

ORIGINAL RESEARCH

p53 protein expression, *TP53* gene mutations, and *in silico* p53 activity as prognostic markers for ovarian cancer

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(Estrid Høgdall)**Abstract**

Background: The frequency of alterations in the tumor protein p53 (*TP53*) gene varies among different cancers. Data suggest an overall occurrence rate of 47.8% of *TP53* gene alterations in ovarian cancer (OC). This study aimed to investigate the prognostic impact of p53 protein expression, *TP53* gene mutations, and *in silico* prediction of p53 activity. **Methods:** Blood and tissue samples from 163 OC cases from the Danish Pelvic Mass/Gynecologic Ovarian Vulva Endometrial and Cervix cancer (GOVEC) study were collected through the Danish CancerBiobank (Bio- and GenomeBank, Denmark). We used immunohistochemistry (IHC) to measure p53 protein expression and sequencing, to detect *TP53* mutations, and *in silico* prediction to estimate p53 activity level. **Results:** p53 protein expression was found to have no prognostic value for overall survival (OS): $\geq 1\%$: $p = 0.09$; $\geq 10\%$: $p = 0.71$. Patients with missense mutations had a shorter OS with a hazard ratio (HR) of 1.80 (95% confidence interval (CI) 1.06–3.04, $p = 0.04$), while patients with *TP53* termination mutation had an even shorter OS with an HR of 2.31 (95% CI 1.13–4.73, $p = 0.02$). Tissue from 11 patients with positive p53 expression ($\geq 10\%$) did not have a *TP53* mutation in the tumour tissue. The percentage of p53 protein expression positivity ranged from 10% to 80%, with only four samples showing p53 expression positivity of more than 30%. *In silico* p53 activity was found to have no independent prognostic value. **Conclusions:** p53 negative tissue may be optimally subjected to *TP53* sequencing. *TP53* termination mutations may represent a subgroup of patients with significantly shorter survival and may therefore be considered for individualized follow-up programs.

KeywordsOvarian cancer (OC); p53; *TP53*; Overall survival (OS), neoplasm proteins

1. Introduction

Ovarian cancer (OC) is the 8th most common cancer among women worldwide, with an incidence of 324,398 cases in 2022. According to Global Cancer Observatory (GLOBOCAN), the yearly mortality rate of OC is 207,252 deaths, making it one of the most lethal gynecological malignancies [1]. The main reason for the high mortality is the number of patients diagnosed at advanced stages of the disease, where radical surgery may not be an option. Additionally, chemotherapy resistance is a significant issue, with around one-third of patients not responding to platin-based chemotherapy [2].

For patients diagnosed with stage I OC undergoing surgery, the 5-year survival rate is approximately 85%, compared to 29% for patients diagnosed at stage IIIc–IV, according to the International Federation of Gynecology and Obstetrics (FIGO) [3, 4]. These findings emphasize the need for a better understanding of OCs and the underlying molecular mechanism to improve diagnostics and treatment, which is

reflected in improved survival.

The tumor protein p53, discovered in 1979, has been routinely measured in clinical and research settings in human cancer. The p53 protein is encoded by the *TP53* gene, located on the human chromosome 17p13.1. The p53 plays a crucial role in maintaining cellular and tissue homeostasis in response to cellular stress. Furthermore, p53 can act as a direct transcription activator and repressor of several hundred genes [5]. p53 also plays an important role in angiogenesis, DNA damage response, oncogene activation, and the prevention of aneuploidy propagation [6]. *TP53* gene mutations are found in approximately 50% of all human cancers and are therefore suggested to have a clinical impact on the patient's prognosis and treatment [7–10]. The frequency of alterations in the *TP53* gene varies among different cancers, and data have suggested an overall mutation rate of 47.8% in OC, compared to only 6% in cervical carcinomas [11]. Mutations in the *TP53* gene can occur throughout the entire gene sequence. However, around 80% of mutations are found in the DNA-binding domain [11–

14]. *TP53* gene mutations occur more frequently in advanced FIGO stages as compared to early FIGO stages [15]. Some studies have associated *TP53* gene mutations with patient outcomes, but findings remain inconsistent [10, 16–20].

Alterations in p53 may be detected through the expression levels of the p53 protein or by mutations in the *TP53* gene. The *TP53* gene mutations can be characterized as missense mutations (including point mutations and in-frame deletions/insertions), termination mutations (nonsense mutations), frameshift deletion/insertion mutations, or disulfide (Cysteine) functional mutations [20]. In clinical settings, p53 protein expression assessed through immunohistochemistry (IHC) is considered a surrogate marker for *TP53* mutations [18, 21, 22]. To our knowledge, no studies reported whether *in silico* p53 activity levels may have any prognostic impact in patients diagnosed with ovarian cancer.

This study aimed to investigate the prognostic impact of p53 protein expression, *TP53* gene mutations, and *in silico* p53 activity levels. Additionally, the study aimed to compare the concordance between *TP53* mutations and p53 protein expression.

2. Material and methods

2.1 Study participants

The patients were included from the Gynaecologic Clinic at Rigshospitalet, Denmark, due to a pelvic mass or pelvic pain potentially caused by OC. All patients included in this study were histopathologically diagnosed as OC by a pathologist specialized in gynaecologic oncology. All patients were registered in the Danish Gynaecologic Cancer Database (DGCD), which is a Danish compulsory research and quality online database. Oral and written informed consent was signed by each patient before inclusion in the Pelvic mass/GOVEC study.

Patients were examined according to the Danish Cancer Fast Track Guidelines, including gynaecological examination, measurement of serum Cancer Antigen125 (CA125), ultrasound examination, calculation of Risk of Malignancy Index (RMI), Positron Emissions Tomography/Computed Tomography (PET/CT) scanning, and evaluation of performance score. Subsequently, each patient was discussed at Multidisciplinary Team conferences, and a primary treatment strategy was planned. All patients underwent surgery conducted by a specialist in gynecologic oncology, and all patients were candidates for primary surgery. All diagnoses were confirmed by a pathologist specialized in gynaecological pathology.

