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Original Research

Comprehensive biomarker analysis of long-term response to trastuzumab in patients with HER2-positive advanced gastric or gastroesophageal adenocarcinoma

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Abstract ***Background:*** A subgroup of patients with HER2-positive metastatic gastric and gastroesophageal junction cancers shows long-term response under trastuzumab maintenance monotherapy. Obviously, HER2 status alone is not able to identify these patients. We

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Prognostic factor;
Gastric cancer;
Trastuzumab

performed this study to identify potential new prognostic biomarkers for this long-term responding patient group.

Patients and methods: Tumour samples of 19 patients with HER2-positive metastatic gastric and gastroesophageal junction cancer who underwent trastuzumab treatment were retrospectively collected from multiple centres. Patients were divided into long-term responding (n = 7) or short-term responding group (n = 12) according to progression-free survival (PFS≥12 months vs. PFS < 12 months). Next-generation sequencing and microarray-based gene expression analysis were performed along with HER2 and PD-L1 immunohistochemistry.

Results: Long-term responding patients had significantly higher PD-L1 combined positive scores (CPS) and CPS correlated with longer progression-free survival. PD-L1 positivity (CPS ≥ 1) was further associated with an increased CD4+ memory T-cell score. The *ERBB2* copy number as well as the tumour mutational burden could not discriminate between short-term and long-term responding patients. Genetic alterations and coamplifications in HER2 pathway associated genes such as *EGFR*, which were connected to trastuzumab resistance, were present in 10% of the patients and equally distributed between the groups.

Conclusion: The study highlights the clinical relevance of PD-L1 testing also in the context of trastuzumab treatment and offers a biological rational by demonstrating elevated CD4+ memory T-cells scores in the PD-L1-positive group.

1. Introduction

Gastric cancer (GC) is the fifth most common cancer with the fourth-highest mortality worldwide [1]. Between 12% and 30% of metastatic gastric and gastroesophageal junction cancers (mGC/mGEJC) show overexpression of the human epidermal growth factor receptor 2 (HER2) caused by *ERBB2* amplification [2].

In 2010, the ToGA study demonstrated that the addition of trastuzumab, a humanised monoclonal HER2-targeting antibody, to chemotherapy significantly improved the survival of patients with HER2-positive mGC/mGEJC in comparison to chemotherapy alone [3]. Even though this was a tremendous success, the overall survival remained poor with a median of 13.8 months [3,4]. However, several case reports and few smaller studies have reported that a subgroup of HER2-positive mGC/mGEJC patients showed prolonged survival for several years under maintenance therapy with trastuzumab alone [5–8]. To date, a biomarker that reliably identifies this long-term responder to trastuzumab treatment remains elusive.

A high level of HER2 gene amplification was proposed as improved predictor of sensitivity to trastuzumab treatment, but the studies defined different thresholds [9–12]. Furthermore, a tumour mutational burden (TMB) of more than 10 mutations per Mb was indicated to be a potential biomarker for trastuzumab efficacy [13]. Moreover, initial resistance to trastuzumab or impaired treatment response was associated with the presence of co-amplifications and mutations of oncogenes or genes related to the HER2 signalling pathway such as *EGFR* and *KRAS* [10,14,15]. In addition, intratumoural heterogeneity of HER2 protein expression, frequently found in GC and GEJC, was associated with poor prognosis [16–18].

In 2021, immunotherapy offered new treatment options for mGC/mGEJC patients and the combination of nivolumab/pembrolizumab and chemotherapy was approved as first-line therapy for programmed cell death ligand 1 (PD-L1) expressing tumours. Since then, PD-L1 expression is routinely assessed in clinical diagnostics in addition to the HER2 status [4,19,20]. Recent studies like the KEYNOTE-811 explored the efficacy of anti-PD-L1 treatment in HER2-positive mGC/mGEJC showing that the combination of pembrolizumab and trastuzumab with chemotherapy leads to improved response rates compared to treatment with chemotherapy and trastuzumab alone [21]. In addition, the combined expression of PD-L1 and HER2 was found to be a positive prognostic factor for survival in GC [22]. Findings from a preliminary biomarker analysis of the DESTINY-Gastric03 trial showed a large overlap between HER2 and PD-L1 expression in 85% of the patients GC or GEJC [23]. In the KEYNOTE-811 study, similar frequencies for double positivity (HER2+/PD-L1+) have been reported [21].

