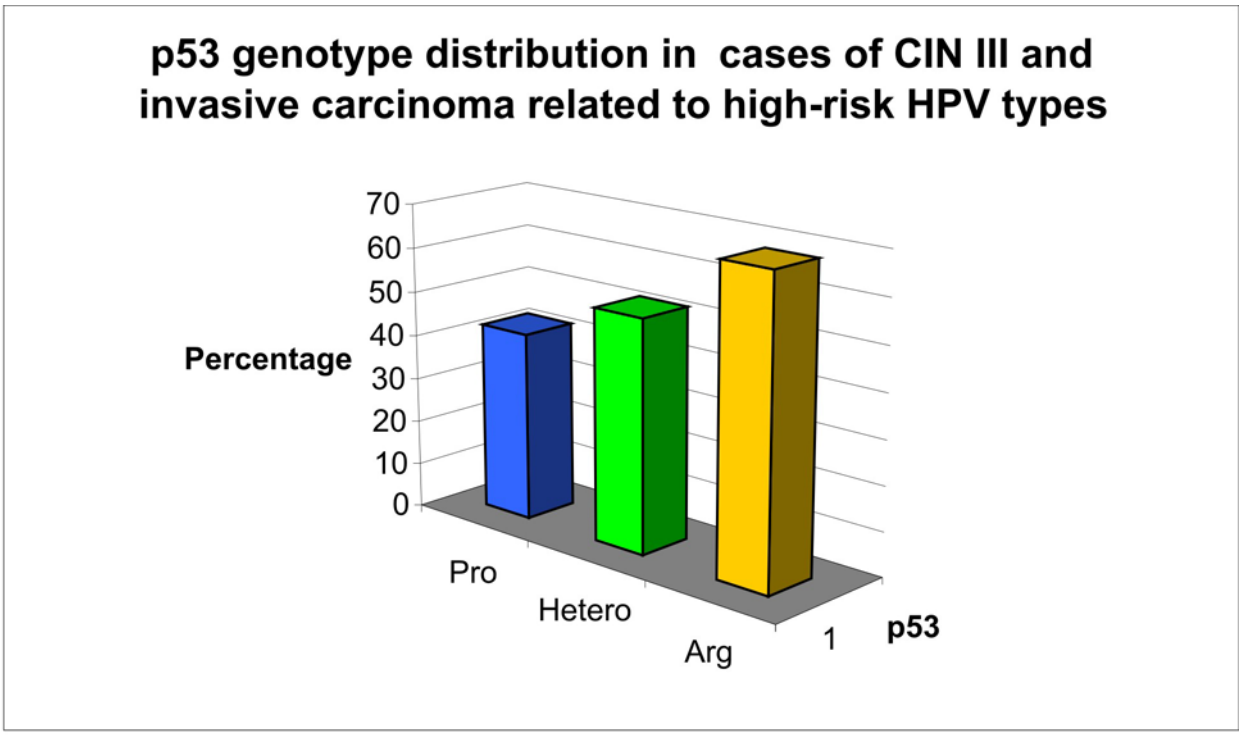


Figure 4.13: p53 genotype distribution in HPV 16/ 18/ 31/ 45/ 87 related high-risk lesions

This figure illustrates the distribution of p53 polymorphism codon72 in cases of CIN III and invasive carcinomas in the case of infection with high risk Human Papilloma Viruses like HPV- 16/18/31/45/87.

The arginine genotype forms the largest subgroup (68.2 %) in this group followed by heterozygous genotypes with 53% and proline with 42.9 %.

5 DISCUSSION

The Arg/Arg genotype of the p53 antioncogene was previously identified as a risk factor for HPV-related cervical cancer (Storey et al., 1998; Zehbe et al. 1999/ 2001; Saranth et al., 2002; Ojeda 2003, Zur Hausen, 2002, Pegoraro et al., 2002, Min- min et al 2006).

Storey and colleagues showed that the polymorphism at codon 72 gene determines the efficiency of HPV-16 or HPV-18 E6 oncoprotein in degrading p53 in vitro.

