# Immunohistochemical Evaluation of the Role of *p53* Mutation in Cervical Cancer: Ser-20 *p53*-Mutant Correlates with Better Prognosis

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**Abstract.** *Background: Cervical cancer is driven by human papillomavirus virus-specific oncoprotein E6. E6 interacts with E3 ubiquitin-protein ligase, resulting in the proteolysis of p53 protein. The aim of this study was to analyze one TP53 mutation in patients with cervical cancer and to correlate it to prognosis. Materials and Methods: A total of 248 paraffin-embedded tumor samples were stained for mutated p53 protein. The distribution and intensity of staining both in the nucleus and cytoplasm were evaluated with a semi-quantitative immunohistochemical score. Results: A total of 66% of studied cervical carcinomas expressed the mutated p53 protein. The overall survival was better for patients expressing the mutated p53 protein in the nucleus. Conclusion: Interestingly, we found a very high mutation rate of TP53 in a cancer type where p53 is initially inactivated via E6 during the development of cervical cancer. An unexpected finding is the correlation of this mutation with better survival, possibly due to better response to therapy.*

Worldwide, cervical cancer is the fourth most common tumor diagnosed and cause of death in women, with an estimated 528,000 cases and 266,000 deaths in 2012 (1, 2). About 80% of cervical cancer cases occur in low-to-medium resource countries (3). Over the past decades, the introduction of tumor screening programs in many high-resource countries contributed to reduced incidence and mortality due to cervical cancer (4).

Oncogenic human papillomavirus (HPV)s, mainly HPV 16 and 18 genotypes, have been strongly associated with the risk of developing intraepithelial lesions and appear to be involved in the development of more than 90% of all squamous cell carcinomas and adenocarcinomas of the cervix (5). However, most individuals who have had HPV infections do not develop cervical cancer since the low-grade squamous epithelial lesions induced spontaneously regress in more than 90% of cases (1). Cervical cancer typically develops from pre-cancerous changes over a period of 10 to 20 years (6). HPV-induced cervical cancer is driven by the virus-specific oncoproteins E6 and E7. The E6 protein of HPV types 16 and 18 interacts with E3 ubiquitin-protein ligase, resulting in the proteolysis of p53 protein (7). In addition, E6 binds E1A binding protein p300 and CREB-binding proteins and reduces the ability to activate p53-responsive promotor elements (7). Variable levels of *E6* mRNA have been found in both cervical intraepithelial neoplasia and cervical cancer. However, the constitutive expression of early viral genes is not in itself sufficient to induce and maintain the transformation status (6, 8). Accumulation of genetic and epigenetic alterations over time may, therefore, be crucial for the ultimate progression to cancer.

Mutations of *TP53* gene are among the most common genetic alterations in many human malignancies (9-11). More than 50% of human tumors contain a mutation or deletion of the *TP53* gene, ranging from 5 to 80% depending on the type, stage, and etiology of tumors (12). Many studies have investigated a genetic link between these variation and cancer susceptibility. Results, however, have been controversial. In 2009, a meta-analysis study of 49 pooled studies failed to show a link between a very common mutation (namely substitution of an arginine for a proline at

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codon position 72) and cervical cancer susceptibility (13). In 2011, the same mutation was found to be associated with higher pancreatic cancer risk among males, but seemed to protect Arab women against breast cancer (14, 15). To date, many other mutations of *TP53* have been described. Mutations that deactivate p53 in cancer usually occur in the central DNA-binding domain. Most of these mutations destroy the ability of the protein to bind to its target DNA sequences, and thus prevent transcriptional activation of these genes. Furthermore, 80% of the most common mutants have the capacity to exert dominant-negative effect over wild-type p53 and thus prevent the activation of transcription. In comparison only 45% of the less frequent mutants studied have this capacity (16). Roles played by other mutations of the p53 protein are much less known. In this study, we focused on one mutation occurring in the *N*-terminal part of p53, which contains one of the two transcription activation domains of the protein involved in the regulation of several pro-apoptotic genes (17).

The aim of the study was to analyze the mutation of the *TP53* gene in patients with cervical cancer and to correlate mutation to clinical parameters and prognosis.

