

Analysis of p53 protein expression levels on ovarian cancer tissue microarray using automated quantitative analysis elucidates prognostic patient subsets

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Background: p53 protein is regarded as a valuable prognostic marker in cancer with a potential use as a molecular target. Here, we sought to determine the prognostic value of p53 in ovarian cancer using a novel method of compartmentalized *in situ* protein analysis.

Patients and methods: A tissue array composed of 141 advanced stage ovarian cancers uniformly treated was constructed. For evaluation of p53 protein expression, we used an immunofluorescence-based method of automated *in situ* quantitative measurement of protein analysis (AQUA).

Results: High nuclear p53 expression levels were associated with better outcome for overall survival (OS) ($P = 0.0023$) and disease-free survival ($P = 0.0338$) at 5-years. High cytoplasmic p53 expression levels were associated with better outcome for OS ($P = 0.0002$). In multivariable analysis, high nuclear and high cytoplasmic p53 level with International Federation of Gynecology and Obstetrics (FIGO) stage were the most significant predictor variables for OS and high nuclear p53 level with FIGO stage were the significant predictor variables for disease-free survival.

Conclusions: Assessment of the prognostic value of p53 protein levels using conventional immunohistochemistry is limited by the nonquantitative nature of the method. AQUA provides precise estimation of p53 protein levels and was able to elucidate the association of p53 protein levels and ovarian cancer prognosis.

Key words: AQUA, ovarian cancer, p53

introduction

Ovarian cancer is the fifth most frequent female cancer type and the leading cause of death among gynecologic cancer patients [1]. There are no proven methods of prevention, and often is a rapidly fatal disease. If diagnosed and treated while the disease is confined to the ovary, the 5-year survival rate is 95%; unfortunately, 70% of cases are diagnosed at late stages and prognosis is dismal [2].

Patients with advanced disease (stages III and IV) are treated using optimal surgical debulking followed by chemotherapy. The current standard chemotherapeutic approach for ovarian cancer patients includes taxane and platinum-based regimens. Although this regimen is highly effective, 50% of women will die of the disease [3]. Traditional clinicopathologic factors do not accurately classify patients in relation with prognosis. The only

validated marker for ovarian cancer is CA 125, which is detectable in the serum of >80% of women with ovarian carcinomas [2]. CA 125 is, however, reliable only in monitoring response to treatment or disease recurrence and not as a diagnostic or prognostic marker. Therefore, considerable interest lies in identifying molecular prognostic indicators to guide treatment decisions.

The cell cycle regulators are particularly interesting because they are frequently altered in human cancer. p53 is a tumor suppressor gene located on 17 p13 and encodes a nuclear phosphoprotein, which has a major function as negative regulator of the cell cycle [3]. p53 is one of the most commonly mutated tumor suppressor genes and it is altered in 50% of advanced cases of ovarian cancer [4]. p53 expression is induced in response to oncogene activation, hypoxia and DNA damage and its activation results in apoptosis or cell cycle arrest. Under normal circumstances, p53 is tightly regulated through its interaction with MDM2, a negative regulatory partner. p53 activity can also be inactivated by viral proteins including human papillomavirus (HPV)E6, simian virus 40 large T antigen, adenovirus E1a and hepatitis B x antigen. Viral E6

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targets p53 for degradation and most HPV-associated cancers have wild-type p53 genotype but are phenotypically p53 inactive [5]. The activated p53 gene has multiple effects on gene expression, the most relevant of which in terms of senescence is transcriptional activation of p21, a pleiotropic inhibitor of different cyclin/cyclin-dependent kinase complexes. p53 protein is also playing a crucial role in apoptosis via pathways involving negative-regulation of antiapoptotic genes and positive-regulation of proapoptotic genes [6].