Zehbe et al., 1999/ 2001, showed that there is an association between codon 72 polymorphism of p53 and infection with high-risk HPV in the development and outcome of cervical cancer in Swedish and Italian women while others failed to confirm this association. So recent studies show controversial results (Storey A et al 1998, Saranth D et al 2002, Pegoraro RJ et al 2002, Rosenthal AN et al 1998).

Malisic et al. 2008 could not prove an association between codon 72 polymorphism of p53 and cervical cancer in Serbian population, however they did not take into consideration the HPV infection of all cases they examined which leads to distorted results.

Also Oliveira et al., 2008, and Fernandes et al., 2008, could not establish a correlation between codon 72 polymorphism of p53 tumor suppressor gene and cervical lesions in their studies analyzing samples from Brazilian and Portuguese women.

Govan et al., 2007, did not observe a relationship between p53 codon 72 genotype and HPV- associated cervical cancer in South African women, however they considered that the frequency of the homozygous Pro/Pro residue at codon 72 is remarkably increased in the South African population compared to Indian, Caucasian or Portuguese population and therefore results can also be distorted by ethnic variety.

In our study we assessed the impact of p53 polymorphism on the validity of the proposed association by investigating the distribution of p53 genotypes in 111 HPV-related cervical lesions and 117 healthy pregnant women.

For this purpose, we examined the single nucleotide polymorphism in codon 72 of the p53 tumor suppressor gene by PCR and sequencing.

DNA was extracted from paraffin-embedded cervical tissues from 111 patients who were all previously HPV-tested by ELISA, 17 with LSIL (CIN I +CIN II),

47 with HSIL (Ca i.s.+ invasive carcinoma) and 47 with benign lesions (Condylomata accuminata, Papilloma etc.) as well as DNA from 117 pregnant females all of whom were tested by PAP 1 with normal negative cell picture in PAP-smears.

As accurate and robust assays are inevitable to produce statistically significant and reliable results, we used cycle sequencing to verify our results.

P53 sequence was detected by PCR using primer pairs p53+/p53- at 55°C annealing temperature. Polymorphism was detected by digestion with restriction enzyme Bsh1236.

HPV-DNA was proven by PCR using not only primer pairs HPV1/2 but also GP 5/6 and subsequently cycle sequencing.

Andersson (Andersson et al., 2001) investigated 111 cases of adenocarcinoma of the cervix collected through the Swedish Cancer Registry and 188 controls (females with normal cytology at organised gynaecological screening) with regard to p53, codon 72, polymorphism using a PCR- and SSCP-based technique. In the controls, 9 % showed pro/pro, 44 % pro/arg and 47 % arg/arg, whereas in the invasive adenocarcinomas, the corresponding figures were 0 %, 29 % and 71 %, respectively. The difference was statistically significant (P = 0.001). HPV DNA was identified in 86 tumours (HPV 18 in 48, HPV 16 in 31 and HPV of unknown type in 7 cases) and 25 tumours were HPV negative. The p53, codon 72, genotypes observed in HPV-positive and HPV-negative cervical adenocarcinomas were not statistically different (P = 0.690). The results indicate that women homozygous for arg/arg in codon 72 of the p53 gene are at an increased risk for the development of cervical adenocarcinomas.

Saranth (Saranth et al., 2002) examined DNA from 337 Indian women with invasive cervical cancers, 164 women with clinically normal cervix, 64 women with low-grade squamous intraepithelial lesions (LSIL), and 5 women with high-grade squamous intraepithelial lesions (HSIL) for the presence of HPV16/18 using consensus primers in a polymerase chain reaction (PCR), and the specific HPV type was identified by Southern hybridization of the PCR product using HPV16/18 type-specific nucleotide sequences as probes. Further, 134 women with cervical cancers and 131 healthy women were used to determine the frequency of p53 genotypes, Pro/Pro, Arg/Arg, and Pro/Arg, using peripheral blood cell DNA to indicate the constitutional genotypes and allele-specific primers, in a PCR-based assay.