## Materials and Methods

*Patient data*. Tissue samples from 248 formalin-fixed and paraffin-embedded cervical neoplasia biopsies from patients referred to the Gynecology Unit at the Department of Obstetrics and Gynecology, Ludwig Maximilian University of Munich, from 1993 to 2002, were included in this study. All cases had been previously characterized in terms of histology (grade, tumor size and lymph-node infiltration by tumor cells) at the Department of Pathology, Ludwig Maximilian University of Munich (Table I).

All material was sampled for diagnostic purposes and research was carried out in accordance with the legal requirements concerning confidential medical communication as well as the data protection act. Consequently, consulting the Ethics Committee of the Medical School, Ludwig Maximilian University of Munich, and written informed consent from the patients prior to participation in the study was not required.

*Immunohistochemistry*. Paraffin wax-embedded tissue sections of 3 μm from samples were deparaffinized in xylol for 20 min, washed in 100% ethanol and then incubated in methanol/$H_2O_2$ (3%) for 20 min. After rehydration in an alcohol gradient to distilled water, the slides were placed in a pressure cooker containing sodium citrate buffer (pH=6.0) and heated for 5 min. Slides were washed twice in phosphate buffer solution (PBS) and blocked using blocking solution (Zytomed, Berlin, Germany) for 5 min. Each slide was separately incubated with a specific antibody against mutated p53 protein (at position 20, serine to asparatic acid - ab32049; Abcam, Cambridge, UK) diluted 1/100 in PBS. Incubation of the sections with the primary antibodies lasted for 16 h at 4˚C. Afterwards, sections were washed twice in PBS before incubation with post-block reagent (Zytomed) for 20 min. Finally, slides were washed in PBS and then incubated with the horseradish peroxidase-polymer (3) (Zytomed) for 30 min. Staining was performed using 3,3-diaminobenzidine (DAB) substrate solution (Dako, Glostrup, Denmark) for 180 sec. Counterstaining was carried out with Mayer's hemalaun for 2 min. Finally, sections were washed in tap water for 5 minutes and afterwards dehydrated in an ascending alcohol series and washed in xylol. Slides were cover-slipped with Eukittquick-hardening mounting medium (Sigma-Aldrich, St. Louis, USA).

The intensity score (IRS) used examines the intensity and distribution of antigen expression and is calculated by multiplying the percentage of positively stained cells (0: no staining; 1 <10% of cells; 2: 11-50%; 3: 51- 80%, 4> 81%) with the intensity of cell staining (0: none; 1: weak; 2: moderate; 3: strong). Two independent investigators examined the sections using a Leitz Diaplan microscope (Leitz, Wetzlar, Germany). The concordance was of 95%. In cases of different staining evaluation, both investigators carried out re-evaluation until an agreement was reached (5% of all cases). Positive controls were carried out with human breast cancer sections. Negative controls were performed by replacement of the primary antibody and alternative incubation of the slides with IgG rabbit or mouse control antibodies (Biogenex, San Ramen, CA, USA).

*Statistical analysis*. Correlation of staining with grading, age, size, nodal status and survival analysis was evaluated with the statistical program R (Version 0.98.1028; RStudio, Inc., Boston, MA, USA). The Mann–Whitney *U*-test was used for evaluation of two independent groups. Values of $p<0.05$ were considered statistically significant.

Table I. *Clinical characteristics of the study population*.

| | No./total. | % |
|---|---|---|
| Age, years | | |
| ≤50 | 143/248 | 58 |
| >50 | 105/248 | 42 |
| No. of positive nodes | | |
| 0 | 149/248 | 60 |
| 1-4 | 97/248 | 39 |
| NA | 2/248 | 1 |
| Tumor size (cm) | | |
| ≤2 | 111/248 | 45 |
| 2-4 | 128/248 | 52 |
| >4 | 9/248 | 3 |
| Tumor grade | | |
| I | 20/248 | 8 |
| II | 141/248 | 57 |
| III | 78/248 | 31 |
| NA | 9/248 | 4 |
| Tumor subtype | | |
| Squamous | 199/248 | 80 |
| Adenocarcinoma | 49/248 | 20 |
| Tumor progression | | |
| No | 190/248 | 77 |
| Yes | 58/248 | 23 |
| Survival* | | |
| Alive | 210/248 | 85 |
| Dead | 38/248 | 15 |

*At 236 months. NA: Not available.