All patient data were continuously registered online in the Danish Gynecologic Cancer Database (DGCD) by gynecologists, pathologists, and oncologists. The OC was staged according to the FIGO classification.

Blood and tissue samples from 163 OC cases from the Danish Pelvic Mass/GOVEC study were included and obtained from the Danish CancerBiobank (Bio- and GenomeBank, Denmark) [23]. Briefly, all blood samples were collected less than 2 weeks before surgery. The samples were centrifuged at 2000×g for 10 min at room temperature and fractionated into serum aliquots of approximately 0.5 mL, which were stored at

−80 °C on the day of collection. The handling of blood samples from sampling to freezing was time-stamped and recorded in the database to ensure adherence to the protocol. The tissue samples were collected as part of routine clinical procedure, representing the clinical diagnosis. The samples were handled in accordance with biobank guidelines, with standardized time stamping from samples withdrawn to storage at −80 °C. Tissue-handling was completed within 1 hour of tissue procurement, according to standard procedure. Fresh frozen tissue was stored at −80 °C, and formalin-fixed, paraffin-embedded (FFPE) tissue was stored at room temperature [23].

2.2 Analyses

2.2.1 Tissue microarray (TMA)

FFPE tissue from primary surgery was used for the TMA production. Two representative areas were marked on a freshly cut Hematoxylin and Eosin-stained tissue section from each selected block. Tissue cylinders with a diameter of 2 mm were punched from corresponding areas in the donor tumour block and placed into 2–4 individual recipient paraffin blocks (TMA blocks), using a custom-made manual Tissue Array Instrument (MTA-1, Beecher Instruments, Silver Spring, MD, USA) [24]. Once the cores were placed into the recipient block, the paraffin was slightly melted in an oven at 60 °C for 10 minutes to bind the cores into the block. A total of 6 TMAs, consisting of 2 cores of corresponding viable and representative tissues from each OC case, were constructed to minimize the risk of intra-tumour variability. Four cores of control tissue (two kidney and two liver) were strategically placed in each TMA to ensure a unique orientation of every TMA. A maximum of 31 different patient tumour samples were placed on each TMA.

2.2.2 p53 IHC

For the IHC staining, 2 µm sections from the TMA blocks were transferred to glass slides (DAKO Chem-Mate Capillary Cap Microscope Slides, 57 mm, DAKO A/S, Glostrup, Denmark) using the Section Transfer System, STS, Ergostar HM200 (MICROM International GmbH, Walldorf, BW, Germany). The slides were stored at 4 °C for a maximum of 8 days until IHC p53 staining. Immediately before staining, the sections were deparaffinized in xylene and rehydrated in graded dilutions of alcohol. To demask antigens within the tissue, the sections were pretreated in TEG Buffer pH 9.0 (Tris 10 mmol/L and EGTA 0.5 mmol/L) by heating in a microwave oven to 98 °C for 15 minutes, and allowing them to cool by leaving them in the buffer at room temperature for an additional 15 minutes. The staining for p53 protein expression was carried out using the monoclonal antibody anti-human p53 Protein (Clone DO-7, code No. M7001, DAKO A/S, Glostrup, Denmark), diluted 1:400 for 1 hour at room temperature. The staining procedure was performed on a DAKO TechMate™ 500^{PLUS} Instrument (DAKO A/S, Glostrup, Denmark), according to the manufacturer's instructions. Scoring for p53 expression was based on the proportion of tumor cells exhibiting distinct nuclear immunopositivity. Two different cut-offs for p53 positivity were used, ≥1% and ≥10%. Representative photomicrographs of tumour tissue showing positive and negative staining for the

specific antigen are presented in Fig. 1.

2.2.3 TP53 mutation detection

DNA extraction was carried out using a Promega Maxwell® RSC instrument (Promega Corporation, Madison, WI, USA) and the Maxwell® RSC DNA FFPE kit (AS1450) according to the manufacturer's instructions. The DNA concentration was measured using a Nanodrop® 2000 (Thermo Fisher Scientific, Waltham, MA, USA) and diluted to 5 ng/μL. Sequencing of *TP53* was conducted using Next Generation Sequencing (NGS) with a customized gene Ion AmpliSeq™ *TP53* Panel, including a panel of two pools with 24 amplicons designed to analyze all coding exons of the *TP53* gene. In brief, 10 ng genomic DNA was amplified with a pre-mixed primer pool and Ion AmpliSeq™ HiFi master mix Ion AmpliSeq™ Library Kit 2.0 (4480441, Life Technologies, San Jose, CA, USA) for 2 min at 99 °C, followed by 22 cycles of 99 °C for 15 s and 60 °C for 4 min, and subsequently maintained at 10 °C. Polymerase chain reaction (PCR) amplicons were treated with 2 μL FuPa reagent at 50 °C for 10 min, followed by 55 °C for 10 min, then 60 °C for 20 min. Amplicons were ligated to adapters with the diluted barcodes of the Ion Xpress™ Barcode Adapters kit (4474517, Thermo Fisher Scientific, Waltham, MA, USA) for 30 min at 22 °C, then 72 °C for 10 min. Adaptor-ligated amplicon libraries were purified using Agencourt® AMPure® XP reagents (A63880, Ramcon, Birkerød, Denmark). The library concentration was adjusted to 100 pM using an Ion Library TaqMan™ Quantitation Kit (4468802, Thermo Fisher Scientific, Waltham, MA, USA). Emulsion PCR was performed on the final library using the Ion One-Touch™ System and Ion PGM™ Template OneTouch™ 200 Kit (4480974, Thermo Fisher Scientific, Waltham, MA, USA) and Template-positive Ion Sphere™ Particles were enriched with Dynabeads® MyOne™ Streptavidin C1 Beads, according to the manufacturer's instructions using an Ion OneTouch™ ES system (4469495, Thermo Fisher Scientific, Waltham, MA, USA). Purified Ion Sphere particles were loaded on an Ion 318 Chip v.2 (4484354, Thermo Fisher Scientific, Waltham, MA, USA). Finally, massive parallel sequencing was conducted on a Personal Genome Machine (PGM) sequencer (Ion Torrent™,

Thermo Fisher Scientific, Waltham, MA, USA) using the Ion PGM Sequencing 200 Kit version 2 (4482006, Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions.