Two types of mutations affect the p53 gene namely null and non-null mutations. It is very important to remark that the mutant (non-null) p53 protein has increased stability and it accumulates predominantly in the nucleus of neoplastic cells whereas null mutations lead to an unstable truncated p53 protein. As a result, mutant (non-null) p53 protein is detectable by immunohistochemistry (IHC), whereas null p53 mutation is not detected [7]. The wild-type p53 protein has a very short half-life and is detected in low levels by IHC. In various studies, cases with wild-type p53 sequence showed overexpression of p53 protein. The accumulation and stabilization of normal p53 protein may be caused by non-mutational events. Physiological stimuli such as DNA damage resulting from the presence of free radicals from tumor-associated macrophages or following therapy—hypoxia, specific oncogenic stresses and other genes interacting with p53 such as MDM2 gene may play a critical role [5]. Various studies investigated the clinical significance of p53 overexpression in patients with ovarian cancer but the results are not consistent [8, 9].

Tissue microarrays is a useful tool for simultaneously studying specimens from hundreds of patients. This tool carries the inherent advantage of uniform handling of all specimens. Another high feature associated with the use of tissue microarrays is the recently developed method of automated, quantitative analysis, which provides precise, reproducible, measurement of antigen levels, free of the subjectivity associated with pathologist-based scoring [10]. Automated, quantitative analysis provides continuous output scores, as opposed to the arbitrary nominal scores obtained with pathologist-based ‘by-eye’ scoring of zero, one, two or three or ‘high’ and ‘low’.

Here, we sought to determine whether p53 protein levels and expression pattern are associated with clinical outcome in a large cohort of uniformly treated patients with epithelial ovarian cancer using a novel *in situ* quantitative method of protein expression.

patients and methods

patient population

Inclusion criteria were primary epithelial ovarian cancer patients [International Federation of Gynecology and Obstetrics (FIGO) stages III and IV] who underwent surgical resection in the Department of Gynecology of Alexandra University Hospital in Athens from 1996 to 2003 and treated postoperatively with carboplatin and paclitaxel (Taxol, BMS; UK) chemotherapy. In all cases, an effort was made for optimal surgical cytoreduction and adequate staging, which included at least total abdominal hysterectomy with bilateral salpingoophorectomy, inspection and palpation of all peritoneal surfaces and retroperitoneal area, biopsies of suspect lesions for metastases, infracolic omentectomy and peritoneal washings. Grading was carried out by evaluation of tumor architecture, the amount of solid

neoplastic areas, nucleus-cytoplasm ratio and nuclear pleomorphism. The tumors were subdivided into three groups, well differentiated (G1), moderately differentiated (G2) and poorly differentiated (G3), according to these criteria.

Chemotherapy was instituted 2–3 weeks after surgery. All patients received platinum–paclitaxel chemotherapy. Gynecological examination, CA 125 assay and radiological investigations, if necessary, were carried out monthly for the clinical assessment of response, which was recorded according to World Health Organization criteria [11]. Follow-up examinations were carried out every month.

tissue microarray construction

A tissue microarray consisting of tumors from each patient in the cohort was constructed at the Yale University Tissue Microarray Facility. Following institutional review board approval the tissue microarray was constructed as previously described [12], including 141 cases. Tissue cores 0.6 mm in size were obtained from paraffin-embedded formalin-fixed tissue blocks from the Department of Pathology archives, Alexandra University Hospital. Hematoxylin and Eosin stained slides from all blocks were first reviewed by a pathologist to select representative areas of invasive tumor to be cored. The cores were placed on the recipient microarray block using a Tissue Microarrayer (Beecher Instrument, Silver Spring, MD). All tumors were represented with two-fold redundancy. As controls normal tissues from ovary, cervix, endometrium, lymphnode and muscle were used. Previous studies have demonstrated that the use of tissue microarrays containing one to two histospots provides a sufficiently representative sample for analysis by IHC. Addition of a duplicate histospot, while not necessary, does provide marginally improved reliability [12]. The tissue microarray was then cut to yield 5- μ m sections and placed on glass slides using an adhesive tape transfer system (Instrumedics, Inc., Hackensack, NJ) with UV cross-linking.