Table II. *Variables taken into account for the proportional hazards regression analysis (Cox model) including the rule used to discriminate, the number of women attributed to each group and the number we could not attribute (NA).*

| Variable | Rule | N | Yes | NA |
|---|---|---|---|---|
| Grade | At least grade 3 | 161 | 78 | 9 |
| Age | Older than 47.5 years | 124 | 124 | 0 |
| Tumor size | Tumor bigger than 2 cm | 111 | 136 | 1 |
| Nodal status | Tumor nodal infiltration | 149 | 97 | 2 |

Table III. *Proportional hazards regression analysis (COX model). Including regression coefficient (R. coef.), standard error (S. error), p-value and 95% confidence interval (CI) for the hazard ratio (lower and higher 0.95%).*

| Variable | R. coef. | S. error | Hazard ratio | 95% CI | *p*-Value |
|---|---|---|---|---|---|
| Mutant p53 | | | | | |
| Cytoplasmic | 0.3629 | 0.2774 | | 0.7283-3.0205- | 0.3942 |
| Nuclear | –1.0188 | 0.4561 | | 0.1477-0.8826 | 0.0255 |
| Tumor grade | 0.4900 | 0.3379 | | 0.8146-3.2707 | 0.3546 |
| Age | 0.6928 | 0.3610 | | 0.9826-4.0681 | 0.3624 |
| Tumor size | 1.3199 | 0.4761 | | 1.4721-9.5171 | 0.0056 |
| Nodal status | 0.4599 | 0.3420 | | 0.8103-3.0963 | 0.1786 |

## Results

*No difference in cytoplasmic expression of mutated p53 protein*. A total of 66% of the studied cervical cancer cases expressed the mutated p53 protein (*i.e.* more than 10% of the cancer cells stained) in the cytoplasm. We found 81 patients with no visible expression for mutated p53 protein; 17 had a score of 1; 20 scored 2; 12 scored 3; 67 scored 4; 16 scored 6; 23 scored 8; five scored 9; four scored 12 and three were non quantifiable. Differential expression of mutated p53 in the cytoplasm of human cervical cancer assessed by immunoperoxidase staining is presented in Figure 1a-f. The expression was highly variable, from no expression at all to very strong and diffuse expression. However, the Kaplan–Meier curves showed no difference in the survival of the two groups (Figure 1g). The 5-year survival rate for the group with no cytoplasmic expression of mutated p53 was 87.3%, comparable to the 91.2% survival rate for the group with cytoplasmic expression of mutated p53. The 10-year survival rate for the group with no mutated p53 cytoplasmic expression was similarly 83.8%, not significantly different from that of the group with cytoplasmic expression of mutated p53 (85.0%). Overall these results show no significant advantage of cytoplasmic expression of mutated p53 protein ($p$=0.718).

*Survival advantage associated with nuclear expression of mutated p53 protein*. A total of 42% of studied cervical cancer cases expressed mutated p53 protein (*i.e.* more than 10% of the cancer cells stained) in the nucleus. We found 141 patients with no visible expression for mutated p53 protein; 19 scored 1; 56 scored 2; four scored 3; 10 scored 4; eight scored 6; one scored 8; one scored 9; five scored 12 and three were non quantifiable. Differential expression of mutated p53 in the nucleus of human cervical cancer assessed by immunoperoxidase staining is presented in Figure 2a-d. The expression was again, highly variable, from no expression at all to very strong and diffuse expression. However, the Kaplan–Meier curves showed there to be a difference in the survival of the group expressing mutated p53 in the nucleus, as shown in Figure 2e. The 5-year survival rate in the group with no nuclear expression of mutated p53 was 88.0%, significantly reduced compared with that of the group expressing nuclear mutated p53 (92.8%). The 10-year survival rate was similarly reduced (81.7% versus 90.4%, respectively). Overall, these results show a significant advantage of nuclear expression of mutated p53 protein ($p$=0.024).