The sequencing quality and data output were evaluated by two experienced molecular biologists. This involved assessing statistics and quality metrics for the specific sequencing on the Ion Torrent. The Ion Torrent Suite Coverage Analysis was used to evaluate the quality of each library. The Hg19 reference genome was used to align the sequences using the Ion Torrent Suite Variant caller. Variants including single nucleotide variant (SNV), insertions/deletions (INDELs), and multi nucleotide variant (MNV) were annotated, and their graphical representation was visualized using the software Integrative Genomics Viewer (IGV). A minor allele frequency (MAF) threshold of 5% and a reading depth of >100× were used when considering a mutation.

2.2.4 p53 activity

The annotation level of p53 activity following *TP53* gene mutation was performed *in silico* using the *TP53* mutant loss of activity database (TP53MutLoad) at the website <https://www.p53.fr/>. The analysis was based on the transactivation of wild-type p53-activated fragment 1 (WAF1) in combination with published recommendations for analyzing and reporting *TP53* gene variants. Data for WAF residual transcriptional activity of mutant p53 on the WAF1 promoter (% compared to wild-type p53) were taken from the publication by Kato *et al.* [25]. For nonsense variants and truncated p53, activity was assumed to be nil ($p53_{mut0}$), residual activity <20 was classified as mutant p53 ($p53_{muta}$), and ≥20 as classified as wild-type p53 ($p53_{wt}$) [25, 26].

2.2.5 Statistics

Statistical comparisons between groups were carried out using the Chi-Square test for categorical data and Rank-Sum tests for continuous variables. Survival curves were estimated using the Kaplan-Meier method. The impact of p53 expression, *TP53* mutation, and *in silico* p53 activity on survival was estimated using the Cox proportional hazards model [17]. Multivariate

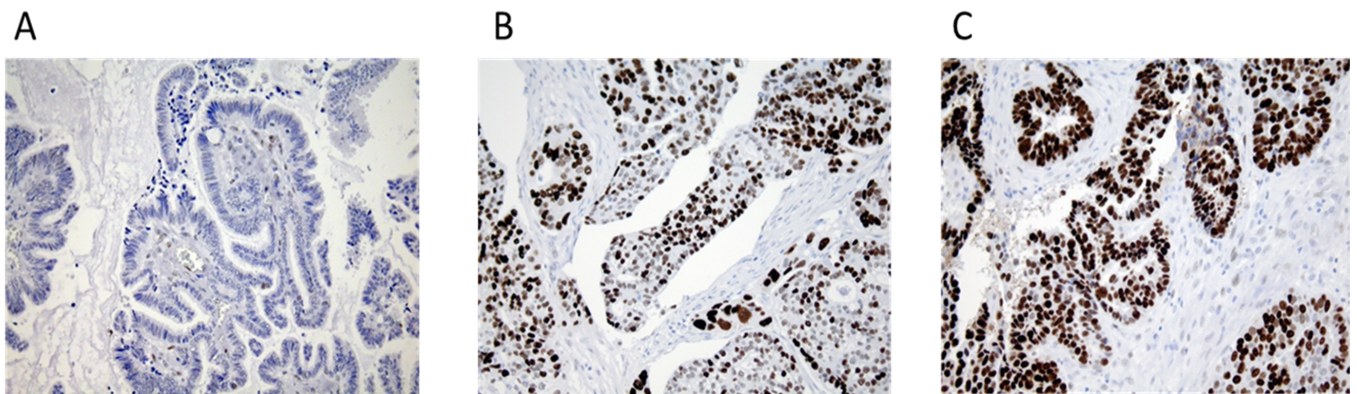


FIGURE 1. Examples of p53 immunohistochemical staining of tumor tissue. (A) Tissue showing p53 negative staining in a highly differentiated serous adenocarcinoma, FIGO stage III (0%) (objective ×40). (B) Tissue showing 60% p53 overexpression in a poorly differentiated serous adenocarcinoma, FIGO stage III (objective ×40). (C) Tissue showing 100% p53 overexpression in a poorly differentiated serous adenocarcinoma, FIGO stage III (objective ×40).

analysis, adjusting for known clinical covariates, were performed. Interaction tests between the covariates of interest, that is p53 activity as well as *TP53* mutation, and the clinical covariates were also done. For p53 protein expression, two cut-offs of $\geq 1\%$ and $\geq 10\%$ were used to designate positivity or negativity of staining. For p53 activity, two different scales were used. In one scale, the p53 activity was divided into three groups ($p53_{wt}$, $p53_{muta}$ with activity >0 , and $p53_{mut0}$ with activity $=0$), and in another scale, p53 activity was divided into three groups using the percentiles: lower ($p53_{lower}$ activity $=0$); median ($p53_{median}$ activity above 0 and below 8.7), and upper ($p53_{upper}$ activity ≥ 8.7).

For *TP53* mutational status, a five-tiered scale was used (*TP53* wild-type (WT), missense *TP53* mutation, *TP53* termination mutation, in-frameshift *TP53* mutation, and *TP53* functional mutation). The assumptions of proportionality and linearity for the Cox model were assessed using martingale residuals as well as graphical methods. The analyses were performed using SAS (v9.4, SAS Institute, Cary, NC, USA) and SPSS 11.5 Statistical Software (IBM, New York, NY, USA). *p*-values less than 5% were considered statistically significant.

3. Results

The characteristics of the patients enrolled in the study are described in Table 1.

