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ORIGINAL ARTICLE

Comprehensive analysis of PD-L1 expression, HER2 amplification, ALK/EML4 fusion, and mismatch repair deficiency as putative predictive and prognostic factors in ovarian carcinoma

Elisa Schmoeckel[1] · Sophie Hofmann[1] · Daniel Fromberger[1] · Miriam Rottmann[2] · Beate Luthardt[1] · Alexander Burges[3] · Udo Jeschke[3] · Thomas Kirchner[1] · Sigurd F. Lax[4] · Doris Mayr[1]

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Abstract

Most ovarian carcinomas (OC) are characterized by poor prognosis, particularly the most frequent type high-grade serous carcinoma. Besides PARP inhibitors, target-based therapeutic strategies are not well established. We asked the question which other therapeutic targets could be of potential value and, therefore, analyzed a large cohort of OC for several predictive factors. Two hundred eighty-eight (288) cases of OC including the major histological types were analyzed by immunohistochemistry for PD-L1HER2, ALK, and the mismatch repair (MMR) proteins MLH1, PMS2, MSH2, and MSH6. HER2 amplification and ALK/EML4 fusion were assessed by fluorescence in situ hybridization. The most frequent finding was PD-L1 expression ≥ 1% in 19.5% of the cases, which correlated with a significantly better overall survival in multivariate analysis ($p < 0.001$). HER2 amplification was detected in 11 cases (4%), all high-grade serous carcinomas. Amplification of HER2 did not correlate with patients' survival. ALK/EML4 fusion was found in two cases (0.74%): one high-grade serous and one endometrioid carcinoma. MMR deficiency was only present in one case of stage IV high-grade serous carcinoma. Subsets of high-grade serous carcinomas show PD-L1 expression and HER2 amplification, respectively, and, therefore, could qualify for immune checkpoint inhibitor therapy or anti HER2 therapy. PD-L1 is also of prognostic impact. ALK/EML4 fusion is very rare in OC and not a putative therapeutic target.

Keywords Ovarian carcinoma · HER2 · ALK/EML4 · PD-L1 · Mismatch repair deficiency · Microsatellite instability

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✉ Elisa Schmoeckel
Elisa.Schmoeckel@med.uni-muenchen.de

[1] Institute of Pathology, Ludwig-Maximilians-University (LMU), Munich, Germany

[2] Munich Cancer Registry (MCR) of the Munich Tumor Centre (TZM), Institute for Medical Information Processing, Biometry, and Epidemiology (IBE), University Hospital of Munich, Ludwig-Maximilians-University, Munich, Germany

[3] Department of Gynaecology and Obstetrics, Ludwig-Maximilians-University (LMU), Munich, Germany

[4] Department of Pathology, Hospital Graz II and Medical University Graz, Graz, Austria

Introduction

Ovarian carcinoma (OC) includes different histological subtypes, which are heterogeneous with respect to underlying pathogenesis, molecular features, and prognosis. This biological diversity of OC is not considered for adjuvant treatment which encompasses relatively uniform chemotherapeutic schemes. In contrast to many other solid tumors, the evaluation of predictive biomarkers and prognostic factors is not an integrated part of the diagnostic procedure of OC, so far. Although a subset of high-grade serous carcinoma (HGSC) can be effectively targeted by PARP inhibitors in addition to chemotherapy, further therapeutic options are of great interest [21, 33]. Currently, the group of "non-HGSC" receives a combination of platin- and taxane-based, adjuvant chemotherapy

depending on stage and grade but regardless of molecular features or histological type [8, 25].

Recently, for several categories of solid cancer, the entity-based therapy has been replaced by a target-based therapy, which addresses to specific tumorigenic pathways. The putative responsiveness of these therapeutic targets, which is usually related to molecular alterations such as mutations, gene fusions, or gene amplifications, may be tested before treatment by molecular pathological analysis. Among the most frequent therapeutic targets, which have not yet been introduced into standard OC therapy, are the immune checkpoint inhibitor molecule programmed cell death 1 ligand 1 (PD-L1), amplified human epidermal growth factor receptor 2 (HER2), fused anaplastic lymphoma kinase/echinoderm microtubule-associated protein-like 4 (ALK/EML4), and mismatch repair deficiency (MMRd).