quantitative IHC

Tissue microarray slides were deparaffinized and stained as previously described [13]. In brief, slides were deparaffinized with xylene followed by ethanol. Following rehydration in dH₂O, antigen retrieval was accomplished by pressure cooking in 0.1 mol/l citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked by incubating in 0.3% hydrogen peroxide in methanol for 30 min. Nonspecific antibody binding was then blocked with 0.3% bovine serum albumin (BSA) for 30 min at room temperature. Following these steps, slides were incubated with primary antibody at 4°C overnight. Primary mouse monoclonal anti-human antibody to p53 (clone DO7, DAKO, Carpinteria, CA) was used at 1 : 100 dilution in 0.3% BSA/tris buffered saline. This antibody has been validated in previous studies using IHC and western blot analysis of normal and neoplastic tissue [14]. This antibody recognizes both wild-type and mutant p53. Subsequently, slides were incubated with goat anti-mouse secondary antibody conjugated to a horseradish peroxidase-decorated dextran polymer backbone (Envision; DAKO Corp., Carpinteria, CA) for 1 h at room temperature. Tumor cells were identified by use of anticytokeratin antibody cocktail (rabbit anti-pancytokeratin antibody z0622; DAKO Corp.) with subsequent goat anti-rabbit antibody conjugated to Alexa546 fluorophore (A11035, Molecular Probes, Eugene, OR). We added 4',6-diamidino-2-phenylindole (DAPI) to visualize nuclei. Target (p53) molecules were visualized with a fluorescent chromogen (Cy-5-tyramide; Perkin Elmer Corp., Wellesley, MA). Cy-5 (red) was used because its emission peak is well outside the green–orange spectrum of tissue autofluorescence. Slides were mounted with a polyvinyl alcohol-containing aqueous mounting media with antifade reagent (*n*-propyl gallate, Acros Organics, Vernon Hills, IL) (Figure 1).

automated image acquisition and analysis

Automated image acquisition and analysis using automated *in situ* quantitative measurement of protein analysis (AQUA) has been described