A total of 163 patients with data on overall survival (OS) were included in this study. Of these, 121 (74%) patients were deceased, and 42 (26%) patients were still alive at follow-up. The median follow-up, calculated using the reverse Kaplan-Meier method, was 48 months, ranging from 0.6 to 189 months. The median age of the patients was 64 years, with an age range of 31 to 88 years and an interquartile range (IQR) of 55 to 73 years. Among all the patients included, 81% (131 patients) were diagnosed with a serous adenocarcinoma; of which 128 were high-grade serous adenocarcinoma (HGSC). Additionally, 72% (117 patients) were diagnosed at FIGO stage III or IV at the time of diagnosis (Table 1).

3.1 p53 protein expression

In our study, using a cut-off of $\geq 1\%$ for positivity, 111 samples (70%) were described as positive for p53 tissue expression. Using a 10% as cutoff, a total of 92 samples (58%) showed positive p53 tissue expression. Fig. 1 provides examples of p53 protein expression in tissue. Among the positive samples, 24 patients (15%) had 100% positivity for p53 tissue protein expression.

However, in our univariate analysis, we found that p53 tissue protein expression using both cut-offs ($\geq 1\%$ or $\geq 10\%$) did not demonstrate any prognostic value, neither reflected in OS, respectively ($\geq 1\%$: OS: $p = 0.09$; $\geq 10\%$: $p = 0.71$).

3.2 *TP53* mutational status

The results of *TP53* mutational status are presented in Table 1. A total of 163 patient samples were analyzed. Among these, 40 samples were *TP53* WT, 53 samples had a missense *TP53* mutation, 15 had a *TP53* termination mutation, 33 samples

TABLE 1. Characteristic of the study cohort (N = 163).

Variables	Values
Age (median/range) yr	64 (31–88)
FIGO stage	
I	28
II	18
III	95
IV	22
Histology	
HGSC	128
LGSC	3
Other	32
Performance score	
0	76
1	60
2	17
3	3
Missing	7
Overall survival	
Dead	121
Alive	42
<i>TP53</i> status	
WT	40
Missense	53
Termination	15
Frameshift	33
F (Cys-disulfide)	20
Del in-frame	2
p53 tissue protein expression	
0%/ $<10\%$	49/68
$>1\%$ / $>10\%$	111/92
Missing	3/3
p53 activity percentile-scale	
WT	40
0	67
>0 to <8.7	21
≥ 8.7	31
Missing	4

FIGO: International Federation of Gynaecology and Obstetrics; *WT*: Wild-type; *HGSC*: High Grade Serous Carcinoma; *LGSC*: Low Grade serous Carcinoma.

showed a frameshift mutation in *TP53*, 2 samples had in-frame deletions, and 20 samples had a functional *TP53* mutation (change in the nucleotide sequencing coding for a cysteine amino acid).

WT *TP53* was found in both early-stage OC (FIGO stage I/II) and late-stage OC (FIGO stage III/IV) with an over-

all frequency of 25% (40/163). *TP53* mutations were more frequently found in late-stage OC, regardless of the type of mutation. Specifically, the frequency of missense mutation was 28% in FIGO stage I/II (15/53 pts), compared to 72% in FIGO stage III/IV (38/53); Termination mutations were found in 20% of FIGO stage I/II (3/15) compared to 80% in FIGO stage III/IV (12/15); frameshift mutations were present in 21% of FIGO stage I/II (7/33) compared to 79% in FIGO stage III/IV (26/33); functional mutations were identified in 15% of FIGO stage I/II (3/20) compared to 85% in FIGO stage III/IV (17/20) and the two patients with identified deletions in-frame were both diagnosed with FIGO stage III disease.

Kaplan-Meier analyses, which included patient information of *TP53* mutational status, reveal differences, although not statistically significant in OS between patients with *TP53* WT and those with identified *TP53* mutations (Fig. 2).

Univariate Cox regression was performed to study the impact of mutational *TP53* status on OS, using *TP53* WT as the baseline. The analysis revealed that patients with missense mutations had a shorter OS with an HR of 1.80 (95% CI 1.06–3.04, $p = 0.04$), while patients with *TP53* termination mutation had an even shorter OS with an HR of 2.31 (95% CI 1.13–4.73, $p = 0.02$). No significant OS differences were observed for

other mutational changes (frameshift mutation and functional mutation) compared to patients with WT survival ($p = 0.06$ and $p = 0.08$, respectively). Multivariate Cox regression analyses, including age, histology, stage, and mutational status, showed sequencing status of no independent prognostic value (Table 2). Tests for interaction between *TP53* mutation were done for PS ($p = 0.23$) and age ($p = 0.65$). Tests for interaction were not performed for FIGO stage and histology due to very low sample sizes for FIGO stage I/II and non-serous histology.

3.3 *TP53* mutation and IHC

Using the cut-off of $\geq 1\%$ for positivity of p53 expression, 30 patients with negative p53 expression were found to have a *TP53* mutation in the tumour tissue. The serous subtype was the predominant histological subtype, accounting for 28 cases, while the remaining two cases were an endometrioid and one mucinous adenocarcinoma. Most mutations ($N = 23$) were found in the DNA-binding domain, 4 samples had a mutation in the transactivation domain, and 3 samples had a mutation in the nuclear localization domain. Regarding mutation type, termination mutations were seen in 12 samples, frameshift mutations in 15 samples, and missense mutations in 3 samples