*Cox Proportional hazards regression analysis*. To confirm these results, we performed a multivariable survival analyses based on the Cox model. We estimated the effect of the nuclear expression of mutated p53 after adjustment for other explanatory variables available at the time of the surgery: higher grade as graded after pathological characterization, age higher than the median (*i.e.* 47.5 years) at the onset of disease, larger size of the resected tumor (2 cm and greater) and presence of infiltrated tumor cells in the local lymph node. All these factors correlated with higher risk of death as presented in Table II. The proportional hazards regression analysis gives a more precise idea of the influence of these variables, as presented in Table III. The grading of the tumor and the presence of infiltrated tumor cells in local lymph nodes did not remain significant factors ($p$=0.167 and $p$=0.179, respectively). Moreover, the effect of age greater than the median at the onset of disease was reduced to a trend ($p$=0.059). However, resected tumor size and nuclear expression of mutated p53 protein were both significant ($p$=0.005 and $p$=0.025, respectively). The regression coefficient for the nuclear expression of mutated p53 protein was found to be negative, consequent with a given survival advantage. Overall these results confirm a significant advantage of nuclear expression of the mutated p53 protein in cervical cancer.

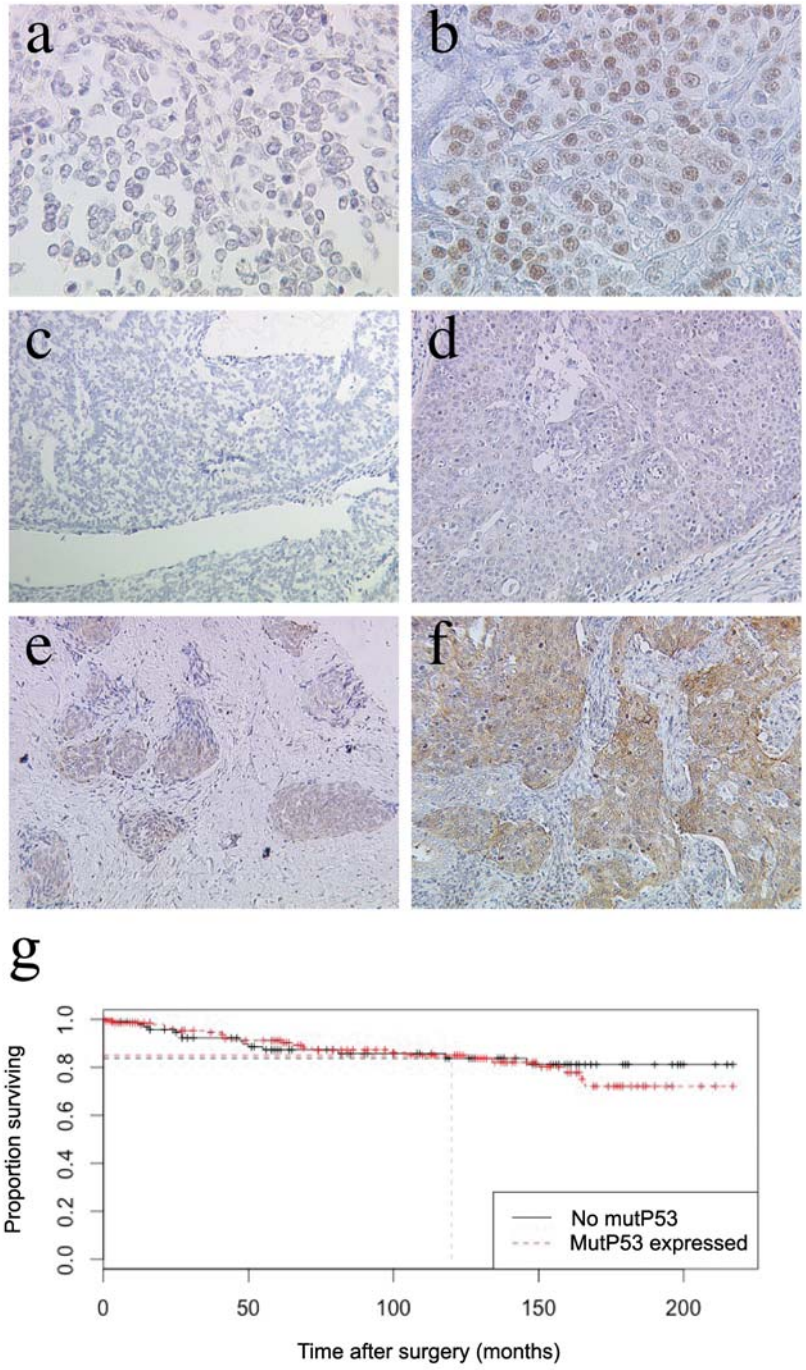