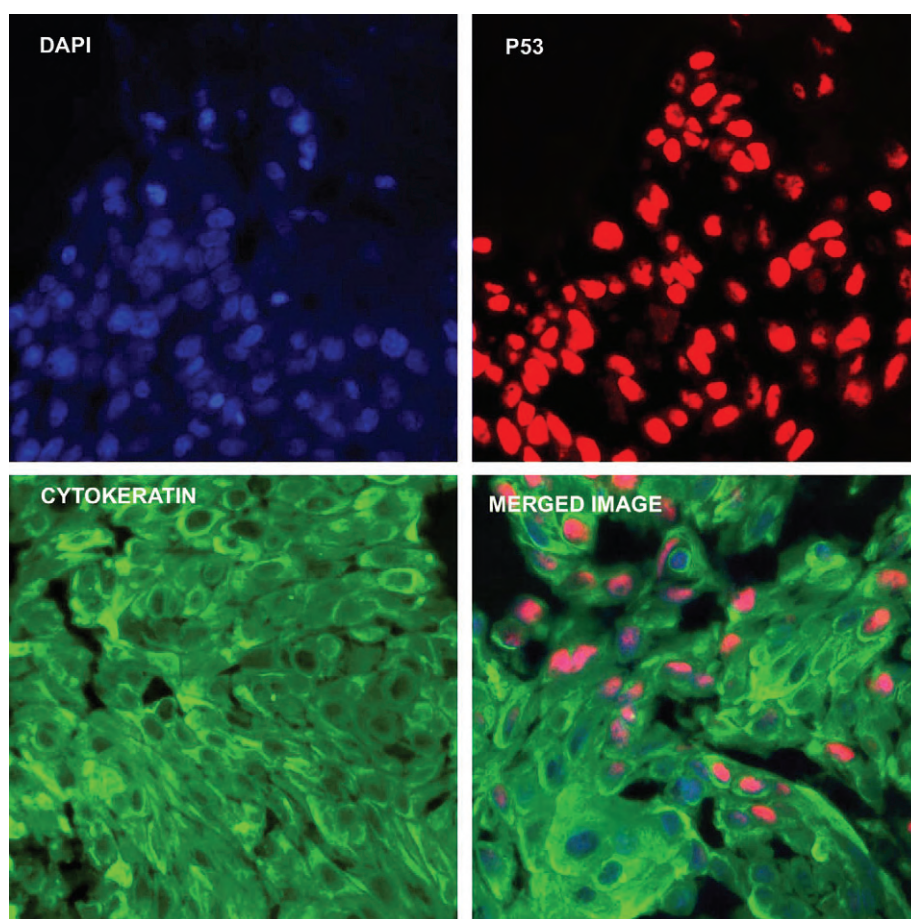


Figure 1. Protein expression of p53 was determined using AQUA analysis on the basis of immunofluorescence. Digital images of each tumor spot were captured using Cy3 anticytokeratin antibody to generate a tumor mask. 4,6-Diamidino-2-phenylindole (DAPI) was used to visualize nuclei and Cy-5 was used to visualize p53. A three-color merged image for each tumor is also shown.

previously [15, 16]. In brief, monochromatic, high-resolution (1024×1024 pixel; $0.5 \mu\text{m}$) images were obtained of each histospot. We distinguished areas of tumor from stromal elements by creating a mask from the cytokeratin signal. DAPI signal was used to identify nuclei and the cytokeratin signal was used to define cytoplasm. Overlapping pixels [to a 99% confidence interval (CI)] were excluded from both compartments. The p53 signal (AQUA score) was scored on a normalized scale of 1–255 expressed as pixel intensity divided by the target area. AQUA scores for each subcellular compartment (nuclear and cytoplasmic p53 signal) were recorded. AQUA scores for duplicate tissue cores were averaged to obtain a mean AQUA score for each tumor.

statistical analysis

Histospots containing <10% tumor as assessed by mask area (automated) were excluded from further analysis. AQUA scores represent expression of a target protein on a continuous scale from 1–255. It is often useful to categorize continuous variable in order to stratify patients into high versus low categories. Several methods exist to determine a cut point, including biological determination, splitting at the median and determination of the cut point which maximizes effect difference between groups. If the latter method (the so-called ‘optimal P value’ approach) is used a dramatic inflation of type I error rates can result [17]. A recently developed program, X-Tile, allows determination of an optimal cut point while correcting for the use of minimum P value statistics [18]. As the AQUA technology is new, there are no established cut points available for quantitative p53 expression.

Therefore, for categorization of p53 expression levels, the X-Tile program was used to generate an optimal cut point. This approach has been successfully applied to AQUA data analysis [15]. Two methods of statistical correction for the use of minimal P value approach were utilized. First, the X-Tile program output includes calculation of a Monte Carlo P value for the optimal cut point generated. Cut points that yield Monte Carlo P values <0.05 are considered robust and unlikely to represent type I error. Secondly, the Miller–Siegmund (MS) minimal P value correction referenced by Altman et al. [17] was utilized. This approach is accepted in the statistical literature, but relatively unknown in the medical/biological research community. Briefly, when making multiple comparisons to find the minimum P value using the log-rank test, the false-high rate (i.e. the percentage of times a marker that has no true prognostic value will be found to have a $P < 0.05$) can approach 40%. Altman’s statistical adjustment generates a minimum P value corrected to yield a true false-high rate of 5%. The corrected P value (P_{cor}) is calculated as follows: $P_{\text{cor}} = \text{phi}(\text{zeta}) [\text{zeta} - (1/\text{zeta})] \log[e] [(1 - \text{epsilon}) < 2 > / \text{epsilon} < 2 >] + 4 \text{phi}(\text{zeta}) / \text{zeta}^\ddagger$. Our calculations were carried out using an epsilon of 0.10. Progression-free survival (PFS) and overall survival (OS) were subsequently assessed by Kaplan–Meier analysis with log-rank for determining statistical significance, and only P corrected was reported. This approach has been successfully