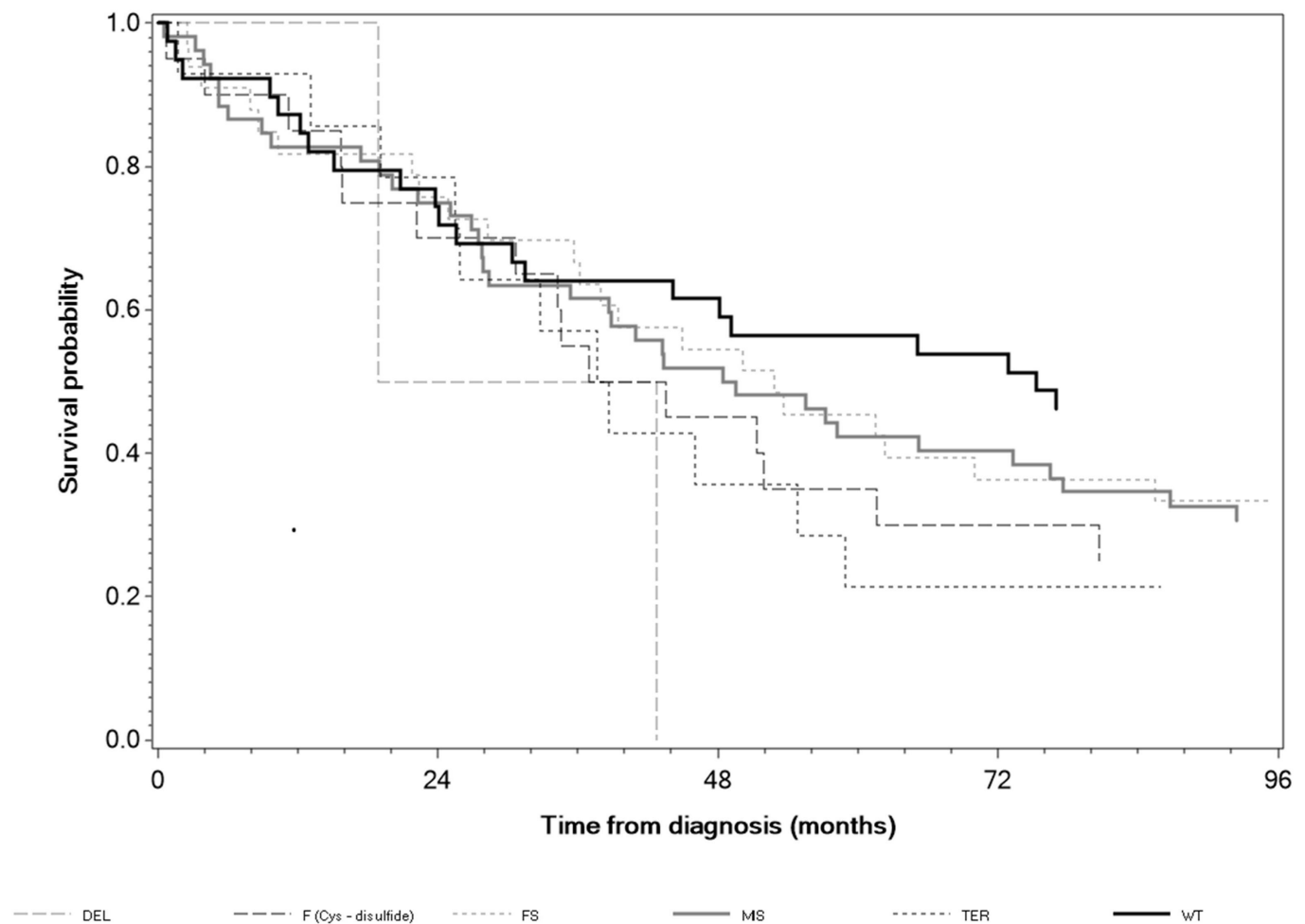


FIGURE 2. Kaplan-Meier overall survival curves for patients being *TP53* wild-type (WT) and *TP53* mutated. ($N = 163$, log rank $p = 0.10$ for F (Cys-disulfide), $p = 0.08$ for FS, $p = 0.039$ for missense, $p = 0.015$ for termination, all versus WT). Note that for deletion, $n = 2$. DEL: Deletion; FS: Frameshift; MS: Missense; TER: Termination.

TABLE 2. Multivariate Cox regression analyses of *TP53* sequencing status and clinical information.

	Hazard Ratio	95% CI	<i>p</i> -value
<i>TP53</i> sekvens_status WT	1.00		
Sekvens_status DEL	2.06	0.46–9.24	0.3436
Sekvens_status F (Cys-disulfide)	1.12	0.56–2.24	0.7531
Sekvens_status FS	0.87	0.46–1.64	0.6649
Sekvens_status MIS	0.76	0.42–1.37	0.3571
Sekvens_status TER	1.27	0.60–2.67	0.5370
FIGO stage I	1.00		
FIGO Stage II	2.00	0.81–4.93	0.1319
FIGO Stage III	4.23	1.98–9.06	0.0002
FIGO Stage IV	3.44	1.40–8.41	0.0069
Histology Serous	1.00		
Histology Non-serous	0.69	0.33–1.46	0.3336
Performance score 0	1.00		
Performance score >0	1.76	1.20–2.59	0.0042
Age (10 years difference)	1.25	1.06–1.48	0.0090

FIGO: International Federation of Gynaecology and Obstetrics; WT: Wild-type; DEL: Deletion; FS: frame-shift; MIS: Missense; TER: Termination; CI: confidence interval.

(Table 3).

Tissue from 11 patients with positive p53 expression ($\geq 10\%$) did not have a *TP53* mutation in the tumour tissue. Among these samples, 5 were serous adenocarcinomas, 4 were clear cell carcinomas, 1 was an endometrioid adenocarcinoma, and 1 was a mucinous adenocarcinoma. The percentage of p53 protein expression positivity ranged from 10% to 80%, with only four samples having p53 expression positivity of 30% or more (Table 4).

3.4 *In silico* predicted *TP53* activity

In this study, 40 patients had wild-type *TP53* ($p53_{wt}$), while 123 patients had mutated *TP53* in the tumour tissue. p53 activity was divided into three groups: $p53_{wt}$, $p53_{muta}$ activity >0 , and $p53_{mut0}$ activity $=0$.

The Kaplan-Meier estimates show a difference between WT and the three groups of p53 activity and OS (Fig. 3).