Anti-HER2-therapy is widely used in breast cancer and in addition in gastric and colorectal cancer [26]. HER2 amplification was studied in OC but by now anti-HER2-therapy was applied only in single cases of OC [28, 29]. Treatment of non-small cell lung cancer by anti-tyrosine kinase inhibitors effectively targets the ALK/EML4 translocation [18, 34], but for OC data are controversial. A few studies suggested a therapeutic application [16, 35] although the TCGA data did not report evidence for ALK/EML4 translocation in OC [4]. Therapeutic inhibition of the immune checkpoint proteins PD-L1 has become a standard procedure for non-small cell lung cancer [37]. Several trials have revealed an improved outcome also for OC [22, 30]. In addition, the responsiveness for immune checkpoint inhibitors is related to microsatellite instability (MSI) due to the stimulation of immunogenicity by the frequent mutations in mismatch repair (MMR)-deficient neoplasms [13, 20, 42]. This has been demonstrated particularly for colorectal carcinoma [2, 41].

We analyzed a cohort of clinically well-documented OC from a single cancer center for alterations in ALK, HER2, PD-L1, and MMRd by immunohistochemistry and molecular analysis. The findings were also correlated to patients' overall survival.

Materials and methods

Study group and clinical data

The study cohort included 288 cases of OC of which 233 were HGSC and the remaining 55 carcinomas consisted of 19 low-grade serous (LGSC), 23 endometrioid (EC), and 13 mucinous (MC) carcinomas. All cases were primarily diagnosed between 2003 and 2007 at the Institute of Pathology, Ludwig-Maximilians-University, Munich, Germany, and underwent a careful histopathological review and reclassification according to the 2014 WHO classification under assistance of immunohistochemistry using PAX8, WT1, p53, Ki67, ER, and PR. Borderline/atypical proliferative tumors were excluded from this study. Complete follow-up data were available for all cases with a mean follow-up time of 44.6 months. One hundred sixty-eight (168) of the 288 patients (58.3%) died of tumor-related disease. Data regarding somatic or hereditary BRCA mutations or Lynch syndrome were not available. The clinicopathological parameters are listed in Table 1. Due to the limited number of cases per group except for HGSC, a detailed subgroup analysis focused on HGSC.

Immunohistochemistry

Immunohistochemical stains were performed using formalin-fixed paraffin-embedded (FFPE) tissues on a tissue microarray (TMA). The TMA was constructed by using two cores of 2.0 mm punched from each donor block and transferred to an empty paraffin block under the guidance of a precision tool.

Serial sections were cut at 4 μm from each paraffin block and mounted on SuperFrost Plus microscope slides (Menzel Gläser, Braunschweig, Germany), deparaffinized, and stained with hematoxylin and eosin (HE). Immunohistochemistry was subsequently performed for ALK, ER, HER2, MLH1, MSH2, MSH6, p53, PAX8, PD-L1, PMS2, ER, PR, and WT1 (Supplementary Table 1). Immunohistochemistry for ALK, ER, HER2, MLH1, MSH2, MSH6, p53, PD-L1, PMS2, and PR was subjected to heat-induced epitope unmasking by heating with a pressure cooker and performed on a Ventana Benchmark XT autostainer (Ventana Medical Systems, Oro Valley, AZ) with the XT UltraView diaminobenzidine kit (Vector Laboratories, Burlingame, CA) and hematoxylin

Table 1 Clinicopathological parameter

Study group ($n = 288$)	
Age (years)	
Mean	62
Minimum	23
Maximum	93
FIGO	*N* (%)
I	28 (9.7)
II	22 (7.6)
III	197 (67.4)
IV	44(15.3)
Histological types	*N* (%)
High-grade serous	233 (80.9)
Low-grade serous	19 (6.6)
Endometrioid	23 (8.0)
Mucinous	13 (4.5)

counterstaning (Vector Laboratories, Burlingame, CA). Positive controls were included.

Evaluation of immunohistochemistry

The interpretation of the immunohistochemical stains for HER2 was based on the three-tiered scoring system according to the guidelines of breast cancer published by the American Society of Clinical Oncology (ASCO) [39]. Scores 0 and 1+ were interpreted as HER2 negative. Score 2+ was interpreted as equivocal for HER2. Score 3+ was interpreted as HER2 positive.

Immunohistochemical staining for ALK was interpreted as positive if tumor cells showed a strong multifocal or diffuse, granular cytoplasmic expression. Weak cytoplasmic immunoreactivity was scored as negative.

PD-L1 staining was graded by using the PD-L1 "Cologne score" [32], referring to the following: score $0 = < 1\%$, score $1 = \geq 1$ and $< 5\%$, score $2 = \geq 5$ and $< 10\%$, score $3 = \geq 10$ and $< 25\%$, score $4 = \geq 25$ and $< 50\%$, and score $5 = \geq 50\%$. A tumor cell was considered "PD-L1 positive" if the cell membrane was partially or completely stained, irrespective the staining intensity. Cytoplasmic PD-L1 staining was disregarded.

For the MMR proteins, complete absence of nuclear staining was considered "loss of expression" and negativity, respectively. Adjacent normal stromal cells served as positive control. Immunohistochemistry was repeated on the original tumor block in all cases of uncertain immunoreactivity.

In order to confirm MSI, the case with immunohistochemical MMRd was further analyzed using a PCR-based technique on a Sanger sequencer according to a standardized protocol.

Fluorescence in situ hybridization

FISH for HER2 and ALK/EML4 was performed according to a standardized protocol. Sections were cut at 3 μm from each TMA block and mounted on SuperFrost Plus microscope slides (Menzel Gläser, Braunschweig, Germany). For HER2-FISH, the dual color fluorescence HER2-specific probe (ZytoLight SPEC ERBB2/CEN 17 Dual Color Probe, Spectrum Green and CEP 17 Spectrum Orange, ZytoVision, Bremerhaven, Germany) was used. The interpretation of the results for HER2-FISH followed the ASCO/CAP guidelines for breast cancer [39]. In all cases of HER2 equivocal status by FISH or technical problems on the TMA, the FISH analysis was repeated on the original large tumor block.

For ALK/EML4 FISH, a triple color break apart single fusion probe (ZytoLight_SPEC ALK/EML4 TriCheck, ZytoVision, Bremerhaven, Germany) was used. The TriCheck probe encompasses two probes (orange and green) flanking the breakpoint cluster region of ALK and third probe (blue) covering the complete echinoderm microtubule-associated protein-like 4 (EML4) gene. ALK/EML4 fusion was defined as the presence of split signals and/or single red signals in ≥ 15% of tumor cells according to the guidelines for non-small lung cancer [36]. The ALK/EML4 FISH was repeated on the original large tumor block in all positive cases and in all cases that could technically not be evaluated on the TMA. For each case, a minimum of 30 cells were analyzed but in most cases 60 cells were evaluated.

The FISH analysis was performed on a ZEISS Axioskop microscope (Carl Zeiss AG, Oberkochen, Germany), equipped with a 100-V OSRAM lamp (OSRAM AG, Munich, Germany) and objectives by ZEISS (Carl Zeiss AG, Oberkochen, Germany). Three independent readers analyzed all samples.

Statistical analysis

For statistical analysis, the SPSS Statistics version 23 (SPSS Inc., Chicago, IL, USA) was used. For testing proportional differences in univariate analysis, the Pearson's Chi-square test or the Fisher's exact test for qualitative variables and unpaired *t* test for quantitative normally distributed variables were used. The survival curve was generated using the Kaplan-Meier technique and differences between these curves were tested by the log-rank test. All tests were two-sided and the level of statistical significance was accepted at $p \leq 0.05$. For multivariate analyses, the Cox regression model for overall survival was used.

Ethical approval

All patients' data were fully anonymized, and the study was performed according to the standards set in the Declaration of Helsinki 1975. All tumor tissue used was leftover material that had initially been collected for histopathological diagnostics. All diagnostic procedures had already been fully completed when samples were retrieved for the study. The current study was approved in writing by the Ethics Committee of the Ludwig-Maximilians-University, Munich, Germany (approval number 18-130). Authors were blinded for clinical information during experimental analysis.

Results

The results of immunohistochemistry and FISH analyses are summarized in Table 2 and correlated to the histological subtypes.

Table 2 Distribution of HER2 amplification (HER2), ALK/EML4 fusion (ALK), mismatch repair deficiency (MMRd), and PD-L1 status (positivity of ≥ 1% of the tumor cells) according to the histological type of ovarian carcinoma

	High-grade serous ($n = 233$)	Low-grade serous ($n = 19$)	Endometrioid ($n = 23$)	Mucinous ($n = 13$)
HER2 ($n = 11$)	11 (4.7%)	0	0	0
ALK ($n = 2$)	1 (0.4%)	0	1 (4.3%)	0
MMRd ($n = 1$)	1 (0.4%)	0	0	0
PD-L1 ($n = 57$)	55 (23.6%)	0	2 (8.7%)	0

PD-L1 immunohistochemistry

Fifty-seven of the 288 cases (19.5%) were PD-L1 positive (≥ 1%), of which 55 were HGSC (23.6%) (Fig. 1). The detailed results are listed in Table 3. Generally, the PD-L1 status based on the "Cologne score" did not correlate with the histological type ($p = 0.271$); however, positivity ≥ 25% was only present in HGSC. In addition, the number of cases in the non-HGSC categories was limited. In univariate analysis, overall survival was significantly better in cases with PD-L1 immunoreactivity, referring to the "Cologne score" ($p = 0.012$; Fig. 2). This was confirmed by multivariate Cox regression analysis showing PD-L1 expression as a significant and independent prognostic factor with hazard ratio of 2.302 if dichotomized for < 1 and ≥ 1% (Table 4).

HER2 status by immunohistochemistry and FISH

By immunohistochemistry, 3 out of 288 cases (1.0%) were scored as 3+, 42 cases (14.6%) as 2+, and 243 cases (84.4%) as 0 or 1+. HER2-FISH could be analyzed in 284 cases (98.6%), 4 cases were technically not adequate for evaluation. Amplification was found in 11/284

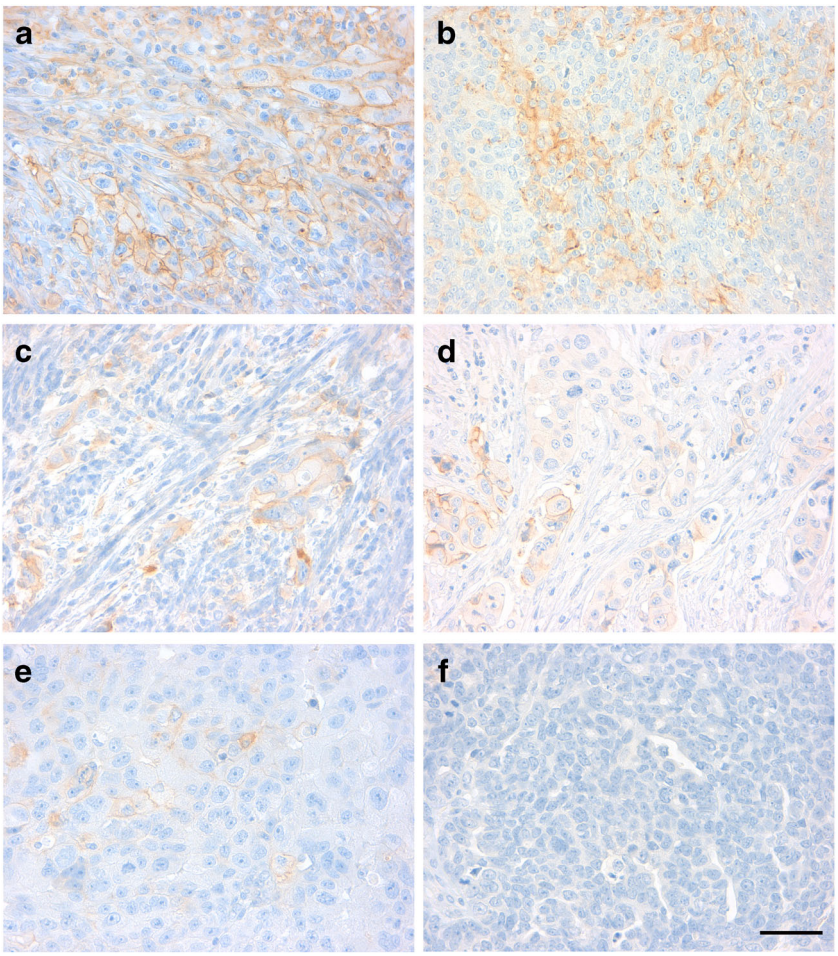

Fig. 1 Immunoreactivity of PD-L1. PD-L1 expression in ≥ 50% (**a**), ≥ 25 and < 50% (**b**), ≥ 10 and < 25% (**c**), ≥ 5 and < 10% (**d**), ≥ 1 and < 5% (**e**), and < 1% (**f**) of tumor cells. For all figures ×400 magnification was used (scale bar refers to 50 μm)

Table 3 PD-L1 score ("Cologne score") related to the histological type of ovarian carcinoma

Histological subtypes		High-grade serous ($n = 233$)	Low-grade serous ($n = 19$)	Endometrioid ($n = 23$)	Mucinous ($n = 13$)	Total (288)
PD-L1	< 1%	178 (76.4%)	19 (100%)	21 (91.2%)	13 (100%)	232 (80.5%)
	≥ 1%	13 (5.6%)	0	1 (4.4%)	0	14 (4.8%)
	≥ 5%	13 (5.6%)	0	0	0	13 (4.5%)
	≥ 10%	21 (9.0%)	0	1 (4.4%)	0	22 (7.6%)
	≥ 25%	7 (3.0%)	0	0	0	7 (2.4%)
	≥ 50%	1 (0.4%)	0	0	0	1 (0.3%)

(3.9%) cases (Fig. 3a, b), and in another 11 cases the HER2 status was originally equivocal by FISH. Based on the guidelines of Wolff et al. 2018, the 11 cases of HER2 equivocal status would be classified as HER2 negative by FISH [40]. The HER2 status was concordant between immunohistochemistry and FISH: The three cases with score 3+ were all amplified. The 42 score 2+ cases consisted of 8 amplified cases and 28 cases without amplification. In five cases with score 2+, the HER2 status remained equivocal by FISH and one case could not be evaluated. The results are detailed in Supplementary Table 2. HER2 amplification was only present in HGSC (4.7% of HGSC), taken into account the small number of cases in the non-HGSC categories. Although 7 of 11 patients with HER2-amplified carcinomas (63.6%) died of disease, HER2 amplification did not correlate with survival ($p = 0.616$).

ALK/EML4 fusion by immunohistochemistry and FISH

ALK/EML4 FISH could be evaluated in 271 cases (94.1%). Seventeen samples were technically not adequate for evaluation. Translocation of ALK/EML4 was detected in 2/271 cases (0.74%), of which one was a HGSC and the other a grade 2 EC (Fig. 3c–f). Both cases presented at FIGO stage III, were HER2- and PD-L1-negative, and showed no MMRd. The patients died of tumor (survival time: HGSC 31.1 months, EC 12.9 months). By immunohistochemistry both cases showed a strong cytoplasmic reactivity, EC additionally nuclear staining (Fig. 3e).

FISH analysis showed for EC ALK split signals in 23.3% and ALK split signals with combined ALK/EML4 inversion in 11.7% of tumor cells; in 75% of tumor cells, ALK signals were normal without breakpoints. The HGSC revealed ALK split signals in 63.3% and ALK split signals with combined ALK/EML4 inversion in 43.3% of the tumor cells; in 16.7% of the tumor cells, ALK signals were normal without breakpoints. ALK immunoreactivity without underlying ALK/EML4 fusion was not found.

Immunohistochemistry for mismatch repair proteins

Loss of immunoreactivity for MSH2 and MSH6 was detected in only one case of HGSC (pT3cpN1pM1; FIGO stage IV)

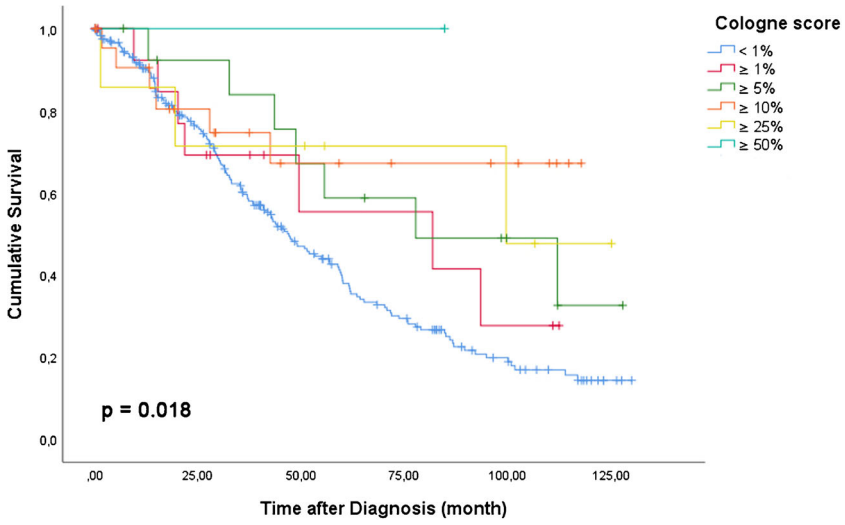

Fig. 2 Kaplan-Meier survival analysis for PD-L1. Censoring events have been marked in the graph

Table 4 Multivariate Cox regression analysis with overall survival for PD-L1 ($n = 288$ for all variables)

Variable	Hazard ratio	95% Confidence interval	p value
Age (per year)	1.209	1.065–1.373	0.003
Grading G1 versus G2/3	1.738	0.831–3.636	0.142
Histological type High-grade serous versus all other histological types versus HGSC	0.989	0.611–1.576	0.707
FIGO (I/II versus II/III)	2.394	1.466–3.535	< 0.001
PD-L1 (≥ 1 versus < 1%)	2.302	1.499–3.535	< 0.001

(Fig. 4) and for this case MSI was confirmed by molecular analysis. Furthermore, this case was negative for HER2, ALK, and PD-L1. The patient was diagnosed at the age of 44 years and died of tumor-dependent death (survival 8.3 months).

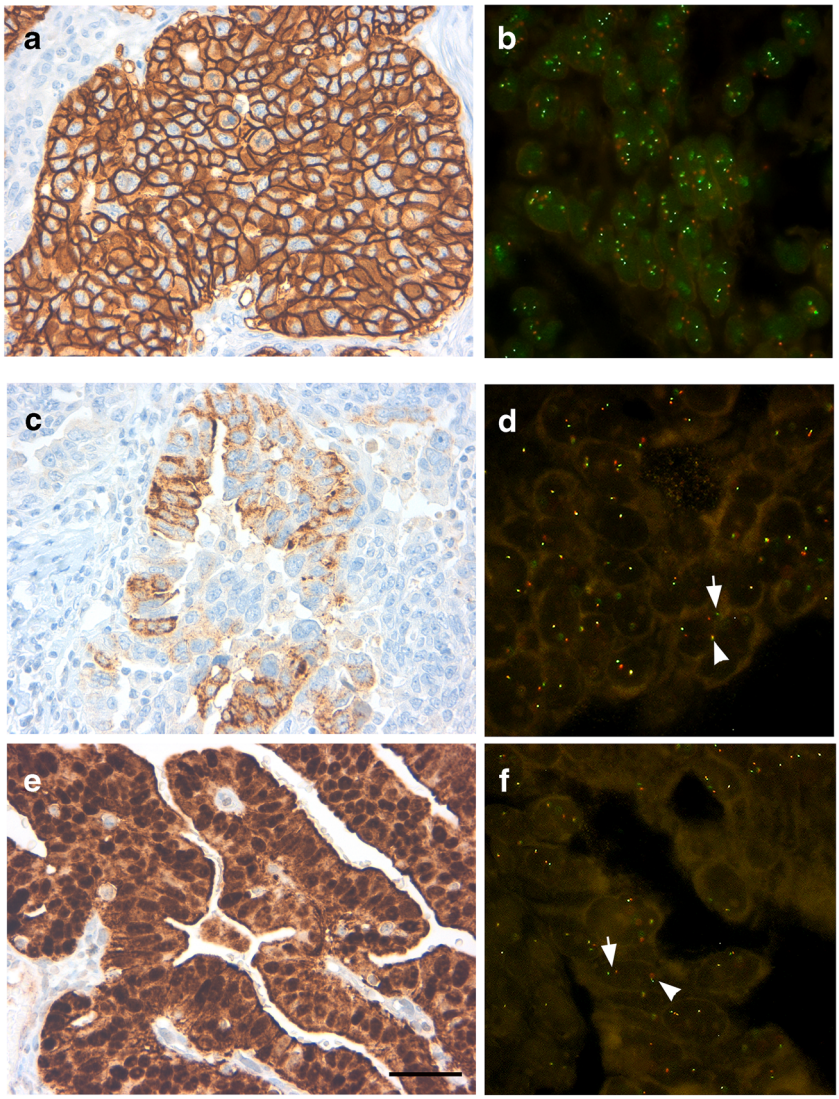

Fig. 3 HER2 amplification and ALK/EML4 translocation: HGSC with strong cytoplasmic and membranous (score 3+) immunoreactivity (**a**) and amplification (**b**) of HER2. HGSC with strong granular cytoplasmic immunoreactivity of ALK (**c**) and ALK/EML4 translocation (**d**). Endometrioid carcinoma with unusual nuclear and cytoplasmic ALK immunoreactivity (**e**) and ALK/EML4 translocation (**f**). Arrows point to ALK split signals and arrow-heads point to ALK fusion signals (**d**, **f**). ×400 magnification was used for all immunohistochemical figures (**a**, **c**, **e**), scale bar refers to 50 μm. ×1000 magnification was used for FISH photomicrographs (**b**, **d**, **f**)

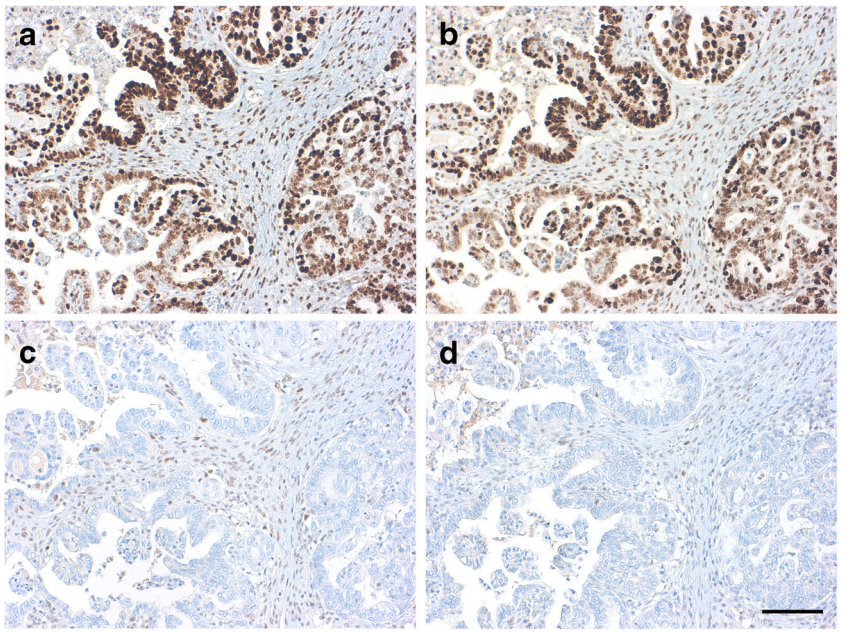

Fig. 4 HGSC with MMRd showing loss of immunoreactivity for MSH2 (**c**) and MSH6 (**d**), while expression of MLH1 (**a**) and PMS2 (**b**) was preserved. ×200 magnification was used for all pictures (scale bar refers to 100 μm)

Discussion

In this study we analyzed various predictive biomarkers for putative targeted therapy in a clinicopathologically well-characterized collective of 288 OC of which 80% were HGSC. We found PD-L1 positivity ≥ 1% in about one quarter of HGSC and in less than 5% of the heterogeneous non-HGSC group. Stronger PD-L1 immunoreactivity (≥ 25%) was limited to HGSC. MMRd was very rare (in < 1% of the cases) and, interestingly, present HGSC. HER2 amplification was found in about 5% of HGSC whereas ALK/EML4 fusion was very uncommon. Therefore, HGSC might be considered for immune checkpoint inhibitor therapy due to frequent PD-L1 expression and less likely for anti-HER2 or tyrosine kinase inhibitor therapy. We would like to stress the strengths of this study represented by the well-characterized patients' collective with long-term follow-up. In addition, this collective underwent systematic histological review and immunohistochemical typing. We are aware that the number of cases in the non-HGSC categories is limited and, therefore, we handled the results of subgroup analysis with caution. Another point of critique could be the use of TMA which contain less amounts of the tumor compared to whole section. Previous studies have shown for several biomarkers, in particular, for PD-L1 that TMA is usually representative for whole sections [10, 17, 23].

Currently available data suggest an increasing importance of immune checkpoint inhibitors in the treatment of OC [3, 14]. So far, it is not clear whether in OC PD-L1 expression of the tumor cells or of the immune cells is predictive for therapeutic response and whether the histological type plays a role. In this study, we analyzed the positivity for PD-L1 in the tumor cells and found positivity in 1% or more in about 20% of all cases and in about 24% of all HGSC, respectively. In addition, PD-L1 expression ≥ 1% turned out to be an independent positive prognosticator. This has been found by others and may be caused by the high numbers of tumor-infiltrating lymphocytes in PD-L1 positive cases [6]. However, the prognostic impact of the PD-L1 status in OC is controversial since PD-L1 expression may correlate with poor prognosis in OC [38, 43].

MMRd and MSI, respectively, have been demonstrated as predictor of an effective immunomodulatory therapy [9, 15]. However, based on our and others' findings, MMRd seems to be very rare in OC. We studied MMRd in all cases and found loss of MSH2 and MSH6 associated with MSI in only one case of HGSC (< 1%). The other OC types showed retained MMR immunoreactivity which is in contrast to the literature [9, 27]. However, these results need to be handled with caution since our EC subgroup was small with 20 cases and clear cell carcinomas were excluded. For our MMRd case, genetic counseling and germ-line testing were not performed. It has been speculated whether this has a favorable prognosis in OC like in colorectal cancer [11, 12], but due to the infrequent occurrence this cannot be assessed [7, 27]. Our single patient was diagnosed at stage IV and survived only 8.3 months.

According to previous studies, HER2 amplification is mainly found in the mucinous type of OC [1, 28]. We found HER2 amplification only in HGSC, whereas all 13 MC were HER2 negative but in this respect the limited number of MC needs to be taken into account. Using the guidance for breast cancer, we were able to show

concurrence between HER2 immunohistochemistry and HER2 FISH and, therefore, immunohistochemistry could be used as primary investigative tool in OC, too. The prognostic impact of HER2 amplification in OC is unclear, which may be related to its rare occurrence. In this and a previous study, there was no correlation between the HER2 status and patients' survival although in a meta-analysis poor outcome was found [24, 29]. Clinical data for anti-HER2 therapy in OC is very limited, although responsiveness was reported for single cases of mucinous type [31].

ALK/EML4 fusion was present in only two cases (0.74%) but it is also an infrequent finding in non-small cell lung cancer by about 4–5% [5]. In non-small cell lung cancer, ALK/EML4 fusion can be very efficiently and reliably detected by immunohistochemistry [19]. Taken a strong, granular cytoplasmic staining as positive, immunohistochemistry for ALK was also in our collective concordant with ALK/EML4 FISH. Interestingly, the case of EC revealed in addition to a cytoplasmic also a strong nuclear immunoreactivity but FISH analysis did not show an uncommon translocation. The prognostic impact of ALK/EML4 fusion is unclear even in non-small cell lung cancer where it is more frequent in younger patients. Both of our OC cases with ALK/EML4 fusion died of tumor. The consideration of ALK rearrangement as a diagnostic and putative therapeutic target needs to be seen with caution. Although the identification of ALK rearrangement is used for therapy in non-small cell lung carcinoma, there is limited evidence for the identification of ALK alterations in OC, only described in few reports and not by the TCGA [4, 16, 35].

In summary, our results show that, particularly, PD-L1 positivity and to a lesser extent HER2 amplification might provide an approach to therapeutic targets in the treatment of OC, which need to be validated in clinical trials. ALK/EML rearrangement is very rare and might, therefore, only be a putative target for selective cases. In addition, the PD-L1 status seems to be an independent positive prognosticator for OC. We are aware that the effort to find a therapeutic target for ovarian cancer needs to be based on solid findings and a proven and demonstrable rationale.

Author's contributions Doris Mayr designed the study. Elisa Schmoeckel designed the study and wrote the first draft of the manuscript. Elisa Schmoeckel, Sophie Hofmann, Daniel Fromberger, and Beate Luthardt carried out the research work and analyzed the data. Miriam Rottmann supported the statistical analyses. Doris Mayr, Alexander Burges, Udo Jeschke, Miriam Rottmann, Thomas Kirchner, and Sigurd F. Lax reviewed and edited the manuscript. All authors gave final approval for publication.

Elisa Schmoeckel takes full responsibility for the work as a whole, including the study design, access to data, and the decision to submit and publish the manuscript.

Compliance with ethical standards All patients' data were fully anonymized, and the study was performed according to the standards set in the Declaration of Helsinki 1975. The current study was approved in writing by the Ethics Committee of the Ludwig-Maximilians-University, Munich, Germany (approval number 18-130).

Conflict of interest There are no business relationships that might lead to a conflict of interest.

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