[‡]Where phi indicates the probability density function. P_{min} is the minimum P value generated by evaluating multiple cut points. Zeta is the $(1 - P_{\text{min}}/2)$ -quantile of the standard normal distribution. Epsilon denotes the proportion of values excluded from consideration as an optimal cut point.

applied to AQUA data analysis [15]. All survival analysis was carried out at 5-year cut-offs. Confidence intervals were assessed by univariate and multivariate Cox proportional hazards model. OS was defined as time from first day of chemotherapy to death from any cause. PFS was defined as time from first day of chemotherapy to the first of either death from any cause or disease progression (assessed by CA 125 increase and/or imaging studies). Performance status (PS) was dichotomized into 'zero' versus all others, histologic type into serous versus all others and clinical response into complete response (CR disappearance of all known disease) versus all others. Although several cut-off values of residual volume tumor have been proposed, it has been reported that gradual gradations of residual disease can affect ovarian cancer prognosis. Our patient population was divided into two groups according to the extent of residual disease at first surgery; ≤ 2 cm and > 2 cm. Comparisons of p53 expression with FIGO stage and grade was made by Mantel-Haenszel chi-square test. Comparisons of p53 expression with PS, histology, clinical response and residual disease were made by Fisher's exact test. Comparison of p53 expression status with age was made using Pearson correlation. All calculations and analyses were carried out with Statview software (version 5.0.1; SAS Institute Inc., Cary, NC).

results

clinical and pathological variable analysis

One hundred and forty-one patients were included in the study. Mean follow-up time (range) for the entire cohort was 34.35 months (range 1–91.7). There were 108 (76.6%) FIGO stage III and 33 (23.4%) FIGO stage IV. One hundred (71%) patients had tumors of serous histology. Initial histologic grade was 12 well differentiated (9%), 46 moderately differentiated (33%)

and 82 poorly differentiated (58%). Following initial surgical debulking, residual disease by size was distributed as follows; 30 (21%) with < 2 cm and 111 (79%) with > 2 cm. For clinical response to initial therapy, CR was recorded in 56 (40%) patients and partial response or stable disease/no response in 85 (62.7%) patients. Demographic and clinicopathological variables for the cohort are summarized in Table 1.

quantitative IHC for p53 protein expression and generation of optimal cut point by X-Tile analysis

Of the 150 patients included in this study, 120 (85%) had sufficient tissue for analysis of p53 protein expression by AQUA. Tissues deemed insufficient had $< 10\%$ tumor mask within the histospot, as represented on the tissue microarrays. As visualized by fluorescent IHC, p53 displayed predominantly nuclear expression pattern whereas cytoplasmic p53 staining was generally weaker (Figure 1). Normalized AQUA scores were represented on a 1–255 scale. p53 expression followed a skewed distribution as expected for a cancer tissue biomarker. Using the X-Tile program, an optimal cut point for nuclear and cytoplasmic p53 expression was determined at 6.07 AQUA units, with a Monte Carlo P value of 0.049 as determined by X-Tile. Monte Carlo P values less than $P = 0.05$ indicate robust and valid cut point selection. Patients with nuclear p53 expression ≤ 6.07 were classified as p53 low ($n = 22$) and patients with nuclear p53 expression > 6.07 were classified as p53 high ($n = 98$). Patients with cytoplasmic p53 expression ≤ 6.07 were

Table 1. Demographic, clinical and pathologic data

Variable	<i>n</i> (with AQUA data)	Nuclear p53-low expressors	Nuclear p53-high expressors	<i>P</i>	Cytoplasmic p53-low expressors	Cytoplasmic p53-high expressors	<i>P</i>
Age							
≤ 60	52	10	42	0.8242	13	39	0.0989
> 60	68	12	56		9	59	
Differentiation							
Poor	67	15	52	0.4160	14	53	0.3870
Moderate	41	5	36		5	36	
Well	11	2	9		3	8	
Initial histology							
Serous	87	14	73	0.3029	15	72	0.6157
All others	33	8	25		7	26	
FIGO stage							
III	90	15	75	0.4138	14	76	0.1732
IV	30	7	23		8	22	
Residual disease (cm)							
≤ 2	21	1	20	0.0768	2	20	0.2507
> 2	99	21	78		19	79	
Clinical response to chemotherapy							
CR	49	13	36	0.0539	13	36	0.039
All others	71	9	62		9	62	
Performance status							
No impairment	78	10	68	0.0334	13	65	0.5202
All others	42	12	30		9	33	