Figure 1. *Expression of mutated p53 in the cytoplasm of human cervical cancer tissue as assessed by immunoperoxidase staining: Negative control, IgG rabbit control antibody (breast cancer) (a); positive control (breast cancer); (b) no expression (c); low expression (d); intermediate expression (e); high expression (f). Magnification: a, b, ×200; c-f: ×80. g: Kaplan–Meier analysis of the overall survival of patients according to cytoplasmic expression of mutated p53.*

## Discussion

The results of this study give a good indication as to where to focus the search for new tumor markers in cervical cancer. The high expression of the mutated version of p53 and the difference observed in survival of patients was not expected by analyzing the relevant literature. Despite its central role of p53 in the hallmarks of cancer, *TP53* mutation and

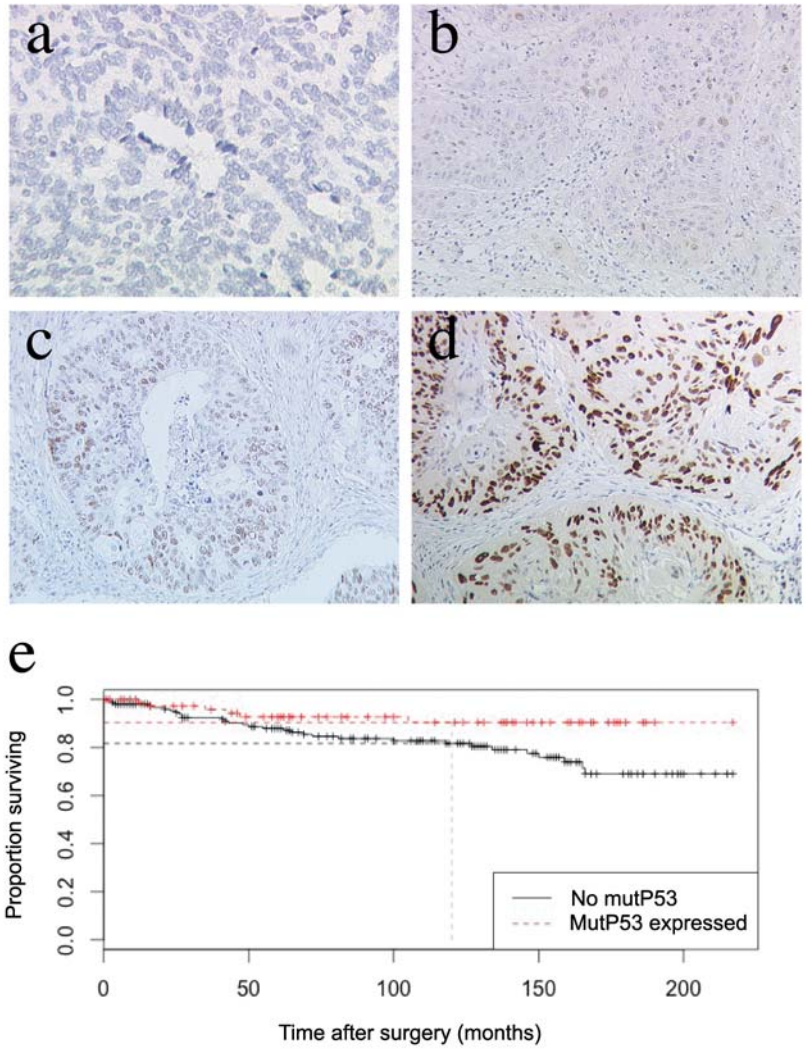


Figure 2. *Expression of mutated p53 in the nucleus of human cervical cancer tissue as assessed by immunoperoxidase staining: No expression (a); low expression (b); intermediate expression (c); high expression (d). Magnification: ×80. e: Kaplan–Meier analysis of the overall survival of patients according to nuclear expression of mutated p53. Survival advantage was shown for patients with tumor with nuclear expressed mutated p53 protein.*

expression status is not used for the prognosis of cervical cancer. Interestingly, we found a very high mutation rate of the *TP53* gene in a cancer type in which p53 is initially inactivated *via* the oncoprotein E6 during its development.