In a univariate Cox regression analysis, it was confirmed that patients with $p53_{mut0}$ activity $=0$ had a significantly decreased OS (HR of 1.94, 95% CI 1.16–3.23, $p = 0.0012$), whereas there was no significant decrease in survival of patients with $p53_{muta}$ activity >0 (HR of 1.78, 95% CI 0.94–3.37, $p = 0.108$) compared to patients with $p53_{wt}$.

Dividing the p53 activity into three other groups using a percentile scale: lower ($p53_{lower} = 0$), median ($p53_{median}$ activity above 0 and below 8.7), and upper ($p53_{upper}$ activity ≥ 8.7) percentile, showed patients with p53 activity $=0$ had a significantly lower OS (Table 5).

Additionally, a multivariate Cox regression analysis was conducted, which included p53 activity (percentile scale), FIGO stage (I, II, III, and IV), histological subtype (serous vs. other), performance status (PS: 1–4), and age (years). This analysis revealed that p53 activity (percentile scale) and

histological subtype of cancer did not independently impact prognosis. On the other hand, FIGO stage, PS, and age were all found to have independent prognostic significance (Table 6). Tests for interaction were done between p53 activity and PS ($p = 0.21$) and age ($p = 0.83$). As above, tests for interaction were not done for FIGO stage and histology.

4. Discussion

In this study, we investigated the prognostic impact of p53 protein expression, *TP53* gene mutations, and the level of *in silico* estimated p53 activity in patients diagnosed with epithelial OC. We also examined the correlation between p53 protein expression and the *TP53* mutations identified through DNA sequencing.

The study cohort consisted of patients consecutively included in the Pelvic Mass/GOVEC study and representing real-world patients. The characteristics of the study cohort are similar to those reported in the Nationwide Danish registry [3, 4].

We observed an overall p53 tissue protein expression in 70% of tumor tissues using a cut-off value of $\geq 1\%$. This is in accordance with other studies that have reported frequencies ranging from 48% to 70% [27–29]. The reported differences in frequency may reflect the heterogeneity of the disease, the specific antibody used, and the cut-off for p53 protein expression, as well as the number of different subtypes included in the studies, as p53 expression is predominantly presented in HGSC.

Several studies have investigated the impact of *TP53* mutations, but their conclusions have been inconsistent [17, 18, 29]. In our study, we investigated the impact of *TP53* mutations by analyzing the different type of mutation identified as well as their locations within different domains of the *TP53* gene.

TABLE 3. Samples with negative p53 expression but harboring *TP53* mutation (N = 30).

Histological type	FIGO stage	p53_IHC (%)	Functional domain	Amino Acid	Allel frequency (%)	Nucleotide change	Sequence status
HGSC	3	0	Transactivation	p.Asp42fs	70.5	c.123_124insAA	FS
HGSC	3	0	Transactivation	p.Glu56fs	70.9	c.166delG	FS
HGSC	3	0	Transactivation	p.Trp91Ter	53.1	c.273G>A	TER
HGSC	3	0	Transactivation	p.Leu93fs	76.0	c.278delT	FS
HGSC	1	0	DNA binding	p.Ser94Ter	76.4	c.281C>A	TER
HGSC	3	0	DNA binding	p.Thr102fs	77.3	c.304_305insA	FS
Endometrioid	1	0	DNA binding	p.Cys124fs	91.1	c.370_371insT	FS
HGSC	4	0	DNA binding	p.Gln144Ter	82.2	c.430C>T	TER
Mucinous	1	0	DNA binding	p.Pro153fs	53.5	c.455_456insC	FS
HGSC	3	0	DNA binding	p.Pro153fs	71.8	c.455_456insC	FS
HGSC	1	0	DNA binding	p.Val172fs	63.2	c.514delG	FS
HGSC	4	0	DNA binding	p.His179Gln	49.2	c.537T>A	MIS
HGSC	1	0	DNA binding	p.Pro190fs	100.0	c.569delC	FS
HGSC	1	0	DNA binding	p.Gln192Ter	76.9	c.574C>T	TER
HGSC	3	0	DNA binding	p.Ile195fs	51.1	c.582delT	FS
HGSC	3	0	DNA binding	p.Arg196Ter	83.5	c.586C>T	TER
HGSC	3	0	DNA binding	p.Arg213Ter	64.9	c.637C>T	TER
HGSC	3	0	DNA binding	p.Glu224Ter	81.4	c.670G>T	TER
HGSC	3	0	DNA binding	p.Glu224Ter	76.3	c.670G>T	TER
HGSC	3	0	DNA binding	p.Asp228fs	32.6	c.681_682insTT	FS
HGSC	4	0	DNA binding	p.Gly244Cys	64.1	c.730G>T	MIS
HGSC	3	0	DNA binding	p.Ile254fs	46.6	c.760_761insCCTCACCA	FS
HGSC	2	0	DNA binding	p.Asp259Tyr	26.4	c.775G>T	MIS
HGSC	3	0	DNA binding	p.Gly262fs	57.8	c.783_784delTG	FS
HGSC	4	0	DNA binding	p.Leu264fs	76.6	c.790delC	FS
HGSC	3	0	DNA binding	p.Glu286fs	75.4	c.857_858delAA	FS
HGSC	3	0	DNA binding	p.Glu286Ter	50.6	c.855_856insT	TER
HGSC	3	0	Nuclear localization	p.Glu294Ter	78.7	c.880G>T	TER
HGSC	3	0	Nuclear localization	p.Glu294Ter	55.1	c.880G>T	TER
HGSC	3	0	Nuclear localization	p.Arg306Ter	91.5	c.916C>T	TER

FIGO: International Federation of Gynaecology and Obstetrics; IHC: immunohistochemistry; HGSC: High Grade Serous Carcinoma; FS: Frameshift; TER: Termination, MIS: Missense mutation.

TABLE 4. Samples with positive p53 expression ($\geq 10\%$) with *TP53* WT (N = 11).