A prevalence of HPV16/18 in 77 % (258/337) of cervical cancer patients, 38 % (24/64) of LSILs, 4 of 5 HSILs, and 15.2 % (25/164) of normal healthy women was observed. The frequency of distribution of the three genotypes of p53 codon 72 in a subgroup of the

HPV16/18-positive cervical cancer patients was Pro/Pro 0.18 and Arg/Arg 0.56, with the heterozygous Pro/Arg 0.26 differing significantly from the genotype frequency in the normal healthy women (chi(2) = 6.928, df = 2, P < 0.05). The prevalence in LSILs confirms HPV16/18 infection as an early event and further indicates a role in progression of lesions.

The p53 genotype distribution indicated that women homozygous for Arg genotype were at a 2.4-fold higher risk for developing HPV16/18-associated cervical carcinomas, compared to those showing heterozygous Pro/Arg genotype (odds ratio 2.4, 95 % confidence interval 1.89 to 3.04).

Pegoraro (Pegoraro et al., 2002) determined the p53 codon 72 status in 281 black South African women with cervical cancer and 340 ethnically matched healthy control subjects. In addition, HPV DNA was confirmed in 190 cervical tumors and the viral type determined. Results showed that overall more cancer patients than control subjects had an Arg allele at codon 72 with respect to both genotype and allelotype (P < 0.05).

Pegoraro et al. furthermore stress that not all studies have taken into account the type of human papillomavirus (HPV) infection present.

Storey (Storey et al., 1998) could show that allelic analysis of patients with HPV-associated tumours reveal a striking overrepresentation of homozygous arginine-72 p53 compared with the normal population, which indicated that individuals homozygous for arginine 72 are about seven times more susceptible to HPV-associated tumorigenesis than heterozygotes. The arginine-encoding allele therefore represents a significant risk factor in the development of HPV-associated cancers.

Ojeda (Ojeda et al., 2003) accomplished a case control study consisting of 60 patients belonging to the Chilean mixed population with newly-diagnosed and histologically-confirmed invasive cervical cancer. 53 matched controls without cervical cancer or known risk factors for this malignancy were recruited and investigated by the Centro de Oncologia Preventiva in Santiago, Chile.

The distribution of p53 codon 72 genotypes in healthy women were as follows:

- Four samples were proline homozygotes (Pro/Pro 7.5 %), 25 arginine homozygotes (Arg/Arg 47.2 %), and 24 heterozygotes (Arg/Pro 45.3 %)
- Among the 60 cervical cancer patients 41 were Arg/Arg (68.3 %),
- Four were Pro/Pro (6.7%) and 15 were Arg/Pro (25 %).

A statistically significant odds ratio of 2.62 was obtained in Ojedas study. Homozygote women for arginine in Santiago have a 2.6-fold increased risk for cervical cancer.

Ojeda (Ojeda et al., 2003) results are strikingly congruent with ours regarding the Arg/Arg group among the cervical cancer patients in Germany (68.2 % versus 68.3 % in Chile).

Min-min (Min- min et al 2006) investigated the relationship between p53 tomur suppressor gene of codon 72 polymorphism in cervical lesions of the Chinese population and they could prove that the frequency of p53 Arg homozygosity in cervical squamous cancer, cervical adenocarcinoma and CIN (II-III) was much higher than that of p53 Arg/Pro heterozygosity and of p53 Pro homozygosity and higher than the frequency of p53 Arg homozygosity in normal samples. Moreover the frequency of p53 Arg homozygosity in high- risk HPV E6- positive cervical squamous samples (64.06 %) is much higher than that in HPV- E6- negative cervical squamous cancer samples (35.29 %). Based on the findings of their study, p53 Arg homozygosity could act as a potential risk factor for the tumorigenesis of the cervix.

Ciotti (Ciotti et al 2006) could establish a relationship between p53 Arg homozygosity of codon 72 polymorphism in HPV infected cervical samples of women from central Italy: Their data show that Italian women homozygous for Arg/ Arg are at significantly increased risk for HPV infection.

Gudleviciene (Gudleviciene et al. 2006) examined the distribution of p53 tumor suppressor gene 72 codon polymorphism in 588 Lithuanian women. They could exemplify in their study that cervical cancer in Lithuanian women is associated with HPV16 infection and with p53 homozygous Arg/Arg allele (OR= 2,10; CI 1,10- 4,19).

