



A retrospective analysis of immunohistochemically determined IRF4 (interferon regulating factor 4) expression in a consecutive cohort of 114 ovarian cancer patients

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Abstract

Background Tumor-infiltrating lymphocytes influence the prognosis of solid tumors, including ovarian cancer (OC). The immunoregulatory transcription factor (IRF4) is mainly expressed in plasma cells and regulates immunoglobulin class switch recombination as well as plasma cell differentiation. Therefore, we analyzed the impact of IRF4 expression in a consecutive cohort of OC patients.

Methods IRF4 expression was evaluated by immunostaining. Differences in IRF4 expression among the subgroups of the established clinical–pathological features like age, histological subtype, tumor stage, histological grading, postoperative tumor burden, and completeness of chemotherapy were determined by χ^2 test. The impact of IRF4 expression on progression-free survival (PFS) and overall survival (OS) was examined by univariate and multivariate Cox analysis adjusted for established clinical–pathological factors and Kaplan–Meier survival analysis.

Results 114 patients entered this study. IRF4 was expressed in 51.7% of the entire cohort. 72.3% patients with high-grade serous OC showed IRF4 expression compared to 37.3% patients with a non-high-grade serous OC ($p < 0.001$). Univariate Cox-regression analysis revealed no prognostic impact of IRF4 expression in terms of PFS ($p = 0.35$) and OS ($p = 0.98$). Kaplan–Meier plots failed to show any prognostic impact for PFS ($p = 0.35$) and OS ($p = 0.98$), too. Established clinical–pathological factors retained their prognostic impact as tumor stage in terms of PFS (< 0.001) and as postoperative residual tumor burden ($p = 0.04$), tumor stage (< 0.001), histological grade ($p = 0.02$), and completeness of chemotherapy ($p < 0.001$) in terms of OS, respectively.

Conclusion Immunohistochemically determined IRF4 expression correlated with high-grade serous OC. However, it failed to show any prognostic impact in this cohort of 114 patients.

Keywords TILs · Ovarian cancer · IRF4 · Antitumor immunity

Introduction

A growing body of knowledge suggests the importance of the immune system in solid tumors, including ovarian cancer (OC) [1]. Some tumors dispose of a high-immunogenic potential caused by a genetic damage resulting in a high-mutational load [2]. The arising tumor-associated neoantigens can evoke an immune response which can—under certain conditions—lead to tumor rejection. In this context, tumor-infiltrating lymphocytes (TILs) have recently been suggested as a prognostic factor in OC [3–5]. TILs consist of different types of immune cells, for example T cells, B cells, plasma cells or macrophages, which have left the vasculature

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or lymphoid structures and were localized in the peritumoral stroma or the tumor epithelium shaping the tumor microenvironment. Many studies have shown that the presence of tumor-infiltrating CD8 positive T cells was associated with the improved survival in OC, particularly in high-grade serous ovarian cancer [3], which are known to be genetically highly unstable [6, 7]. However, the precise impact of tumor-infiltrating B cells, in particular plasma cells, in tumor development still remains unclear, requiring further clarification. On one hand, B cells were shown to act as a synergistic player with CD8 + T cells shaping the microenvironment towards an immunostimulatory milieu leading to tumor suppression. In the study of Milne et al., CD20 + B cells were found to be associated with the improved outcome [3]. On the other hand, the presence of CD138 positive tumor-infiltrating plasma cells correlated with reduced overall survival (OS) in a study of Lundren et al. [8]. Antibody responses produced by tumor-infiltrating plasma cells may induce and potentiate chronic inflammation, and thus promoting tumor development [9]. In contrast, we could demonstrate that the presence of tumor-infiltrating B cells, in particular immunoglobulin kappa C (IgκC) positive plasma cells were associated with the improved outcome (longer MFS) in node-negative breast cancer [10]. Moreover, we were able to determine an independent prognostic impact of the transcription factor IRF4, which is mostly expressed in plasma cells, in node-negative breast cancer [11]. To elucidate the role of tumor-infiltrating plasma cells in OC, we evaluated in this project the impact of the immunoregulatory transcription factor IRF4 in an unselected cohort of 114 OC patients using immunostaining. The interferon regulating factor 4 (IRF4) is constitutively expressed in plasma cells and regulates immunoglobulin class switch recombination as well as plasma cell differentiation [12]. IRF4 is inducible by antigen stimulation in T cells and Toll-like receptor (TLR) signaling in macrophages and negatively regulates TLR-dependent induction of pro-inflammatory cytokine genes [13].