AQUA, automated *in situ* quantitative measurement of protein analysis; FIGO, International Federation of Gynecology and Obstetrics; CR, complete response.

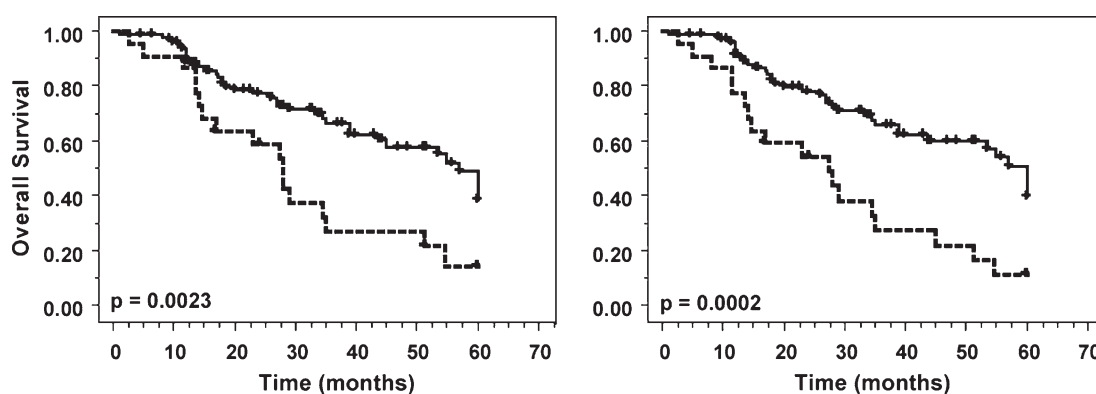


Figure 2. Kaplan–Meier survival analysis for overall survival by nuclear (left) and cytoplasmic (right) p53 expression levels as determined by automated *in situ* quantitative measurement of protein analysis. Patients with high nuclear or cytoplasmic p53 had improved overall survival ($P =$ Miller–Siegmund P values).

classified as p53 low ($n = 22$) and patients with cytoplasmic p53 expression >6.07 were classified as p53 high ($n = 98$).

association of p53 expression and clinicopathological variables

Patients with high nuclear p53 expression levels were more likely to have excellent PS ($P = 0.0334$). Otherwise, there was no other association between nuclear and cytoplasmic p53 and clinicopathological variables including age, histological type, histological grade and FIGO stage (Table 1).

univariate survival analysis

Nuclear and cytoplasmic AQUA expression levels of p53 were examined for association with 5-year OS and PFS using Kaplan–Meier survival analysis with log-rank for determining significance. As use of an optimized cut point can result in increased type I error, the MS correction method was applied to all Kaplan–Meier analyses. Kaplan–Meier survival curves generated for nuclear and cytoplasmic p53, high versus low expression, are given in Figure 2. High nuclear and cytoplasmic p53 expression was associated with better outcome for OS ($P = 0.0023$ and $P = 0.0002$, respectively). There was also a significant association between high nuclear p53 expression and PFS ($P = 0.0338$) (Table 2) (Figure 3), whereas there was no correlation between cytoplasmic p53 expression and PFS (data not shown).