In this retrospective study, we divided HER2-positive mGC/mGEJC patients into two groups based on progression-free survival (PFS) under trastuzumab-based treatment and performed an explorative analysis of biomarkers. We applied targeted next-generation sequencing (NGS) using a 409 gene panel, Affymetrix gene expression analysis and immunohistochemistry to evaluate biomarkers and investigated their prognostic impact in this cohort.

2. Material and methods

2.1. Patients

In this retrospective study, formalin-fixed paraffin-embedded (FFPE) tumour samples from locally tested HER2-positive advanced and/or metastatic adenocarcinoma of the stomach or gastro-oesophageal junction prior to trastuzumab therapy were collected. Diagnoses were established between 2010 and 2021. Patients with available survival and therapy information as well as available tumour tissue were included (Fig. 1). All patients received a platinum and 5-FU-based chemotherapy in combination with trastuzumab (Supplementary table 1). Tumour FFPE samples were received from the archives of the Institutes of Pathology of the University Medical Center Mannheim, Nord-West Krankenhaus Frankfurt, Technical University Munich, and University of Luebeck. Tumour-adjacent normal tissue and five healthy gastric mucosa samples were collected from the archive of the Institute of Pathology of the University Medical Center Mannheim. Samples were reviewed histologically and tumour areas were marked by two board-certified pathologists (TG, DH). This study was approved by the local ethics committee of the Medical Faculty Mannheim of Heidelberg University (2016-080R-MA).

2.2. Grouping

Patients were separated into two groups according to PFS under trastuzumab-containing therapy. The first group includes patients with PFS longer or equal to 12 months. Patients with short-term response (PFS<12 months) are in the second group.

2.3. Immunohistochemistry and in-situ hybridisation

FFPE tissue sections with 2–3 µm thickness were used for all immunohistochemistry (IHC), silver and fluorescence *in-situ* hybridisation (SISH/FISH) experiments.

2.4. PD-L1

PD-L1 IHC was performed on a VENTANA BenchMark ULTRA autostainer utilising OptiView DAB Detection kit (Roche Diagnostics GmbH, Mannheim, Germany), and Biocare PD-L1 antibody (clone CAL10, 1:50, ACI 3171 C, Biocare Medical, Den Haag, Netherlands). Testing was done alongside with a tonsil section as on-slide positive control. PD-L1-stained slides were interpreted according to combined tumour and immune cell score (combined positive score, CPS) by two board-certified pathologists (T.G.; D.H.) who received appropriate training.

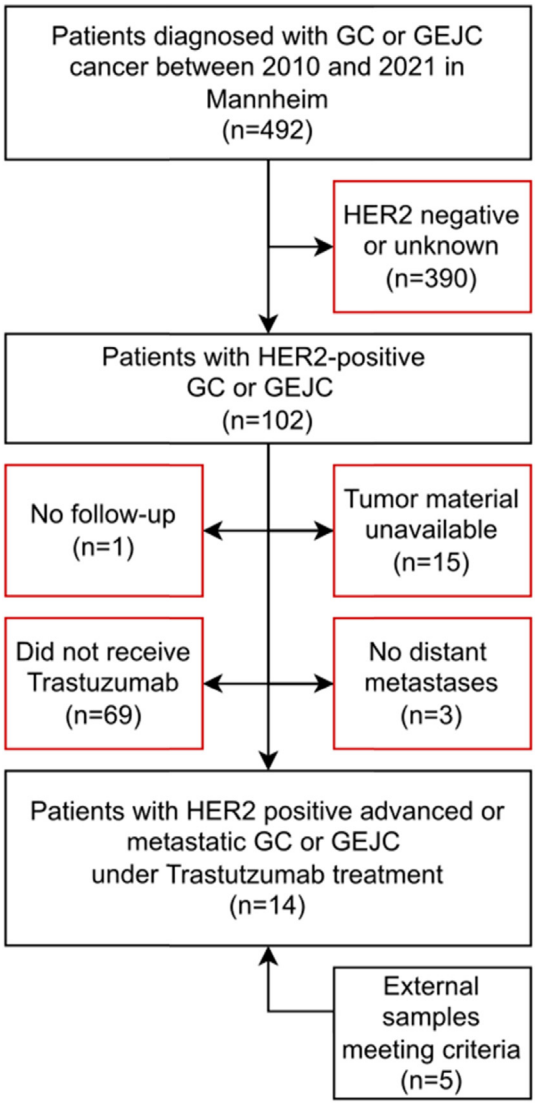

Fig. 1. Flowchart of the selection process of the retrospective study cohort. Criteria for exclusion are shown in red. FFPE, Formalin-fixed paraffin-embedded; HER2, human epidermal growth factor receptor 2; gastric and gastroesophageal junction cancers, GC/GEJC. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.5. HER2