Histological type	Histological grade	Stage	p53_IHC procent	Amino Acid
Serous	1	1	10	WT
Serous	2	3	10	WT
Serous	2	3	30	WT
Serous	3	3	70	WT
Serous	3	4	80	WT
Clear cell	2	3	10	WT
Clear cell	3	3	10	WT
Clear cell	2	1	10	WT
Clear cell	2	1	50	WT
Mucinous	1	3	10	WT
Endometrioid	1	1	10	WT

IHC: immunohistochemistry; WT: Wild-type.

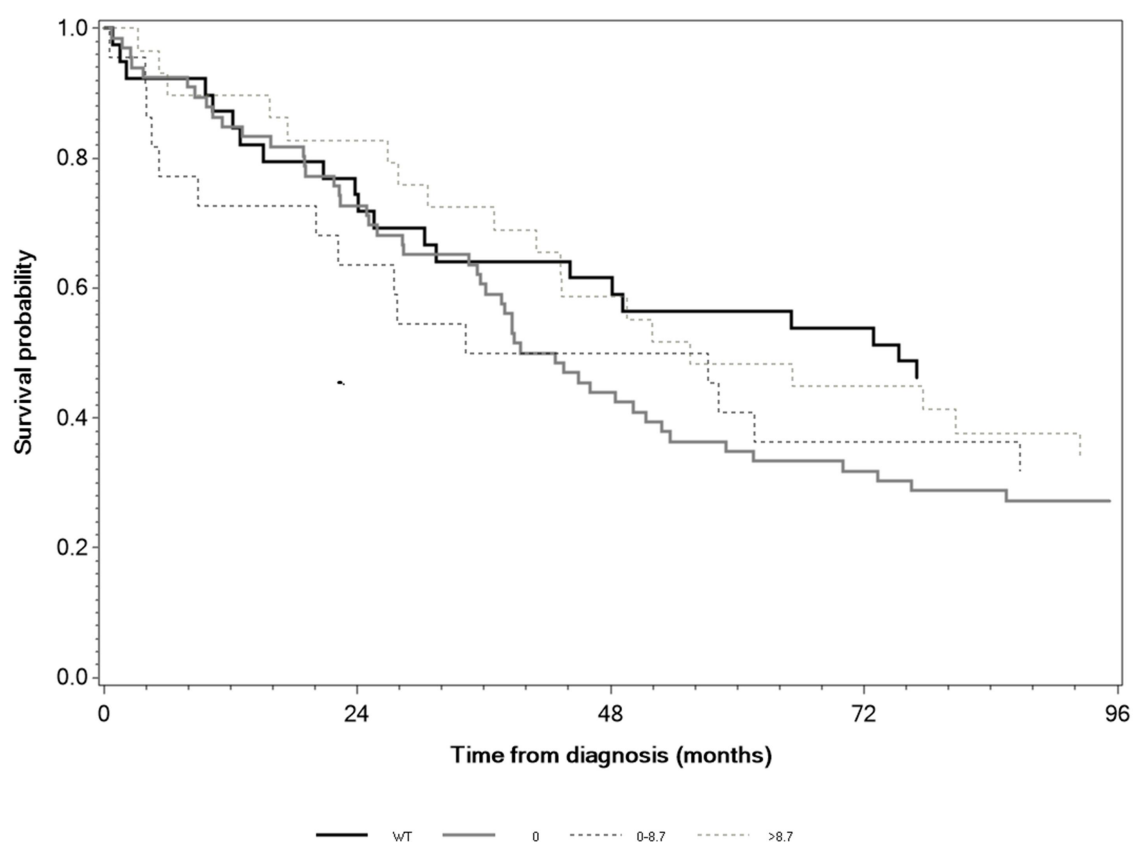


FIGURE 3. Kaplan-Meier analyses of p53 activity. (N = 163, log rank $p = 0.01$ for 0 activity, $p = 0.10$ for activity >0 up to 8.7, $p = 0.18$ for activity >8.7 , all versus WT). The number of samples in each stratum is given in Table 1. WT: Wild-type.

TABLE 5. Univariate Cox regression analyses of p53 activity using the percentile scale.

Overall survival	Hazard Ratio	95% CI	p -value
p53 Activity WT	1.00		
p53 _{lower} : Activity = 0	1.91	1.16–3.16	0.012
p53 _{median} : Activity 0–8.7	1.72	0.91–3.22	0.100
p53 _{upper} : Activity ≥ 8.7	1.50	0.82–2.71	0.180

WT: Wild-type; CI: confidence interval.

TABLE 6. Multivariate Cox regression analyses of p53 activity.

Overall survival	Hazard Ratio	95% CI	p-value
p53 Activity WT	1.00		
p53 _{lower} : Activity = 0	1.00	0.56–1.78	1.000
p53 _{median} : Activity 0–8.7	0.83	0.10–1.68	0.600
p53 _{upper} : Activity >8.7	0.85	0.44–1.64	0.630
FIGO Stage I	1.00		
FIGO Stage II	1.99	0.81–4.94	0.140
FIGO Stage III	4.14	1.92–8.95	<0.001
FIGO Stage IV	3.71	1.48–9.31	0.005
Histology Serous	1.00		
Histology non-serous	0.82	0.32–1.72	0.590
Performance score 0	1.00		
Performance score >0	1.71	1.17–2.54	0.008
Age (10 years difference)	1.25	1.06–1.49	0.009

FIGO: International Federation of Gynaecology and Obstetrics; WT: Wild-type; CI: confidence interval.

In univariate analyses, we found that *TP53* termination mutations, which result in the most substantial structural modification, and *TP53* missense mutations were associated with the worst prognosis for these patients. Due to the different results published, it may be important to categorize patients according to the type of *TP53* mutation for potential individualized follow-up. This supports the conclusion by Tuna *et al.* [18] that each *TP53* mutation should be carefully evaluated for its impact.