multivariable survival analysis

Using the Cox proportional hazards model, we carried out multivariable analysis to assess the predictive value of nuclear and cytoplasmic p53 expression. Nuclear and cytoplasmic p53 expression by AQUA was analyzed for OS and nuclear p53 expression was analyzed for PFS. We also included the following known prognostic variables in the regression model: age, FIGO stage, grade, residual disease, response to chemotherapy and initial histology. High nuclear p53 level (95% CI 0.189–0.645, $P = 0.0008$) along with FIGO stage (95% CI 1.613–5.365, $P = 0.0004$) and clinical response (95% CI 1.085–2.278, $P = 0.0247$) was significant predictor variables of OS. High cytoplasmic p53 level (95% CI 0.168–0.563, $P = 0.0001$) along with FIGO stage (95% CI 1.569–5.227, $P = 0.0006$) and clinical response (95% CI 1.074–3.333, $P = 0.0274$) was significant

Table 2. Univariate 5-year survival analysis

P53 expression class	Relative risk (95% confidence interval)	P
Disease-free survival		
p53 nuclear hi/lo	0.594 (0.362–0.974)	0.0391
Overall survival		
p53 nuclear hi/lo	0.423 (0.237–0.752)	0.0034
p53 cytoplasmic hi/lo	0.363 (0.206–0.641)	0.0005

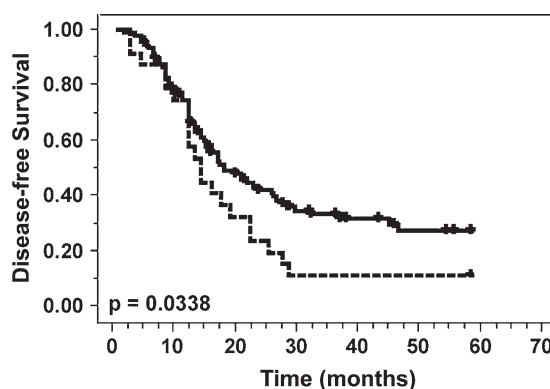


Figure 3. Kaplan–Meier survival analysis for disease-free survival by nuclear p53 expression levels as determined by automated *in situ* quantitative measurement of protein analysis. Patients with high nuclear p53 had improved outcome ($P =$ Miller–Siegmund P value).

predictor variables of OS. High nuclear p53 level (95% CI 0.347–0.975, $P = 0.0399$) along with FIGO stage (95% CI 1.412–3.737, $P = 0.0008$) was significant predictor variables for PFS. Results of multivariable survival analyses are summarized in Tables 3 and 4.

discussion

In the present study, using quantitative IHC, we were able to demonstrate that high p53 expression levels are associated with improved outcome in epithelial ovarian cancer. In multivariable analysis, adjusting for well-recognized prognostic indicators, higher nuclear p53 expression level was the most significant predictor for OS and PFS.

Table 3. Multivariate 5-year survival analysis by Cox regression

Variable	Nuclear p53		Cytoplasmic p53	
	Hazard ratio (95% confidence interval)	P	Hazard ratio (95% confidence interval)	P
Overall survival				
Age (≤60/>60)	1.010 (0.566–1.805)	0.9718	1.116 (0.620–2.008)	0.7149
FIGO stage	2.942 (1.613–5.365)	0.0004	2.864 (1.569–5.227)	0.0006
Grade	0.883 (0.504–1.547)	0.6636	0.915 (0.521–1.607)	0.7576
Histology (serous/all others)	0.920 (0.498–1.695)	0.7887	0.919 (0.499–1.692)	0.7857
Residual disease (≤2/>2 cm)	1.329 (0.561–3.144)	0.5178	1.251 (0.527–2.967)	0.6112
Clinical response to chemotherapy (CR versus all others)	1.915 (1.085–2.278)	0.0247	1.894 (1.074–3.333)	0.0274
p53 expression (high/low)	0.349 (0.189–0.645)	0.0008	0.307(0.168–0.563)	0.0001

FIGO, International Federation of Gynecology and Obstetrics.