The HER2 status of all samples was centrally re-evaluated. HER2 IHC was carried out with primary HER2 antibody (polyclonal, 1:500, A0485, Dako/Agilent). For heat-induced antigen retrieval, the sections were immersed in pre-heated Epitope Retrieval Solution, pH 6 (1:10, RE7113, Leica Biosystems, UK) and incubated at 95 °C in a water bath for 40 min. For detection, the EnVision Detection System, Peroxidase/DAB, Rabbit/Mouse (Agilent, Santa Clara, CA, USA) was used. The HER2 status was evaluated according to CAP guidelines [24]. In cases with equivocal HER2 status (2+), either SISH or FISH was used to detect

chromosomal amplification of HER2. For SISH, the VENTANA HER2 dual ISH Assay (Roche Diagnostics GmbH, Mannheim, Germany) was executed on a VENTANA BenchMark ULTRA autostainer using Ventana HER2 Dual ISH DNA Probe cocktail with Ventana silver ISH DNP and Ventana Red ISH DIG Detection kits (Roche Diagnostics GmbH, Mannheim, Germany). For FISH, a CEN17/*ERBB2* Dual Color Probe was used (ZytoVision GmBH, Bremerhaven, Germany). Pre-treatment, denaturation, hybridisation, and detection were done with the ZytoLight FISH-Tissue Implementation Kit (ZytoVision GmBH, Bremerhaven, Germany) according to the manufacturer's protocol. Slides were evaluated using an Olympus A0485 fluorescence microscope with UPlanSApo 60× objective. In addition, the uniformity of HER2 expression in all samples was microscopically evaluated by two board-certified pathologists (TG, DH). HER2 heterogeneity was assessed for all staining intensities as described by Motoshima *et al.* [25].

2.6. EBV

To detect Epstein–Barr virus-encoded small RNAs (EBER), automated *in-situ* hybridisation was performed on VENTANA BenchMark ULTRA autostainer with Ventana EBER Probe and VENTANA ISH iview Blue Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany).

2.7. MSI

Microsatellite status was determined using Idylla MSI Test (Biocartis NV, Belgium) according to the manufacturer's protocol.

2.8. Nucleic acid isolation

For DNA and RNA isolation, one to five 10 µm FFPE tissue sections were cut for tumour and normal samples. The tumour areas were macro-dissected, guided by prior markings on a HE-stained slide, to ensure high tumour content. Nucleic acid isolation was performed with the RecoverAll™ Multi-Sample RNA/DNA Isolation Workflow according to the manufacturer's protocol. Concentrations were determined using a Qubit™ 3 fluorometer with the Qubit™ RNA high sensitivity (HS) Assay-Kit and Qubit™ dsDNA HS Assay-Kit. Devices and reagents were purchased from Thermo Fisher Scientific, Waltham, MA, USA.

2.9. Target panel sequencing

Before library preparation, 20 ng DNA was treated with uracil DNA glycosylase according to the manufacturer's protocol. Then, the DNA was used to prepare the sequencing library for the Oncomine™ Tumor Mutation Load Assay (TML) with an Ion Chef Instrument. Libraries were diluted to 50 pM and loaded with an Ion 540 chip for sequencing on an Ion GeneStudio™ S5 Prime system. The TML panel covers 409 full-length genes and 1.65 Mb. Sequencing and library preparation were performed in duplicates for all samples. Devices and reagents for library preparation and sequencing were obtained from Thermo Fisher Scientific, Waltham, MA, USA.