It is widely accepted that p53 protein expression detected using IHC can serve as a surrogate marker for *TP53* mutations. However, the reported concordance between p53 protein expression (positive and negative) and *TP53* mutational status may vary [14, 21, 22, 30]. A study by Na *et al.* [31] describes five cases where p53 protein expression was negative, despite the presence of *TP53* mutations. Another study examined targeted sequencing results and p53 IHC results. They reported 12 samples with WT p53 protein expression, all of which had *TP53* mutations characterized as splice site variants, termination mutations, or frameshift mutations [29]. Some of the reported mutations were identical to those found in our study. Our results reinforce the importance of focusing on the confirmation of WT p53 expression in negative samples, but also point toward careful interpretation of patients' *TP53* WT.

In all studies, the same antibody clone was used, although it came from different companies and was used in different solutions. It may be important in the future to investigate whether using the same clone from different companies yields consistent results. The used antibody recognizes p53 terminus, specifically amino acid 18–25, which is a part of the transactivation domain 1. This region overlaps with the murine double minute 2 (MDM2) binding site and phosphorylation sites that regulate p53 stabilization and activation. There are several post-translational modifications occur near the DO-7 epitope, which may decrease DO-7 binding affinity. Furthermore, our results highlight the importance of deep knowledge of the epitope position in the antibodies used, as using p53 IHC staining

may not cover the whole sequence of *TP53*, and therefore, p53 protein expression negative should be considered carefully and maybe supported by a conclusive *TP53* sequencing. This is important because IHC is often performed before sequencing in a routine workflow and is considered sufficient for standard use only. Additionally, the selected cut-off value may be important. In the above studies, only p53 protein expression negative, defined as no staining at all, were analyzed, allowing for meaningful comparisons.

In our study, we investigated whether the activity of p53 may impact survival. We observed, in univariate Cox analyses, that patients with p53 activity = 0 had a significantly lower OS. However, when we included p53 activity alongside known clinical prognostic factors in multivariate models, p53 activity did not show independent prognostic impact. This suggests that p53 activity may be a surrogate for mutation type. Overall, our findings support the idea that *TP53* mutations have prognostic value. The biological implications of p53 expression, *TP53* mutations, and *in silico* p53 activity are numerous. The biological roles of dysfunctional p53 in OC can be described as failure to trigger apoptosis in response to DNA damage, which could indicate genomic instability and genomic rearrangements. Depending on the mutation type, it could contribute to chemotherapy resistance. *TP53* mutations are associated with massive chromosomal instability, is described as a hallmark of HGSC. *TP53* mutant cells tolerate more replication stress, and mutant p53 proteins can interact with transcription, such as Signal Transducer and Activator of Transcription 3 (STAT3), to promote immune evasion. The computational prediction (*in silico* p53 activity) refers to the prediction of the functional impact of *TP53* mutations and downstream p53 transcriptional activity, and thereby may be used as models to identify p53-inactivated tumours even when the mutation is ambiguous. For clinical use, abnormal p53 expression is used to support diagnoses of HGSC and for *TP53* mutations, it may be relevant for the identity of tumour cells in the analyzed samples as well as for targeted therapy in an experimental setting (p.Y220C).

TP53 mutations alone cannot predict response to Poly-ADP-Ribose-Polymerase (PARP) inhibitor treatment, but p53 dysfunction contributes to genomic instability, which co-occurs with breast cancer genes (*BRCA1/2*) or Homologous Recombination Deficiency (HRD) positivity. If *in silico* p53 activity had clearly correlated with OS, it could have potential use in stratifying patients for trials testing p53-directed therapies.

Our study has certain limitations, as well as some strengths. Instead of using whole tissue slides, we utilized TMAs for the p53 expression analyses, which may only give information about a selected tumour area. This approach could potentially overlook intra-tumoral heterogeneity or introduce staining bias. To address this issue, we used two 2 mm tissue cylinders from each tumour for all patient samples, which likely minimized this limitation. The results of the IHC were in concordance, probably due to the relatively large tissue cylinders used. Of benefit, the TMAs give the possibility to stain tissue from many samples at low cost and it also may minimize the day-to-day variability, that can occur in the laboratory. Although the overall sample size is relatively large, the number of rarer subtypes is limited, so findings related to these histological subtypes should be considered exploratory. Most ovarian cancers are serous adenocarcinomas, the subtype most frequently harboring *TP53* mutations, and through testing samples with other histological subtypes did not change the results and were therefore included. On the strengths side, the study benefits from a real-world cohort that reflects the general OC population and from complete follow-up data, enabled by the Danish national registries.

5. Conclusions

In our study, we found that tissue lacking p53 expression may be considered for *TP53* sequencing to enable optimal interpretation. *TP53* termination mutations may be considered for careful follow-up programs as they may present a subgroup of patients with significantly shorter survival. These patients may therefore benefit from potential additional biological treatment earlier in their treatment course, which is possible by doing comprehensive sequencing of genes with targets for biological treatment.

AVAILABILITY OF DATA AND MATERIALS

Due to sensitive information and EU data protection legislation, we are unable to disclose the protein expression/next-generation sequencing datasets for this study readily available, unfortunately. Nonetheless, if a researcher has an interest in our data, they are welcome to contact us and collaborate. The data that support the findings of this study can be requested from The National Secretariat for Bio- and Genome Bank Denmark, RBGB.sekretariat.herlev-og-gentofte-hospital@regionh.dk, Herlev Hospital, Borgmester Ib Juuls Vej 73, 2730 Herlev, Denmark.

AUTHOR CONTRIBUTIONS

EH—conceived the idea for the study and drafted the manuscript; was responsible for the biobank data and the p53 data. IJC—conducted the statistical analysis. TSP—annotated the molecular data. CH—was responsible for the clinical data. All authors edited the manuscript. All authors have read and approved the final manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Study approval statement: The Danish Ethical Committee has approved the protocol according to the rules used in International Conference on Harmonization/Good Clinical Practice (ICH/GCP) recommendations and the Helsinki and Tokyo conventions No. KF01-227/03. The study was conducted in accordance with the ethical standards, according to the Declaration of Helsinki, and according to national and international guidelines. Consent to participate: Written informed consent was obtained from all patients included in the study.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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