Materials and methods

Patients and tissue samples

Patients with OC, who were treated by primary surgery in our institution between 1997 and 2005, entered the study, if paraffin-embedded (FFPE) tissue and plausible follow-up data were available. Follow-up was performed as previously reported in [14] and included data until May 2013. Follow-up data contain the following events: (a) death from OC or from other reasons unrelated to cancer and (b) recurrence of disease, which included metastasis and local relapse. Patients' data in regard to the tumor stage of disease,

postoperative tumor burden, completeness of chemotherapy, and age at diagnosis were collected by reviewing patients' charts as it was previously described in [14].

One of the authors (JJ) reassessed the histological grade and histological subtype. In accordance with Kurman et al. [15], we classify the high-grade serous OC as type II OC and the other types as type I OC. The study was approved by the Research Ethics Committee of the University Medical Center Mainz, Germany. Informed consent was obtained from all the patients, and all the clinical investigations were conducted according to the ethical and legal standards.

Immunostaining of IRF4

For immunohistochemistry, 4-μm-thick formalin-fixed and paraffin-embedded tumor sections were stained with a monoclonal IRF4 antibody (Clone KP-53; Santa Cruz Biotechnology Company, Santa Cruz, California, USA) according to the standard procedures as it was previously described in [11]. All the slides were analyzed using a Leica light microscope (Leica Microsystems Vertrieb Company, Wetzlar, Germany) by two of the authors (ASH, JJ).

Evaluation of immunostaining

For the analysis of immunostaining, we used a semiquantitative scoring method according to the recommendations of an International TILs Working Group 2014 [16]. We evaluated the percentage of the stromal surface area that is occupied by IRF4-positive plasma cells as previously described in [11]: 0, no IRF4-positive infiltrate; 1+, weak IRF4-positive infiltrate: 10–20% of the stromal surface area is occupied by IRF4-positive plasma cells; 2+, moderate IRF4-positive infiltrate: 20–30% of the stromal surface area is occupied by IRF4-positive plasma cells; 3+, strong IRF4-positive infiltrate: 30–40% of the stromal surface area is occupied by IRF4-positive plasma cells. Cases with IRF4 score 0 were considered as negative whereas cases with 1+, 2+, and 3+ were regarded as positive IRF4 expression, respectively. Figure 1 demonstrates representative examples of photomicrographs of different scores of IRF4 staining, positive (tonsil) and negative (human liver tissue) controls.

Statistical analyses

Statistical analyses were performed using the SPSS statistical software program, version 23.0 (SPSS Inc, Chicago, IL, USA). Patients' characteristics were given in absolute and relative numbers (Table 1). Association between clinical–pathological factors and IRF4 expression was analyzed using the χ^2 test. The significance of IRF4 expression and other explorative variables on progression-free survival (PFS) and overall survival (OS) was examined by univariate and multivariate Cox

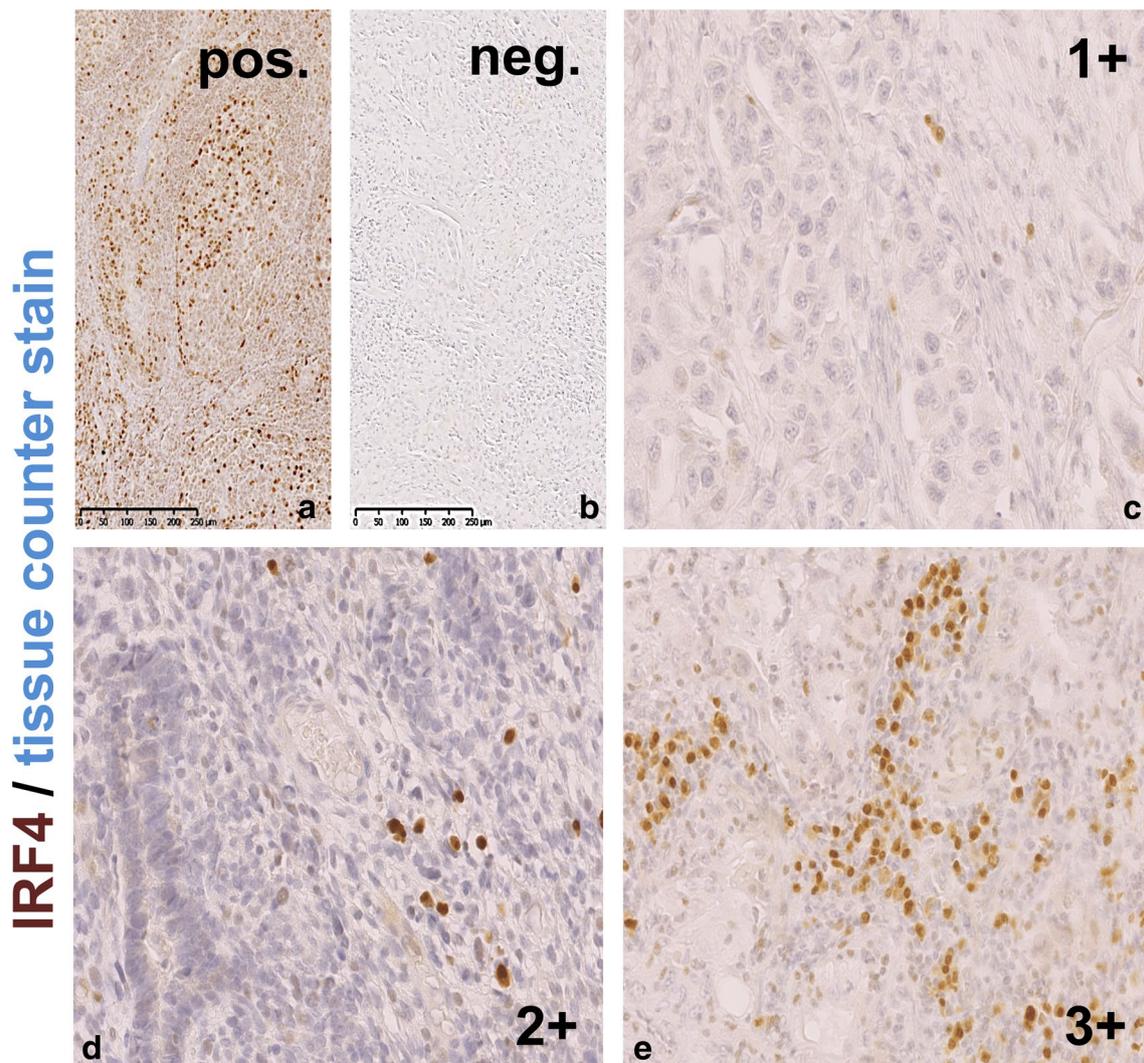


Fig. 1 Representative examples of IRF4 staining in OC and the respective controls. **a** Positive control: normal human tonsil tissue. The antibody strongly labels plasma cells, mainly located in the secondary follicles. **b** Negative control: normal human liver tissue. **c**

Weak IRF4-positive infiltrate (score: 1+). **d** Moderate IRF4-positive infiltrate (score: 2+). **e** Strong IRF4-positive infiltrate (score: 3+). Scale bars **a, b**: 250 μm . Scale bars **c, d**: 100 μm

analysis. First, univariate Cox-regression analysis for every single variable was performed. Second, variables with a p value < 0.05 entered the multivariable Cox-regression analysis with a variable selection via backward elimination. All the associations were given as hazard ratios (HR) including their 95% confidence interval (CI) and p values. For survival analysis, Kaplan–Meier plots were performed and significance levels were calculated using Log-rank test.

Results

114 patients entered this study. IRF4 expression was observed in 51.7% of the whole cohort, among them 18.4% scored 1+, 14.9% scored 2+, and 18.4% scored 3+,

respectively (Table 1). In the entire study cohort, 46 (40.4%) relapses and 62 (54.4%) deaths occurred. Patient's characteristics are given in Table 1. Regarding the histological type, IRF4 expression occurred more frequently in patients with high-grade serous OC: 34 out of 47 (72.3%) cases with high-grade serous OC showed a positive IRF4 expression whereas 25 out of 67 (37.3%) patients suffering from type I OC showed a positive IRF4 expression ($p < 0.001$) (Table 2). Furthermore, results of χ^2 tests demonstrated that the positive IRF4 expression is correlated with the histological grade ($p = 0.006$): among 78 patients suffering from G2 or G3 tumors, IRF4 was expressed in 56 (57.1%). In contrast, only 3 (18.8%) out of the cases with G1 tumors showed a positive IRF4 expression ($p = 0.006$) (Table 2). Moreover, positive IRF4 expression was more frequently observed

Table 1 Patients' characteristics

Parameter	(<i>n</i> = 114) <i>n</i> (%)
Mean age (years) (\pm SD)	62.05 (\pm 12.97)
Tumor stage (FIGO)	
I	30 (26.4)
II	11 (9.6)
III	60 (52.7)
IV	13 (11.3)
Histological grade	
G1	14 (12.3)
G2	42 (36.8)
G3	58 (50.9)
Histological type	
Serous	70 (61.5)
Low-grade serous	25
High-grade serous	45
Mucinous	7 (6.1)
Endometrioid	18 (15.8)
Clear cell	3 (2.6)
Mixed	6 (5.3)
Missing data	10 (8.7)
Postoperative residual tumor burden	
R0	60 (52.6)
R1	29 (25.5)
R2	25 (21.9)
Chemotherapy	
Complete	89 (78.1)
Incomplete	3 (2.6)
Missing data	22 (19.3)
Events	
Relapse	46 (40.4)
Death	62 (54.4)
IRF4 score	
0	55 (48.3)
1	21 (18.4)
2	17 (14.9)
3	21 (18.4)

in cases with positive residual tumor burden (R1 + R2) as compared to patients without residual tumor burden (R0) ($p=0.01$) (Table 2).

Univariate Cox-regression analysis showed that the IRF4 expression neither correlated with PFS ($p=0.35$) nor with OS ($p=0.98$) (Table 3). In multivariate Cox-regression analysis in terms of OS postoperative residual tumor burden (HR 1.91, 95% CI 1.04–3.52 $p=0.04$), tumor stage (HR 5.64, 95% CI 2.18–14.63, $p<0.001$), histological grade (HR 4.40, 95% CI 1.27–15.24, $p=0.02$), and completeness of chemotherapy (HR 0.23 95% CI 0.12–0.46, $p<0.001$) had independent prognostic significance (Table 3). Regarding PFS, only the tumor stage retained its prognostic impact

Table 2 Clinico-pathological factors in relation to the IRF4 expression

Clinico-pathological factor	Negative (IRF score 0) vs. <i>p</i> value positive (IRF4 score 1–3) <i>n</i> (%) vs. <i>n</i> (%)
Tumor stage (FIGO)	
I–IIa	20 (58.8%) vs. 14 (41.2%) 0.152
IIb–IV	34 (43.0%) vs. 45 (57%)
Histological grade	
G1	13 (81.2%) vs. 3 (18.8%) 0.006
G2–G3	42 (42.9%) vs. 56 (57.1%)
Histological type	
High-grade serous	13 (27.7%) vs. 34 (72.3%) <0.001
Others	42 (62.7%) vs. 25 (37.3%)
Age	
< Median	34 (57.6%) vs. 25 (42.4%) 0.042
> Median	21 (38.2%) vs. 24 (61.8%)
Postoperative residual tumor burden	
R0	36 (60%) vs. 24 (40%) 0.014
R1 + R2	19 (35.8%) vs. 34 (64.2%)

Significant *p*-values < 0.05 are shown in bold

(HR 20.30, 95% CI 4.86–84.60, $p<0.001$) (Table 3). Kaplan–Meier plots demonstrated no influence of IRF4 expression on PFS and OS rates ($p=0.35$ and $p=0.98$, respectively) in the whole study cohort (Figs. 2 and 3) as well as in the subgroup of high-grade serous OC ($p=0.66$ and $p=0.92$) (Figs. 4 and 5).

Discussion

The aim of this study was to evaluate the impact of IRF4, which is mainly expressed in plasma cells, and also in macrophages and activated B cells, in an unselected cohort of OC patients using immunostaining.

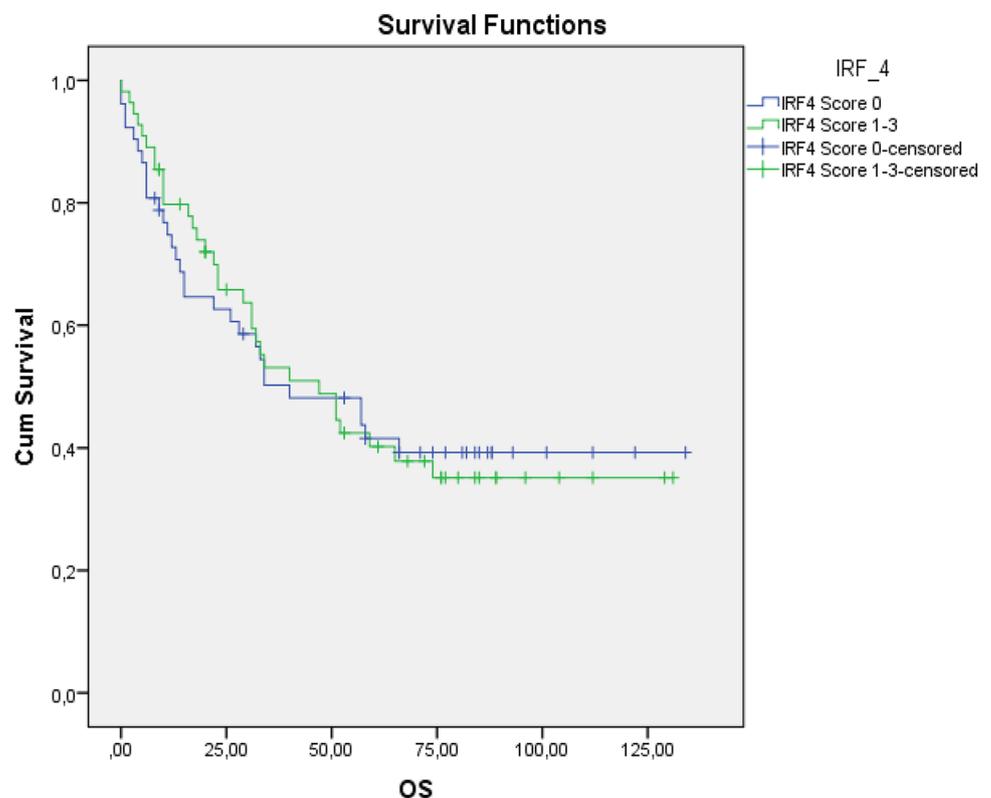
Our results showed that the IRF4 expression occurred more frequently in high-grade serous OC as compared to the other histological subtypes ($p<0.001$). These findings support the hypothesis that the histological subtype of high-grade serous OC disposes of a high-immunogenic potential. This finding is in line with the results of Milne et al. They demonstrated that the immune infiltrates were more prevalent in high-grade serous OC as compared to the other histological subtypes [3]. This could rely on a high-mutational load by genetic alterations within the tumor genome: high-grade serous OCs are characterized by a high frequency of p53 mutations and are genetically unstable [7, 15, 17]. New insights into the tumorigenesis of OC resulted in a dualistic classification of OC: tumor type I is represented by low-grade serous, low-grade endometrioid, clear cell, and mucinous carcinomas [18]. Mutations

Table 3 Univariable and multivariable Cox-regression analysis for disease-free survival, disease-specific survival, and overall survival

Parameter	Univariable		Multivariable	
	HR (95% CI)	<i>p</i> value	HR (95% CI)	<i>p</i> value
OS				
Age	1.76 (1.07–2.91)	0.03	1.11 (0.62–1.99)	0.72
Tumor stage (FIGO)	7.64 (3.27–17.89)	< 0.001	5.64 (2.18–14.63)	< 0.001
Histological grade	3.20 (1.16–8.84)	0.02	4.40 (1.27–15.24)	0.02
Histological type	1.02 (0.61–1.69)	0.945	–	–
Postoperative residual tumor burden	3.06 (1.81–5.17)	< 0.001	1.91 (1.04–3.53)	0.04
Completeness of chemotherapy	0.52 (0.30–0.92)	0.02	0.23 (0.12–0.46)	< 0.001
IRF4	0.99 (0.60–1.63)	0.98	–	–
PFS				
Age	0.94 (0.52–1.70)	0.84	–	–
Tumor stage (FIGO)	20.88 (5.01–87.01)	< 0.001	20.30 (4.87–84.60)	< 0.001
Histological grade	3.20 (0.99–10.36)	0.05	1.43 (0.44–4.70)	0.55
Histological type	1.60 (0.90–2.87)	0.11	–	–
Postoperative residual tumor burden	4.48 (2.35–8.56)	< 0.001	1.51 (0.77–2.86)	0.23
Completeness of chemotherapy	1.20 (0.54–2.68)	0.66	–	–
IRF4	1.32 (0.73–2.37)	0.35	–	–

Significant *p*-values < 0.05 are shown in bold

Fig. 2 Kaplan–Meier plot showing that there is no significant association between immunohistochemically determined IRF4 score and OS in the whole cohort of OC patients ($N=114$, $p=0.349$). To dichotomize the patients, cases with IRF4 score 0 were considered as having low IRF4 expression and cases with IRF4 score 1+ to 3+ as high IRF4 expression, respectively



in KRAS, BRAF, ERBB2, CTNNB1, PTEN, and PIK3CA signaling pathway are characteristic of type I OC [6]. In contrast, type II OCs consist of high-grade serous, high-grade endometrioid, malignant mixed mesodermal tumors (carcinosarcomas), and undifferentiated tumors. Type II

OCs are highly proliferating and aggressive tumors, which are correlated with poor prognosis [15]. Caused by their genetic instability resulting in a higher mutational load, type II OC might dispose of a higher immunogenic potential as compared to type I tumors [18].

Fig. 3 Kaplan–Meier plot showing that there is no significant association between immunohistochemically determined IRF4 score and PFS in the whole cohort of OC patients ($N=114$, $p=0.98$)

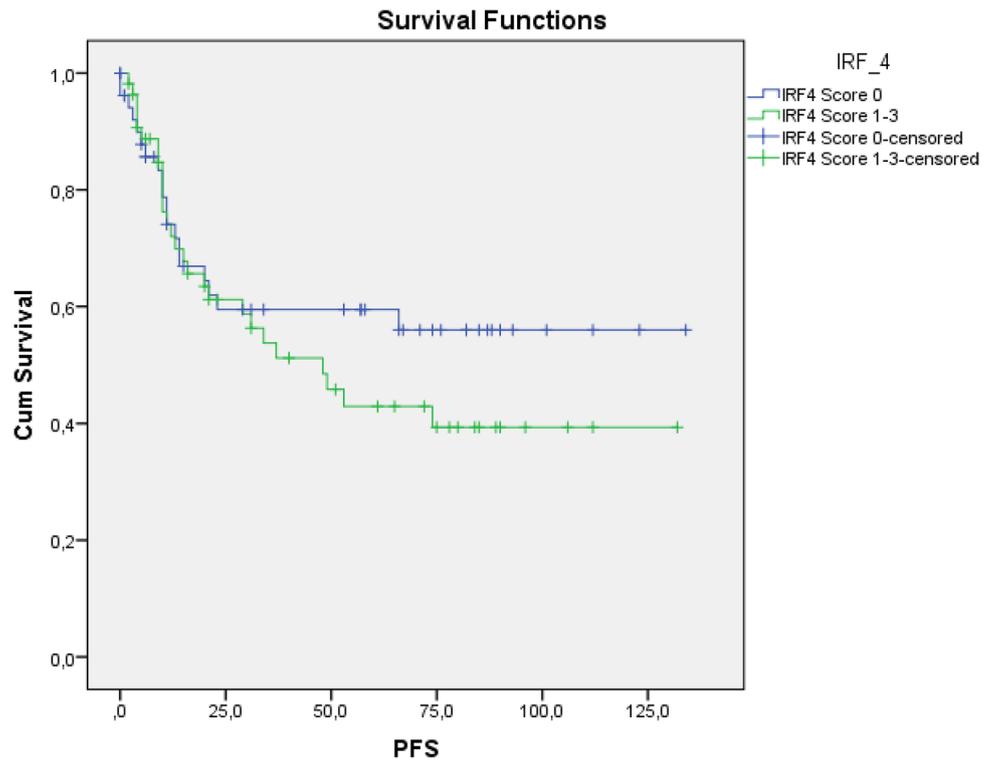
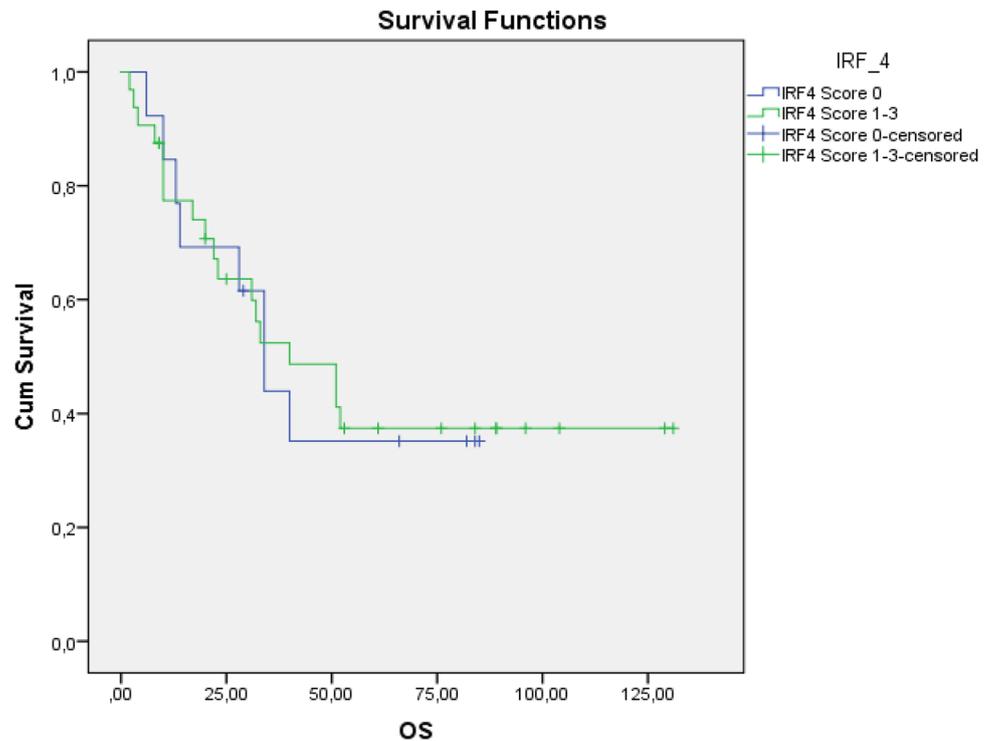


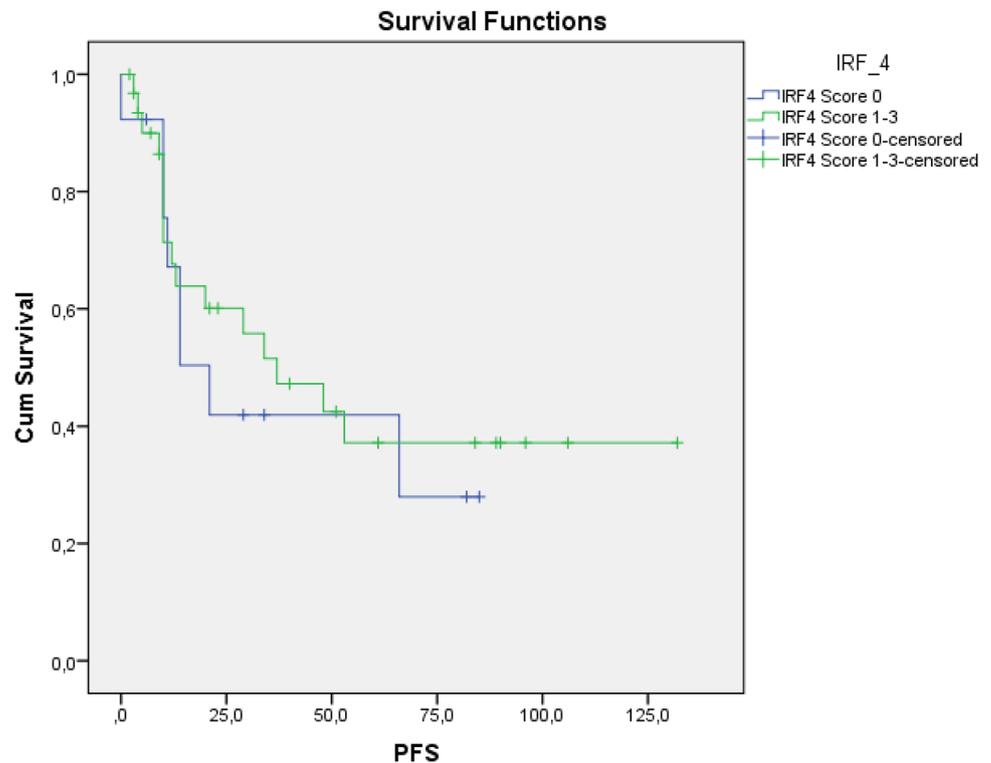
Fig. 4 Kaplan–Meier plot showing that there is no significant association between immunohistochemically determined IRF4 score and OS in the subgroup of high-grade serous OC patients ($N=45$, $p=0.92$ Log-rank)



In the preliminary work, we could show that the IRF4 expression was significantly associated with the improved outcome in node-negative breast cancer [11]. However, here we report no significant correlation between IRF4 expression

and PFS or OS. While the favorable role of tumor-infiltrating CD8 + T cells on prognosis of OC has been shown in many studies, the precise role of tumor-infiltrating B cells or plasma cells remains open, requiring further clarification.

Fig. 5 Kaplan–Meier plot showing that there is no significant association between immunohistochemically determined IRF4 score and PFS in the subgroup of high-grade serous OC patients ($N=45$, $p=0.66$ Log-rank)



In a study of Chapoval et al., B cells antagonized the tumor-suppressing T cell and NK cell-mediated immune responses in a mouse model [19]. In contrast, B cells positively influence the development of an antitumor response by supporting synergistically CD8 + T cell-mediated immune reaction in patients with ovarian cancer [20–22]. This is to conform the findings of Iglesia et al. showing a positive correlation between expression of B cell-associated immune markers and PFS in immunoreactive OCs [23]. Similar results were reported by Montfort et al., they could demonstrate that the tumor-infiltrating B cells enhanced the development of antitumor responses in omental metastases of HGSOc [24]. Lundgren et al. recently evaluated the prognostic impact of tumor-associated B cells and plasma cells in a cohort of 154 OC cases using immunostaining of IgκC, CD20, and CD138: CD20 and IgκC had no prognostic impact whereas CD138 expression was correlated with worse prognosis [8].

Limitations of the study accrue from the low number of analyzed cases and the retrospective design. This may mainly alter the information of follow-up. To cope with this bias, we include only patients with full information of the course of their disease.

In conclusion, we could show in a retrospective study that the IRF4 expression might occur more frequently in the histological subtype of high-grade serous OC. Whether this finding is related to the hypothesis that the histological subtype of high-grade serous OC disposes of a higher immunogenic potential as compared to the other histological

subtypes remains speculative. Finally, our findings failed to show any impact of IRF4 in term of PFS or OS in the consecutive cohort as well as in the subgroup of high-grade serous OC.

Author contributions ASH: project development, data collection, immunostaining, statistical analysis, manuscript writing. MS: project development, manuscript editing, supervision. KA: data curation, support. JJ: evaluation of immunostaining, manuscript editing. SG: technical support, immunostaining. VWE: support statistical analysis. TE: methodology support. KK: formal analysis support. WB: protocol/project development, supervision. AH: project development, manuscript editing, supervision. MJB: data collection, protocol development, manuscript editing, supervision.

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Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest.

Ethical approval The study was approved by the Research Ethics Committee of the University Medical Centre Mainz, Germany. Informed consent was obtained from all the patients, and all the clinical investigations were conducted according to the ethical and legal standards, and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed consent We confirm that this manuscript has not been published elsewhere and is not under consideration by another journal. All the authors have approved the manuscript and agreed with the submission to Archives of Gynecology and Obstetrics.

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