Bam files of tumour and normal samples were downloaded from the IonReporter for analysis of single nucleotide and copy number variants (SNV/CNV) using an automated snakemake pipeline [26]. Detailed information and the pipeline code are available on github (https://github.com/IPorth/TMLflow). In CNV calling, shallow amplification, amplification, deletion and deep deletion were defined at the following copy number (CN) thresholds: $CN \geq 3$, $CN \geq 4$, $CN \leq 1$ and $CN = 0$, respectively. SNVs were filtered for allele frequency (AF) > 0.1 and read depth (DP) > 250. All mutations were visually inspected using the Integrative Genomics Viewer [27]. TMB was calculated by dividing the number of mutations, including non-synonymous and frameshift mutations, by the Mb size of the TML panel.

2.10. Microarray

In total, 18 tumour and 5 healthy gastric mucosa RNA samples were used for gene expression profiling using GeneChip® Human Transcriptome 2.0 Arrays (Thermo Fisher Scientific, Waltham, MA, USA). Biotinylated antisense cDNA was then prepared according to the standard labelling protocol with the GeneChip® WT Plus Reagent Kit and the GeneChip® Hybridization, Wash and Stain Kit (Thermo Fisher Scientific, Waltham, MA, USA). Next, the hybridisation on the chip was performed in a GeneChip® Hybridization oven 640, then dyed in the GeneChip® Fluidics Station 450 and subsequently scanned with a GeneChip® Scanner 3000. All of the equipment used was from the Affymetrix-Company (Affymetrix, High Wycombe, UK). A Custom CDF Version 22 with ENTREZ-based gene definitions was used to annotate the arrays [28]. The raw fluorescence intensity values were normalised by applying quantile normalisation and RMA background correction. Pat1 could not be included in the Affymetrix microarray analysis as the patient sample arrived after the experiment.

2.11. Bioinformatics immune cell analysis

For evaluation of immune cell populations and activity, normalised expression values were used as input for xCell [29], CIBERSORTx [30] and for calculation of the cytolytic activity (CYT) score [31]. With CIBERSORTx, the absolute cell fractions of 22 immune cell types were

calculated. The gene signature enrichment of 64 cell types was analysed with xCell.

2.12. Statistical analysis

Statistical analysis was performed with GraphPad Prism 9 (GraphPad Software; www.graphpad.com) and R4.0.5 (https://www.r-project.org/). The Wilcoxon rank sum test was applied for comparisons of continuous variables between patient groups. Categorical variables were tested with Fisher's exact test. The association between data series was calculated using the Spearman correlation coefficient. Progression-free survival was statistically evaluated using Kaplan–Meyer analysis and compared with the Mantel–Haenszel test. Statistical significance was accepted at $p \leq 0.05$. The significance levels were defined as follows: ns not significant, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$.

2.13. Data availability

The Affymetrix gene expression microarray data of 18 tumour and 5 normal samples are on GEO under accession number GSE220917. Sequencing data will be provided upon request.

3. Results

Nineteen patients with HER2-positive mGC/mGEJC adenocarcinomas obtained from four German medical centres were included in this retrospective study. The patients were grouped according to PFS under trastuzumab-based therapy into a short-term responder group (n = 12) and a long-term responder group (n = 7). At a median follow-up of 18 months, the median PFS was 6 months and 22 months in the short-term responding and long-term responding patients, respectively. There were no significant differences in the clinicopathological characteristics or the HER2 status between patient groups (Table 1). However, there was a slight gender and age imbalance, as patients in the long-term responding group were all male (p = 0.2), and they tend to be younger at diagnosis (p = 0.1).

Immunohistochemical staining of PD-L1 was performed on tumour samples prior to trastuzumab treatment (Fig. 2A). A positive PD-L1 status (CPS≥1) was detected in 47.3% of the patients. Although CPS values were in general very low, CPS was significantly higher in long-term responders compared to patients with short-term response (p = 0.005, Fig. 2B). Further statistical analysis revealed a correlation between CPS and PFS, when the patients were not grouped (r = 0.596, p = 0.007, Fig. 2C). PD-L1 positivity was statistically associated with longer PFS (hazard ratio 0.27, 95% CI 0.084–0.841, Fig. 2D) compared to PD-L1 negative patients. In addition, PD-L1 expression was significantly correlated with younger age at diagnosis (p = 0.019).

To investigate immune cell populations and activity in the patient cohort, the microarray data were analysed with xCell and CIBERSORTx. Both cell population analyses did not exhibit differences in CD8+ and CD4+ T-cells between the patient groups (Supplementary figure 1), but the analysis detected an increased abundance of CD4+ memory T-cells in PD-L1-positive

Table 1
Clinicopathological patient characteristics in long-term responding versus short-term responding patients.