Table 4. Multivariate 5-year analysis by Cox regression

Variable	p53 nuclear hi/lo	
	Hazard ratio (95% confidence interval)	P
Disease-free survival		
Age (≤60/>60)	0.966 (0.613–1.521)	0.8802
FIGO stage	2.297 (1.412–3.737)	0.0008
Grade	0.826 (0.529–1.290)	0.3999
Histology (serous/all others)	1.127 (0.681–1.863)	0.6424
Residual disease (≤2/>2cm)	0.658 (0.331–1.307)	0.2315
Nuclear p53 expression (high/low)	0.582 (0.347–0.975)	0.0399

FIGO, International Federation of Gynecology and Obstetrics.

p53 is a tumor suppressor gene with complex biology and there is a debate about its predictive and prognostic significance in ovarian cancer. Several investigators have studied the association of p53 expression assessed by conventional IHC with outcome in ovarian cancer with conflicting results. Several investigators [8, 19] reported that p53 mutations' status in ovarian cancer as assessed by IHC was associated with poor prognosis. To the contrary, others [20–22] found that p53 expression status was not correlated with prognosis in ovarian cancer patients.

p53 is a tumor suppressor gene with complex biology and a varied record of predictive and prognostic significance in ovarian cancer. Mutation of both p53 alleles often results in overexpression of nonfunctional forms of p53 in an attempt to compensate for the loss of p53 function. Such overexpression is often attributable to missense mutations in exons 5–8. When assessed with standard IHC, these tumors frequently exhibit intense p53 nuclear staining. In contrast, mutations outside of these exons generally result in undetectable levels of p53 protein (p53 null mutations). These tumors are therefore not detected by standard IHC. Theoretically, one should be able to distinguish among p53-negative (null mutant), p53-low (wild type) and p53-overexpressing (non-null mutant) tumors; however, such distinctions are beyond the sensitivity of traditional manually read 'brown-stain' IHC. One of the problems with by-eye scoring of immunohistochemical stains is

the difficulty humans have in translating a continuous marker into a nominal four-point scale. Specifically, the human eye cannot discern between 'low' and 'very low' as is the case with the wild-type p53 and null p53 mutants, respectively. Therefore, ovarian tumors bearing null mutant p53 proteins are scored as 'zero' by standard IHC. As a result, using standard IHC, p53 null mutants are grouped together with wild-type p53. Therefore, some investigators find that 'low p53' is a good prognostic marker while others report that low p53 is a poor prognostic marker, depending on the percentages of null mutants and wild-type p53 in the low p53 category. Our group of 'low' p53 tumors probably represents those bearing null mutants whereas wild-type p53 and non-null mutants were classified as 'high'.

The prognostic significance of non-null mutations or p53 overexpression in ovarian cancer is controversial [23–29]. To the contrary, null mutations are related to early, distant metastases and poor prognosis and it is clear that they represent an independent predictor of poor survival in ovarian cancer [29–31]. The significantly inferior outcome observed for patients with null mutations, but not with missense mutations could be explained by the fact that null mutations lead to a truncated dysfunctional protein. To the contrary, in missense mutations the DNA-binding ability of p53 is retained, so that a part of the gene function is intact [32].

Our analysis demonstrates the power of continuous automated assessment to define subclasses of tumors not achievable using standard pathologist-based assessment. Using this technology, we were able to demonstrate an association between p53 expression levels and outcome consistent with the biological role of p53 in tumor behavior. AQUA has been validated as an *in situ* proteomic technique in multiple tumor types where we were able to demonstrate associations between biomarker levels and outcome not discernable with the standard pathologist-based scoring. Camp et al. [18] validated p53 immunostaining in breast cancer using the AQUA method.

We divided our patients into two distinct subpopulations: p53-low tumors and p53-high tumors. We speculate that the p53-low tumors are true double-negative mutants for p53 and therefore comprise a poor prognosis subset. In contrast, the p53-high tumors most likely represent non-null mutants and wild-type p53. Our results that higher nuclear and cytoplasmic p53 expressers have a better outcome indicate that null mutations may play a more important role in tumor

progression than non-null mutations. This group of patients which carries worse prognosis should be targeted for novel therapeutic strategies such as p53 gene replacement.

In summary, in this case, AQUA has elucidated an intriguing subpopulation of tumors that calls for further biochemical investigation.

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