The *TP53* mutation is strongly associated with cervical cancer and was not detectable in normal tissues surrounding the tumor. The etiological role of infection with high-risk HPV in cervical cancer is well established (18). However, development of cancer, only occurs 10 or 20 years after the first infection and could be one explanation for the high rate of mutation associated with the pathology. Of all *TP53* mutations known, 90% are non-synonymous substitutions and concern single amino acid changes in the DNA-binding region (10). However, we herein analyzed one mutation occurring in the *N*-terminal part of p53, one of the two transcription activation domains of the protein involved in the regulation of several pro-apoptotic genes (17). In its normal functions, p53 senses DNA damage and can arrest growth by holding the cell cycle at the $G_1$/S regulation point on DNA damage recognition, or initiate apoptosis if DNA damage proves to be irreparable (11, 19-21).

The antibody we used detects a mutation at position 20 (serine into aspartic acid), which abolishes one phosphorylation site (Abcam personal communication). The phosphorylation of this serine on detection of DNA damage weakens the interaction of p53 with mouse double minute 2 (MDM2), also known as E3 ubiquitin protein ligase, thereby stabilizing p53 (22-24). Thus, the mutation abolishes the

normal increase in p53 protein level after DNA damage. Overall, the mutation was detected in the cytoplasm of 66% of cancer cases and in 42% in the nucleus. Since this mutation alters the cell's ability to stop the cell cycle on DNA damage recognition, it could work synergistically with E6 to reduce the ability of p53 to activate responsive promoter elements. Together, these two blockades could lead to mutation accumulation and, ultimately, to cervical cancer. Interestingly, MDM2 as an E3 ubiquitin protein ligase could also be directly targeted by E6 protein of HPV (7). MDM2 is also linked with increased risk of cancer and potential of treatment (25-27).

In accordance with its role as a transcription factor, we found mutated p53 had an influence on survival only if expressed in the nucleus. An unexpected finding is the correlation of *TP53* mutation alone with a better survival. *TP53* mutation is often observed in cancer. However, it is commonly associated with severely compromised tumor suppression due to an increased likelihood for uncontrolled cell division (28). One limitation of this study is the absence of information about other possible simultaneous *TP53* mutations, possibly destroying the ability of the protein to bind to its target DNA sequences, and thus preventing transcriptional activation of genes. However, we observed a significant effect of mutated p53 on patient survival, thus we assume that other mutations are less important. As a matter of fact, the capacity of p53 to bind to DNA promoters is of less importance if the protein stays bound to MDM2 and is therefore degraded. It remains to be assessed whether the milder prognosis associated with several mutants may be ascribed to specific functional properties and directly correlated to E6 protein expressed after HPV infection.

Patients included in this study were treated at our Institute and received radiotherapy targeting the DNA of tumor cells. The absence of an increase of p53 protein level after DNA damage due to mutation could be an advantage for tumorigenesis, since it would simplify the transmission of small nonsynonymous mutation; but it should also be a disadvantage in the case of great DNA damage such as that caused by radiotherapy, since the cell cycle would not be stopped and DNA would not be repaired, ultimately leading to more cell death. Indeed, phosphorylation of p53 at Ser-20 seems to be involved in cell radiosensitivity (29). That this kind of mutation often occurs in cervical cancer might be due to the constant E6-dependant p53 deactivation, making tumorigenesis less dependent on a dominant-negative mutation.

In summary, we found a very high mutation rate of the *TP53* gene in cervical cancer. According to this finding the overall survival was better in patients expressing the mutated p53 protein in the nucleus. Our results indicate that p53 mutation might serve as a useful biomarker to predict response to therapy.

## Conflicts of Interest

The Authors declare no conflict of interest exists in regard to this study.

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