Characteristics	Long-term responder (n = 7)[a]	Short-term responder (n = 12)[a]	p-value[b]
Age	59 (58,62)	71 (63,72)	0.1
Gender			0.2
Male	7 (100%)	8 (67%)	
Female	0 (0%)	4 (33%)	
PFS	22 (19,40)	6 (4,9)	<0.001
Location			>0.6
GEJC	3 (43%)	7 (58%)	
GC	4 (57%)	5 (42%)	
Differentiation			>0.9
G2	4 (57%)	6 (45%)	
G3	3 (43%)	5 (45%)	
Unknown	0	1 (9.1%)	
No. of metastatic sites			0.5
1	3 (43%)	4 (33%)	
2	2 (29%)	7 (58%)	
>2	2 (29%)	1 (8.3%)	
Perioperative chemotherapy[c]	1 (14%)	3 (25%)	>0.9
HER2 Status			>0.9
2+ (ISH positive)	2 (29%)	5 (36%)	
3+	5 (71%)	7 (64%)	

[a] n (%); Median (IQR).
[b] Fisher's exact test; Wilcoxon rank sum test.
[c] Without trastuzumab.

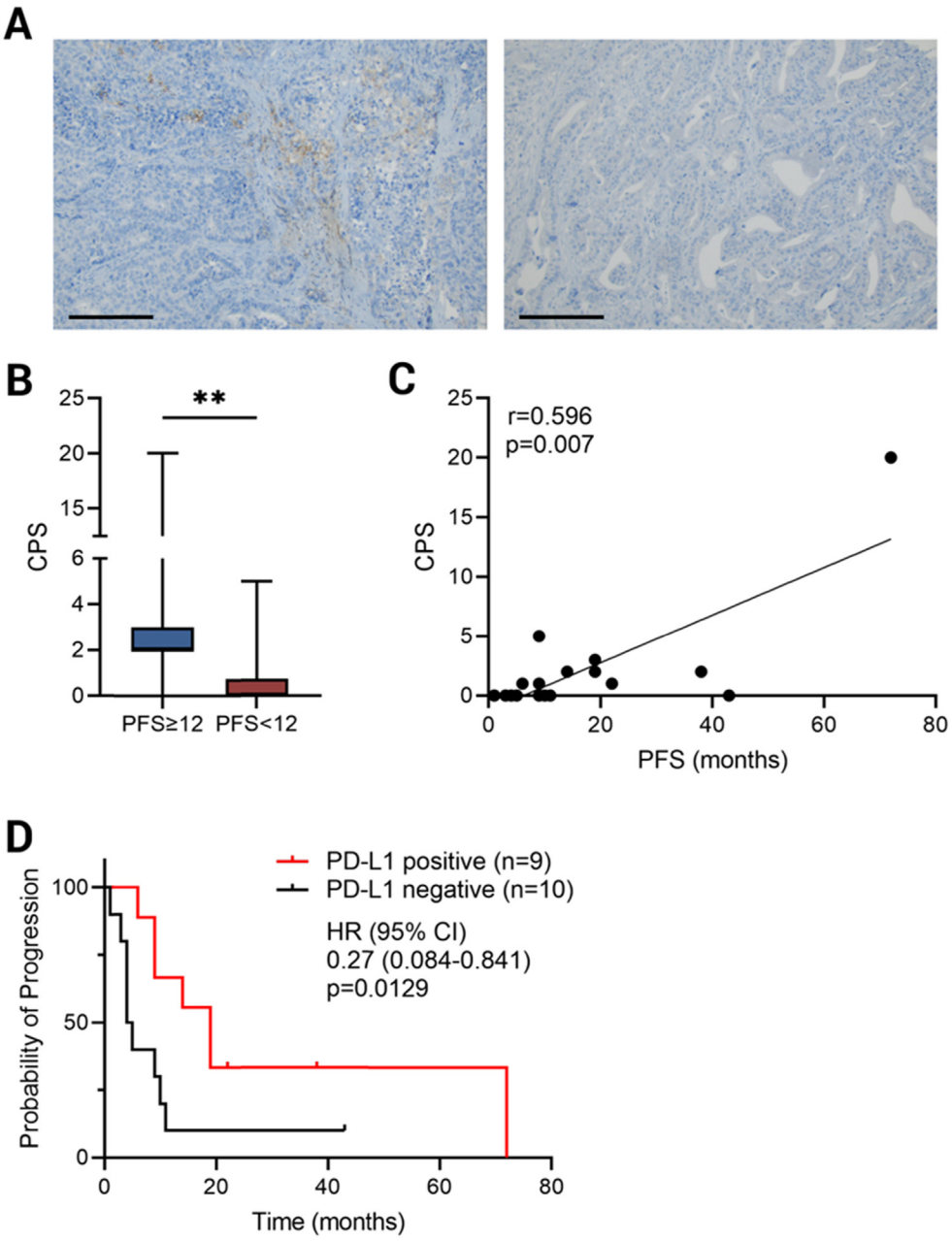

Fig. 2. Evaluation of clinical relevance of PD-L1 expression in HER2-positive mGC/mGEJC. A: Exemplary PD-L1 IHC stainings of mGC/mGEJC sections of patients with high (left) and low combined positive score (CPS, right). Scale bar: 50 μm. B: Comparison of PD-L1 CPS between long-term responding (blue) and short-term responding (red) patients. C: Correlation analysis of PFS and PD-L1 CPS using the Spearman rank coefficient and linear regression; D: Kaplan-Meyer survival curve comparing PD-L1 positive and negative patients.

patients (Fig. 3). XCell additionally determined higher CD4+ T central memory (Tcm) and CD8+ T effector memory (Tem) cells in PD-L1 negative patients (Fig. 3A). Furthermore, the CYT score was significantly increased in the long-term responder patients but was not associated with positive PD-L1 status (Supplementary figure 2). There was also no correlation between TMB and PD-L1 status (Supplementary figure 3). As a side note, all tumour samples were negative for EBV and microsatellite stable (data not shown).

All included patients had a positive IHC and/or ISH HER2 status according local pathology and centrally confirmation was achieved in 95% (18/19) of the patients. One locally HER2 IHC 3+ tested patient did not show any HER2 staining or FISH amplification at central testing. The reason for this could not conclusively be clarified but intratumoural heterogeneity could be a factor. Determining *ERBB2* CN with NGS revealed an amplification in 68% of the patients. The level of amplification did not differ significantly between patient