Jee (Jee SH et al 2004) performed a meta-analysis investigating 37 studies to quantitatively summarize the evidence for a relationship between p53 Arg/ Arg homozygosity of codon 72 polymorphism and cervical cancer: the meta-analysis showed that the overall OR (95 % confidence interval) for cervical cancer among patients homozygous for Arg/ Arg was 1.2 (1.1- 1.3, P= 0.001) compared with the heterozygous mutant (Arg/ Pro). The p53 gene was associated with increased risk for cervical cancer varying by country and HPV type.

Zehbe (Zehbe et al.,1999 / 2001) could also prove a statistically significant correlation as the authors mentioned above concerning high risk-HPV16 when comparing cervical cancer of Italian and Swedish populations.

Significant differences between cases and controls were observed in the following countries:

- UK: $X^2 = 11.19$, $p < 0.001$ (Storey et al., 1998),
- Sweden: $X^2 = 1680$, $p < 0.0001$ (Andersson et al., 2001),
- South Africa: $X^2 = 57.99$, $p < 0.0001$ (Pegoraro et al., 2002),
- India: $X^2 = 6.34$, $p < 0.01$ (Saranath et al., 2002),
- Chile: $X^2 = 5.19$, $p < 0.02$ (Ojeda et al., 2003).

Concerning Ojeda methodological shortcomings such as the lack of representativeness or genetic heterogeneity of some samples, improper selection of control groups, and technical problems related to genotyping may be partially responsible for some inconclusive results observed when comparing reviewed studies.

It is interesting to note that populations showing lower Arg frequencies for numerical reasons manifest on the average higher odds ratios, and a lower mean arginine homozygote frequency was found in population in which significant associations between the p53 codon 72 polymorphism and cervical cancer were found, regarding populations in which these associations were not found to be significant.

We agree with Ojeda that therefore some negative results may be a consequence of high Arginine frequencies in control populations and not necessarily a lack of association.

Finally we summarize that others who failed to prove this association (Rosenthal et al , Lancet Vol 352, 1999) and who did not pay attention to the above mentioned coherencies did not take into consideration the high risk HPV infection, distribution and relation nor did they perform sequencing which this research showed is absolutely essential to avoid distortion of results needed for drawing accurate conclusions.

6 CONCLUSIONS

- 63 out of 96 HPV-pos. cases were high risk infections (65 %).
- Using primer pair GP5/6 (150 bp) increases sensitivity in HPV-PCR undetected by PCR with HPV1/2 primer pair (450 bp).
- Out of 105 HPV-PCR 36 did not show a band- were negative with primer pair HPV1/2, while the same samples were positive when PCR was performed with primer pair GP5/6 – This means that more than one third, exactly 34.3 % of the HPV-PCR were false negative or undetected by PCR using only primer pair HPV1/2.
- Sequencing detects *high-risk HPV* ignored by ELISA which distorts results needed for accurate conclusion (8.2 % were added to *high-risk HPV-group*) 68.2 % of homozygous Arg in the high-risk HPV-group developed CIN III and invasive carcinoma compared to only 53 % of the heterozygous and 42.9 % homozygous Pro.
- Only 4.5 % of Arg/Arg were at low-risk (CIN I+II) in the above mentioned HPV-group and 27.7 % of Arg/Arg had benign lesions.
- These data suggest that women homozygous for Arginine are more susceptible to developing *HPV 16/18 -related* high–risk cervical lesions.
- Others who failed proving this association (Rosenthal et.al, Lancet Vol 352, 1999) did not take into consideration the *high-risk HPV* infection, distribution and relation nor did they perform sequencing.
- Future steps are to evaluate the clinical importance of these findings with a greater population regarding the histological changes in cervical lesion, which could be detected *earlier* if females at higher risk (homozygous for Arginine) are examined in shorter intervals. The PCR test for p53 polymorphism codon 72 is cheap and fast, results are available within a few hours in any PCR laboratory.

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"Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht."