Published in final edited form as:

Gynecol Oncol. 2009 July ; 114(1): 12–17. doi:10.1016/j.ygyno.2009.03.028.

p53 autoantibodies, cytokine levels and ovarian carcinogenesis

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Abstract

Objective—To address the hypothesis that type II ovarian carcinoma, mutation of p53 and plasma levels of particular cytokines are associated with the generation of p53-specific serum autoantibody (AAb) responses in patients.

Methods—Levels of CA125, 17 cytokines and AAbs to tumor-associated antigens including p53 were measured in plasma of 130 gynecologic tumor patients and 84 healthy controls. *TP53* exons 4–9 were sequenced in tumor specimens.

Results—p53 AAbs are associated with high grade, but not low grade ovarian carcinoma. Seropositivity for p53 AAb occurred only in those ovarian carcinoma patients whose tumors contained mutated *TP53*, regardless of the exon targeted. Higher p53 AAb levels were detected in ovarian carcinoma patients who had higher stage disease, but p53 AAb levels were not correlated with CA125 levels. Among high-grade carcinoma patients, there was no relationship between p53 AAb seropositivity and seropositivity to other tumor-associated antigens tested, CA125 level or survival outcome. Both high and low grade ovarian carcinoma patients exhibited elevated levels of IL6, IL8 and IL10 as compared to healthy volunteers, although increased levels of IL5, MCP1, MIP1 and TNF α were associated only with high grade and advanced disease. Higher levels of p53 AAb responses were correlated with elevated circulating IL4 and IL12, but reduced IL8 levels.

Conclusion—Type II, but not type I, ovarian carcinoma patients had elevated serum levels of p53 AAb. p53 AAb is associated with mutation of *TP53*, higher plasma IL4 and IL12 but lower plasma IL8 levels and no survival advantage.

INTRODUCTION

There are numerous subtypes of ovarian tumors and their genesis is controversial. However, two broad types of ovarian carcinoma with distinct molecular pathogenesis and clinical outcomes have been proposed (1). Type I ovarian carcinoma originates from within the normal surface epithelium, progressing via benign cysts to atypical proliferative tumors, that can evolve into low grade carcinoma. These type I ovarian carcinomas are linked to activating K-ras/B-raf or inactivating PTEN/ β -catenin mutations, but exhibit relatively little genomic instability. In contrast to the putative stepwise development of the comparatively indolent type

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Conflict of Interest: The authors declare none.

I disease, type II ovarian carcinoma has been suggested to arise *de novo* (i.e. with no as yet validated histologic precursor lesion) from the surface epithelium of the ovary, peritoneum and/or fallopian tube, and is not associated with K-ras/B-raf or PTEN/ β -catenin mutations (2). Type II serous carcinoma follows an aggressive course, exhibits a high degree of genomic instability, and significantly, exhibits somatic mutation in *TP53* in ~80% of cases (3).

Patients carrying *BRCA1* or *BRCA2* germline mutations are at significantly higher risk for ovarian carcinoma, and p53 mutations are found in these cases at high frequency, even in very early stage I high grade serous carcinoma identified upon prophylactic TAH/BSO (4–6). Notably, over-expression and mutation of p53 was observed not only in these microscopic invasive carcinomas, but also in adjacent dysplastic epithelium. Early serous carcinomas occur predominantly in the fimbriae of women with *BRCA* germline mutations. Recently, a “p53 signature”, described as normal tissue morphology but p53 immunostaining positive (possibly associated with p53 mutation) in the secretory epithelial cells of the fimbriae, has been proposed as the precursor of pelvic serous carcinoma (i.e. encompassing ovarian, pelvic and fallopian tube disease) (6,7). The p53 signature is also associated with DNA damage, as observed using γ -H2AX immunostaining as a biomarker. Tubal intraepithelial carcinomas are not considered as precursor lesions, but rather frank malignancies that will eventually spread if they remain undetected (7).

The tumor suppressor p53 is a nuclear phosphoprotein which plays a critical role in regulating cell proliferation and differentiation (8). *TP53* is the most frequently mutated gene observed in human tumors, including ovarian cancer (3). Mutant p53 typically has a longer half-life than the wild type protein, accumulates in the nucleus and can trigger the development of p53 autoantibodies (AABs) in cancer patients, including ovarian cancer (9). p53 AABs can occur in patients with localized disease, including DCIS and stage I lung or ovarian carcinoma (10–12). The presence of p53 AABs in the sera of asbestosis patients predicted subsequent development of lung malignancy with a positive predictive value of 0.76 and an average lead time to diagnosis of 3.5 years (13). Only 20 to 40% of ovarian cancer patients develop serum p53 AABs (9,12,14). Indeed, while mutation of p53 appears a seminal event in carcinogenesis and is present in ~80% of type II carcinoma (3), it is still unclear why only a subset of these cases generate p53 AABs (9). Here we assess the potential relevance of several biological, clinical and immunological factors that may contribute to the generation of p53 AAB responses in ovarian carcinoma patients.

MATERIALS AND METHODS

Patient samples

Samples and clinical information were obtained with informed consent and this study was performed with the prior approval of the Johns Hopkins University’s Institutional Review Board. Plasma was obtained from heparin-treated blood of patients prior to surgery at the Johns Hopkins Hospital for gynecologic tumors and stored at -80°C . The study utilized plasma samples from 130 ovarian carcinoma patients and as controls, 40 women with invasive cervical carcinoma, 40 with cervical dysplasia, 20 with atypical proliferative ovarian tumors, 37 with benign ovarian disease and 84 healthy female blood donors. Among the 130 ovarian cancer patients, there were 14 low grade and 116 high grade cases (10 at stage I, 7 at stage II, 79 at stage III and 34 at stage IV).

CA125 Assay

Plasma levels of CA125 units were measured with a Cancer Antigen CA125 Enzyme Immunoassay Test Kit, using the manufacturer’s protocol and standards (Panomics).

P53 Autoantibody ELISA

Plasma levels of antibodies to p53 were measured by p53 ELISA Autoantibody Kit, using the manufacturer's protocol and standards (Calbiochem).

ELISA Assay for AAbs to other tumor -associated antigens

The cDNAs for K-ras2A G12V (Biomyx P1020), B-rafV599E (Biomyx P1060), NYESO1 (prepared by direct synthesis by Blue Heron Biotech), mesothelin (provided by Dr Chien-fu Hung), and MAGEA4 (amplified by RT-PCR) were subcloned into pET28a in *E. coli* DH5 α to generate hexahistidine (6His)-tagged fusion protein expression constructs. For protein expression, Rosetta DE3 cells (Novagen) were transformed with each expression construct, and selected with Kanamycin (50 μ g/ml) and Chloramphenicol (34 μ g/ml). After picking an individual colony and growing in 1L Super broth with antibiotics to an OD of 0.8 at 600nm, 1 mM IPTG was added for induction. After a 4 hr induction, the bacteria were harvested by centrifugation (4000 $\times$ g for 15 min). The cell pellet was re-suspended in Solution I (100mM NaH₂PO₄, 10mM Tris-HCl, 8M Urea, atpH8) and protease inhibitor cocktail (Roche EDTA - free cocktail), and clarified by centrifugation (10,000 $\times$ g for 15 min). The clarified supernatant was then mixed with 1 ml Ni-NTA agarose slurry (Qiagen) for overnight at 4 °C. The Ni-NTA agarose slurry was packed in a polypropylene mini-column (Qiagen) and was exhaustively washed with Solution II (same as Solution I but at pH 6.3). Finally, bound antigens were eluted with Solution III (same as Solution I but at pH 4.5) in 1 mL fractions. These fractions were analyzed by SDS-PAGE and Coomassie staining and Western blot. Protein concentrations were estimated with BCA assay (Pierce) using a BSA standard.

Nickel NTA-coated 96-well plates (Pierce) were coated with 0.2 μ g of purified 6His-tagged fusion protein per well overnight at 4 °C. After washing plates with PBST, either human serum samples or a standard antibody were added, and the plates were incubated for 2 hr at RT. Several antibodies were used as standards including anti-p53 mouse mAb (PAb240, Calbiochem), anti-NY-ESO-1 mAb(E978, Invitrogen), anti-mesothelin mAb (K1, Invitrogen), anti-MAGEA4mAb (3D12, Novus Biologicals), K-RasAb (F234, Santa Cruz Biotechnology), and anti-B-Raf mAb (M01, Santa Cruz Biotechnology). The plates were washed with PBST, probed with enzyme-conjugated labeled secondary antibody, incubated for 2 hr at RT, washed with PBST, and finally added TMB substrate and incubated plates for 15 min at 37°C for color development.

P53 mutation

TP53 mutations were determined. Genomic DNA isolated from enriched tumor samples was analyzed for mutations in *TP53* by sequencing of the most commonly mutated exons 4–9 using primers and methods listed in (3). Briefly, an aliquot of 1 μ L of the purified DNA was used in a 25 μ L PCR mixture containing PCR buffer, 10 μ M deoxyribonucleotide triphosphate, and 0.25 U/ μ L Platinum Taq (Invitrogen, Carlsbad, CA). The PCR protocol included denaturation for 2 min at 94°C followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 57°C for 30 sec, and extension at 70°C for 5 min. The PCR products were then purified and sequenced (Agencourt Inc., Beverly, MA). The nucleotide sequences were then analyzed using the Mutation Surveyor program (Soft Genetics LLC, State College, PA). The sequencing data were analyzed by two investigators independently. To confirm the mutations, we resequenced the exons with p53 mutations using reverse primers. The *TP53* polymorphism at codon 72 (Arg/Pro) was not considered as a mutation in this study.

Multiplex Cytokine Bioplex Assays

A multiplex cytokine bead array kit (Bio-Rad) was used to simultaneously quantify 17 cytokines/chemokines (IL-1 β , IL2, IL4, IL5, IL6, IL7, IL8, IL10, IL12, IL13, IL17, G-CSF,

GM-CSF, IFN- γ , MCP-1 (MCAF), MIP-1 β , and TNF- α) in human serum according to the manufacturer's protocol. Briefly, serum samples were first diluted at 1:4 in serum sample diluent solution, and then added into 96-well plate containing beads. Next, the plate was then incubated for 30 min with shaking, washed, detection antibody was added and incubated 30 min with shaking, washed, streptavidin-PE was added and incubated 10 min with shaking, washed, and finally the beads re-suspended with Bio-Plex assay buffer. Lastly, the plate was read by Bio-plex array reader and the data was analyzed by Bio-Plex Manager Software 4.0.

Statistical Methods

We performed analysis comparing p53 antibodies level and survival performance with possible correlated factors. We present Pearson correlation coefficients using the actual level of p53 AAbs and also examine the association of possible correlated factors with p53 seropositivity by Mann Whitney test. To define p53 seropositivity, we introduce a cut-off of 5.4U/ml. We used logrank test to compare survival distributions. The analyses were done using (Graphpad Prism v4 software). The level of significance was set at $p < 0.05$.

RESULTS

Detection of p53 AAb levels by disease status

Since p53 mutation is associated with type II carcinoma (1), we hypothesized that the sera of patients with type II carcinoma but not type I or its precursors would contain p53 specific antibodies. Figure 1 shows that the levels of p53 antibodies in the sera of patients with high grade serous (type II) carcinoma is significantly elevated as compared to healthy female blood donors ($p < 0.001$, ANOVA with Bonferroni's multiple comparison test). In contrast, levels of p53-specific antibodies in sera of women with low grade (type I) serous carcinoma were similar to those of female blood donors, or women with benign ovarian cysts or even atypical proliferative ovarian tumors ($p > 0.05$, ANOVA with Bonferroni's multiple comparison test). K-ras or B-raf mutations are associated with low grade (type I) serous carcinoma. However, we did not observe evidence of AAb specific to either K-ras or B-raf in any patient (not shown) or any difference in reactivity between patient groups.

To derive a cutpoint for the p53 AAb assay, we tested the sera of healthy volunteers that exhibited the six highest values by ELISA, using a Western blot assay (ranging from 4.449–5.392U/ml). Known positive ovarian cancer patient sera reacted with p53 in this Western blot assay, whereas none of the six healthy volunteer sera with the highest reactivity by p53 ELISA react specifically with p53 by Western blot, suggesting the reactivity in the ELISA was non-specific. These cases were therefore considered as negative for p53AAb, and a cutpoint of 5.4 U/ml was established. Utilizing this cutpoint, p53 AAb seropositivity was detected in 25% of the type II carcinoma patients, whereas no p53 AAb seropositivity was seen among 14 women with low grade (type I) carcinoma, 20 with atypical proliferative tumors, and 39 with benign ovarian cysts (Chi-square, $P < 0.0001$). Seropositivity for p53 AAb was not significantly associated with serous carcinoma versus non-serous histotypes (Fisher's exact test, $p = 0.19$). Cervical cancer is typically not associated with somatic *TP53* mutations, and only 1/15 cervical cancer patients and 1/40 cervical intraepithelial neoplasia patients were p53 AAb seropositive.

Correlation between p53 AAbs and TP53 mutations

We sought to confirm that the presence of p53 autoantibody was correlated with a somatic *TP53* mutation within the tumor. When restricting the analysis to high grade ovarian carcinoma, patients whose tumor contained any p53 mutation exhibited significantly higher levels of p53 AAb than those whose tumor contained only wild type *TP53* (Figure 2, $p = 0.0019$, Unpaired T test with Welch's correction). A multitude of distinct mutations, spanning *TP53* exons 4–9, were found in the type II carcinoma patients. Although the numbers of cases is limited, there

was no obvious association between mutation in a particular exon and the development of p53 AAb (Figure 3, $p=0.3$ One way ANOVA).

Moreover, distant metastatic spread of type II carcinoma may be required to trigger p53 AAb. A comparison of the p53 AAb in the sera of women bearing early (stage I/II) versus advanced (stage III/IV) ovarian carcinoma revealed a significantly higher level of p53AAb in the presence of advanced disease ($p<0.001$, Unpaired T test with Welch's correction). However, when using a cutpoint of 5.4 U/ml, there was no significant difference in the presence of p53 AAb in early versus late stage type II carcinoma patients ($p=0.44$, Fisher's exact test).

Correlation of p53 AAbs and CA 125 levels

Since there is some concern that early stage and advanced stage disease are biologically different, we used another independent marker of disease burden. CA125 is a licensed serum biomarker for ovarian carcinoma, and is significantly elevated in women with ovarian carcinoma as compared to healthy volunteers (Table 1). Therefore, to examine the influence of disease burden on p53 AAbs, we compared the CA125 and p53 AAbs levels in the sera of patients with advanced type II carcinoma. No significant correlation was observed between CA125 and p53 AAb levels ($P=0.8$, Pearson).

Correlation between p53 AAbs and plasma levels of 17 cytokines

Changes in the serum levels of various cytokines have been associated previously with ovarian cancer (15,16). We examined the levels of cytokines in the plasma of healthy volunteers as compared to patients with type II carcinoma (Table 1). Of the 17 cytokines tested, only IL5, IL6, IL8, IL10, MCP1, GM-CSF and TNF α levels were significantly increased in high grade ovarian carcinoma patients as compared to healthy controls. The levels of IL6, IL8 and IL10 were also significantly elevated in low grade ovarian carcinoma as compared to healthy volunteers, but not in women with atypical proliferative ovarian tumors, suggesting that these increases in circulating cytokine levels might be associated with carcinogenesis. No significant differences were noted in the plasma levels of each of the 17 cytokines or CA125 in the plasma of ovarian carcinoma patients with wild type versus mutated *TP53*, with the exception of IL6 ($p=0.02$, Mann Whitney) and IL10 ($p=0.007$, Mann Whitney), which were significantly higher in the plasma of those with mutant *TP53* detected in their tumor. Since cervical cancer is not typically associated with *TP53* mutation, we therefore tested the plasma cytokine levels in this patient group. However, similar increases in IL5, IL6, IL8, IL10, MIP-1, GM-CSF and TNF α levels were observed in cervical and high grade ovarian cancer patients (Table 1), suggesting that mutation of *TP53* is not required for the elevation of these cytokines in gynecologic cancer patients.

The production of p53AAbs results presumably from a T helper cell (Th)-dependent humoral immune response to class II carcinoma. Such immune responses are typically associated with the production of cytokines that might potentially be detected in the patient's plasma. We hypothesized that the presence of cytokines might be correlated with the production of p53 AAbs. An examination of correlation was performed between the levels (pg/ml) of each of the 17 cytokines and p53 AAbs in the sera of women with advanced class II carcinoma. Only the absolute levels of IL4 and IL12 exhibited a significant correlation with the levels of p53 AAbs ($p=0.019$ and $p=0.024$ respectively, Pearson). Since elevation of IL12 is typically associated with a Th1 response, and IL4 is with Th2 responses, our finding is therefore suggestive of a balanced Th1/2-type response to p53 in women with advanced type II carcinoma (and no evidence of a Th17 response was observed, $p=0.29$, Pearson) (17). However, upon comparison of cytokine levels and p53 AAb seropositivity based upon the cutpoint of 5.4 U/ml, there was no correlation with the levels of any cytokine, with the exception of IL8. Women with advanced

high grade carcinoma who were also p53 AAb seropositive exhibited lower serum levels of IL8 than seronegative cases (Figure 4, $p=0.002$, Mann Whitney).

Correlation between p53 AABs and other AABs

It is possible that the induction of an AAb response specific to one tumor-associated antigen is correlated with responses to other tumor-associated antigens. However, neither seropositivity nor absolute levels of p53 AABs were correlated with ELISA values for AAb to NYESO-1, K-ras2A, B-raf, mesothelin, or MAGEA4 ($p=0.56$, $p=0.83$, $p=0.56$, $p=0.32$, $p=0.77$ respectively, Pearson), suggesting that autoantibody responses are independent for these tumor-associated antigens.

Correlation between p53 AABs and survival outcome

Tumor antigen-specific immunity might be associated with an improved prognosis. We examined the relationship between p53 AAb and survival of women with advanced type II carcinoma. No association between p53 AAb seropositivity and survival was observed ($p=0.29$, Logrank) among type II carcinoma cases.

Since elevated levels of cytokines could also potentially reflect anti-tumor immunity, or its suppression, we analyzed the relationships between cytokine levels and overall survival. There was no correlation between the survival of women with advanced class II carcinoma and any of the 17 cytokine levels tested. However, improved survival was correlated with early stage at diagnosis ($p=0.0037$, Logrank), and even for stage 3 versus stage 4 cases of advanced class II carcinoma ($p=0.025$, Logrank). Women with low grade ovarian cancer survived significantly longer than those diagnosed with high grade disease ($p=0.0074$, Logrank with a Hazard ratio of 0.19 [95% CI 0.16–0.75]). Longer survival of women with ovarian cancer was associated with a lower CA125 level at diagnosis ($p=0.007$, Logrank using a cutoff of 340 U/ml), although this association was lost when analyzing only those with advanced class II carcinoma ($p=0.098$, Logrank).

DISCUSSION

This study provides seroepidemiologic support that the development of type II ovarian carcinoma is associated with mutation of p53, whereas type I ovarian carcinoma is not. Our findings that p53 AABs are associated with mutation of *TP53*, albeit in no particular exon, are consistent with studies in other cancers (9). Previous studies have indicated that p53 AABs were present at a similar frequency or possibly slightly lower frequency in early as compared to late stage ovarian carcinoma patients (18–21). Our findings are consistent with the earlier data. Although the magnitude of the p53 AAb responses were significantly lower in early versus late stage ovarian carcinoma patients, low grade (type I) disease is more frequently diagnosed at an early stage than high grade (type II) disease, so caution is warranted in interpreting these findings. Another approach to this issue is to compare CA125 as a marker of disease volume with p53 AAb response. There was no evidence that CA125 level was correlated with p53 AAb response in high grade ovarian carcinoma cases, although we note that another study did find such a correlation (21). Such p53 AAb responses have been detected in high risk patients several years prior to the initial diagnosis of lung cancer (13). We speculate that the detection of p53 AABs might be useful in examining epidemiologic factors potentially correlated with the 'p53 signature' and intraepithelial carcinoma in the fallopian tube, and its potential association with subsequent onset of 'ovarian' carcinoma if these lesions are sufficient to trigger a detectable AAb response (6,7). Indeed, a meta-analysis indicated that 35/2404 currently healthy individuals had p53 AABs (9), but the significance of the seropositive findings in presumptive healthy subjects is unclear.

Several studies have observed increases in the levels of cytokines in the serum of ovarian cancer patients, and we have replicated in plasma many of these findings with respect to IL6, IL8, IL10, MIP-1, TNF α and MCP-1, although we observed a reduction rather than an increase in IL7 (15,16,22). Most of these associations were also maintained for early stage disease as compared to healthy volunteers, and were absent from benign tumors. A recent study found that not only ovarian cancer cells, but also the stromal cells and tumor-derived antigen-presenting cells produce high levels of IL6, IL8 and MCP-1 in culture, whereas only the latter produce TNF α (23). IL10 is likely the product of regulatory T cells (24,25). The IL6, IL8 and IL10 cytokine levels were significantly elevated in the class I ovarian carcinoma but not atypical proliferative ovarian tumors as compared to healthy volunteers, consistent with the biological difference between benign tumors and either type of ovarian carcinoma.

In addition, a lower level of IL8 was observed in the plasma of p53 AAb seropositive advanced high grade ovarian carcinoma patients. This was surprising since TP53 mutation is associated with increased IL8 transcript levels in this and other cancer types (26,27), and high grade ovarian carcinoma patients show similarly elevated plasma levels of IL8 as low grade patients ($p=0.28$, Mann Whitney). Interestingly, a recent study identified IL8 as an autoantigen in ovarian cancer patients that triggers the production of IL8AAb in a fraction of patients. This suggests the possibility that human IL8 AAb are produced by patients whose tumors contain p53 mutations and also generate p53AAb, and that the human IL8 AAb interfere with the assay of free plasma IL8 (28).

A weak correlation was observed between the level of p53 AAb response and the serum levels of IL4 and IL12, but this needs additional validation. These cytokines are suggestive of a balanced helper T cell response (17,29), but we observed no evidence that a p53-specific humoral immune response is associated with an improved outcome for high grade ovarian cancer patients. This is consistent with some other studies (11,12,30), while others indicate better (31), or worse (32,33), prognosis is associated with p53AAb. We speculate that the differences in some these studies may result from differing fractions of type I carcinoma patients in the various study cohorts. While early stage and low grade of ovarian carcinoma and lower CA125 values were associated with longer survival, none of the AAb responses or plasma cytokine levels correlated with survival. This may reflect the importance of the intratumoral rather than peripheral cytokine environment and the requirement for CD8 T cell-mediated rather than humoral immunity (34,35). Indeed, intratumoral accumulation of T cells has been associated with improved survival outcomes (36), whereas NK and B-cell infiltration was correlated with a poor prognosis (37). Notably, the type of T cells that accumulate within the tumor are important; a high ratio of CD8+ to CD4+FOXP3+ TILs was correlated with a significantly longer survival, suggesting that the potential benefits of cytotoxic T cells are being tempered by regulatory T cells (38). Indeed, we observed an increased level of IL10 in ovarian carcinoma patients which might be produced by regulatory T cells, but this was not associated with a change in survival (17). Sato *et al* found that neither NY-ESO1 nor MAGEA4 expression was associated with TIL, and herein we observed no correlation between AAb to these or the ovarian tumor-associated antigens such as p53, K-ras2A, B-raf or mesothelin and the survival of ovarian carcinoma patients (38). This implies that the spontaneous immune responses generated to these antigens are inadequate to control disease progression, possibly reflecting the suppressive tumor environment and/or loss of MHC expression by the tumor cells and an overall weak response (35,39). However, the presence of p53 AAb does suggest a break in immune tolerance to the mutant antigen, and supports the potential of mutant p53 as a target for cancer immunotherapy. Indeed, there many potential approaches to enhance p53-specific immune responses by vaccination (40,41), and combat suppressive factors in the tumor microenvironment (42).

Acknowledgments

Grant support was provided by the US PHS Grants RO1 CA122581 (RBSR), P50 CA098252 (RBSR, REB), and the HERA foundation (AS). We thank the Mei-Cheng Wang for manuscript review and the Tissue bank of the Johns Hopkins SPORE in Cervical Cancer for tissue specimens. This manuscript is dedicated to the memory of Sean Patrick in recognition of her friendship, tireless encouragement and commitment to help others with ovarian cancer.

Abbreviations

TAA	tumor associated antigen
AAb	autoantibody
T	tumor-infiltrating lymphocytes

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Figure 1. AAb to p53 by disease status

Reactivity of antibody to p53 by plasma of patients with high grade serous carcinoma (HGS), low grade serous carcinoma (LGS), atypical serous tumors (APST), healthy volunteers (HV) or women with benign ovarian cysts (OVbenign).

Figure 2.
Comparison of p53AAb levels in the plasma of advanced, high grade serous carcinoma patients with respect to TP53 mutation status.

Figure 3.
Plasma p53 AAb of high grade serous carcinoma patients with respect to the *TP53* exon in which the mutation occurs.

Figure 4. Relationship between plasma IL8 levels and *TP53* mutation status and the presence of p53 AAbs

IL8 levels in the plasma of ovarian cancer patients stratified by (A) the presence of *TP53* mutation in the tumor (not significant, Mann Whitney), or by (B) the presence of p53 AAb >5.4 U/ml ($p=0.002$, Mann Whitney).

Table 1

Cytokine, chemokines and CA125 levels by disease status

Median cytokine, chemokine and CA125 levels [and interquartile range] in plasma of patients by disease state as compared to 70 healthy female volunteers (Kruskal-Wallis).

Variable/Median [25%-75% percentiles]	Healthy volunteers (n=70)	Invasive cervical carcinoma (n=38)	Atypical proliferative ovarian tumor (n=20)	Low grade ovarian carcinoma (n=14)	High grade ovarian carcinoma (n=114)	Stage I/II ovarian carcinoma n=16	Stage III/IV ovarian carcinoma (n=113)
β	0.05[0.05-0.05]	0.705[0.22-1.35]	0.05[0.05-0.05]	0.05[0.05-0.05]	0.06[0.05-0.51]	0.05[0.05-0.28]	0.05[0.05-0.57] ***
α	0.05[0.05-0.05]	0.05[0.05-0.05]	0.05[0.05-1.84]	0.05[0.05-0.05]	0.05[0.05-2.62]	0.05[0.05-0.05]	0.05[0.05-2.7]
γ	0.05[0.05-0.05]	0.05[0.05-0.05]	0.05[0.05-0.05]	0.05[0.05-0.925]	0.05[0.05-2.58]	0.05[0.05-0.05]	0.05[0.05-2.58]
δ	0.05[0.05-0.05]	0.76[0.61-0.9] ***	0.05[0.05-0.38]	0.05[0.05-0.36]	0.51[0.05-0.69] ***	0.05[0.05-0.28]	0.51[0.05-0.75] ***
ϵ	0.05[0.05-1.67]	5.23[0.05-57.2] **	0.05[0.05-41.4]	10.2[1.51-63.6] *	8.56[1.41-49.3] ***	17.6[7.25-90.9] ***	7.69[1.29-48.6] ***
ζ	3.44[3.12-4.63]	0.81[0.59-1.725] ***	4.58[0.05-15.5]	0.48[0.05-4.33]	2.67[0.65-9.09]	0.05[0.05-0.57] ***	0.51[0.05-0.75] ***
η	4.2[1.41-49.3]	5.89[0.05-21.6]	4.22[1.52-31.4]	22.6[7.88-138] **	8.56[1.41-49.3] **	16.6[7.84-95] **	11.4[3.73-44.5] ***
θ	0.05[0.05-0.05]	1.71[0.785-3.9] ***	0.05[0.05-2.5]	1.54[0.32-8.26] **	1.64[0.63-4.2] ***	2.88[1.1-4.18] ***	1.49[0.61-4.25] ***
ι	0.05[0.05-0.05]	0.05[0.05-1.94]	0.05[0.05-0.05]	0.12[0.05-1.74]	0.05[0.05-2.41]	2.92[0.5-5.89] *	2.67[0.63-9.45] ***
κ	0.05[0.05-0.05]	0.05[0.05-0.38]	0.72[0.05-23.4]	0.63[0.05-3.0]	0.05[0.05-8.56]	0.05[0.05-0.05]	0.05[0.05-2.41]
λ	0.05[0.05-0.05]	0.05[0.05-0.05]	0.05[0.05-33.4]	0.05[0.05-0.05]	0.05[0.05-0.05]	0.05[0.05-0.05]	0.05[0.05-0.05]
IP-1	131[87-176]	165[119-297]	128[82-227]	230[149-336]	209[146-358] ***	207[149-423] **	210[150-351] ***
IP-1	49.4[39.3-64.1]	67.8[53.6-137] **	76.3[25.2-111]	89.9[43.2-252]	73.1[48.3-127] ***	90.4[53.2-132] *	73.5[48.3-130] ***
CSF	0.05[0.05-8.38]	0.05[0.05-2.3]	0.05[0.05-13.8]	0.05[0.05-0.05]	0.05[0.05-2.62]	0.05[0.05-25.4]	0.05[0.05-1.34]
CSF	0.05[0.05-0.05]	0.05[0.05-26.1] *	0.05[0.05-0.05]	0.05[0.05-0.05]	0.05[0.05-29.6] *	0.05[0.05-13.7]	0.05[0.05-26.9] *
Fa	0.05[0.05-0.05]	0.17[0.05-0.78] ***	0.05[0.05-0.05]	0.05[0.05-0.22]	0.17[0.05-0.78] ***	0.09[0.05-0.65]	0.09[0.05-0.78] ***
125	0[0-29]	0[0-189]	15[10-37]	16[29-330]	236[42-957] ***	21[4-378]	238 [54-957] ***

Significant differences are in bold font

0.05

<0.01

<0.001.

Significance level of detection are set at 0.05pg