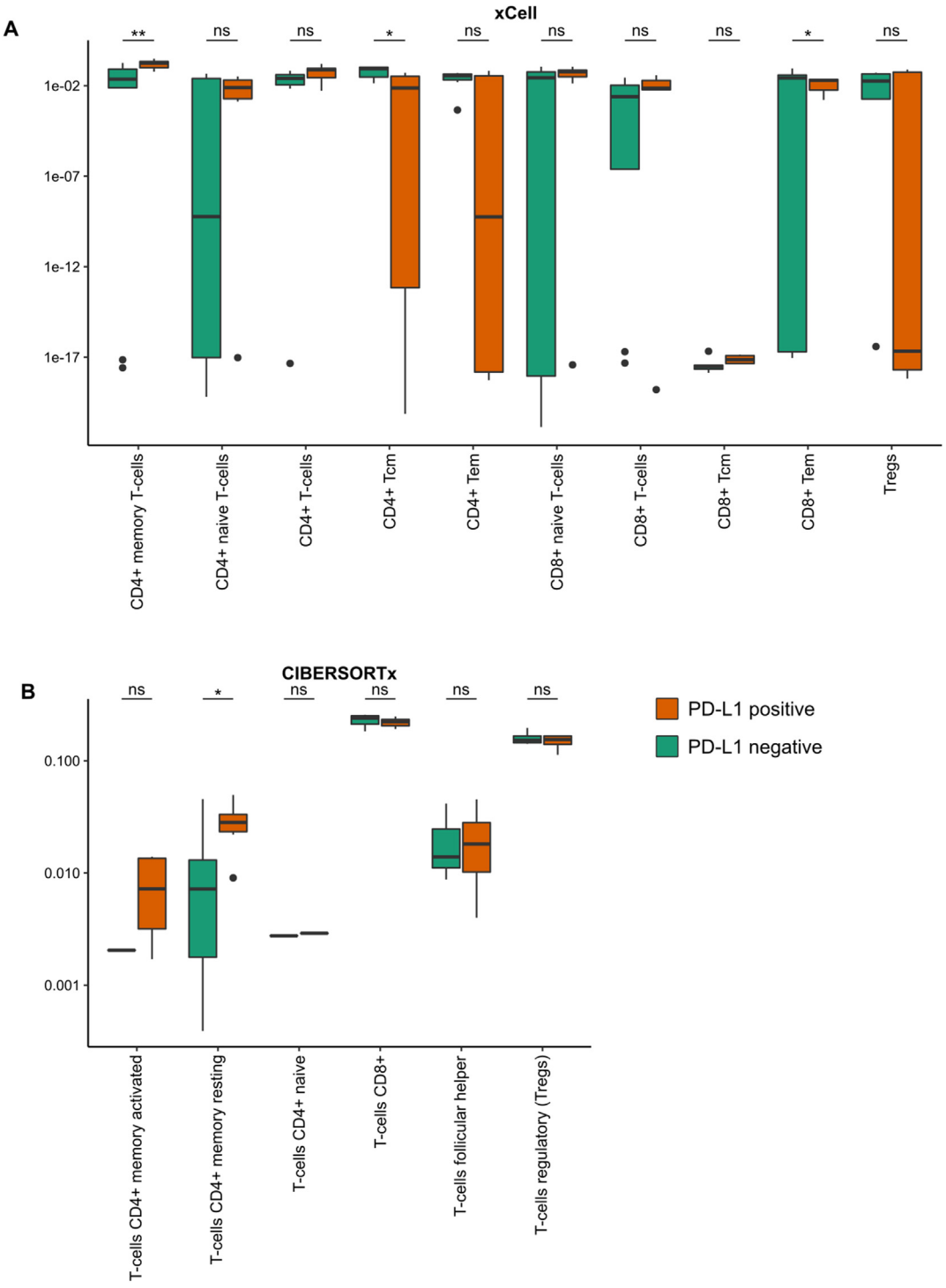

Fig. 3. Comparison of T-cell scores between PD-L1-positive and PD-L1-negative patients using A) xCell and B) CIBERSORTx.

PFS groups (Fig. 4A). The detected *ERBB2* CN was lower in resection specimen, which made up 32% of the tumour samples, but the trend was not statistically significant (Fig. 4B). Moreover, higher *ERBB2* CN were determined in samples with homogenous HER2 expression compared to samples with heterogeneous expression pattern (Fig. 4C).

Two patients in the long-term responder group had missense mutations in *ERBB2* (Fig. 5A). In Patient 5, the *ERBB2* p.P489L mutation was detected together with an *EGFR* R408Q mutation. In this case, *ERBB2* was not amplified based on NGS analysis. Patient 4 carried the *ERBB2* mutation p.V777L in addition to the *ERBB2* amplification. Mutations in HER2 signaling associated genes occurred solely for EGFR (2 of19 patients) and were evenly distributed among the different prognostic groups. Mutations in *KRAS*, *MET* and *PIK3CA* were not detected. *ERBB2* was frequently co-

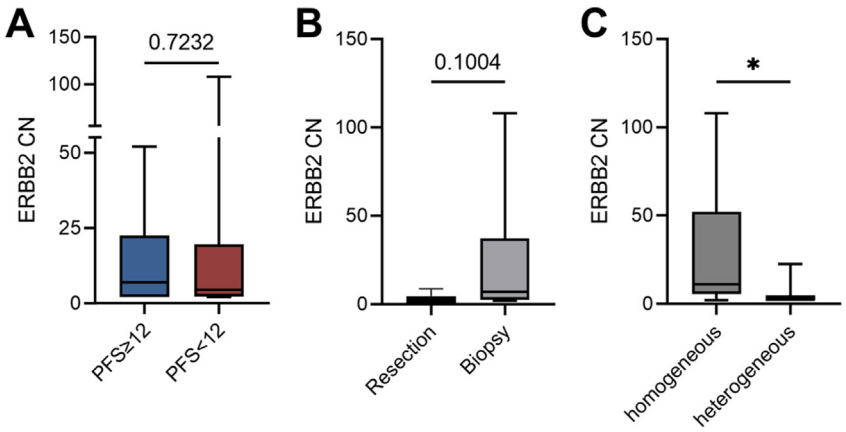

Fig. 4. Evaluation of ERBB2 CN in context of progression-free survival (A), tumour sample type (B) and HER2 expression pattern (C).

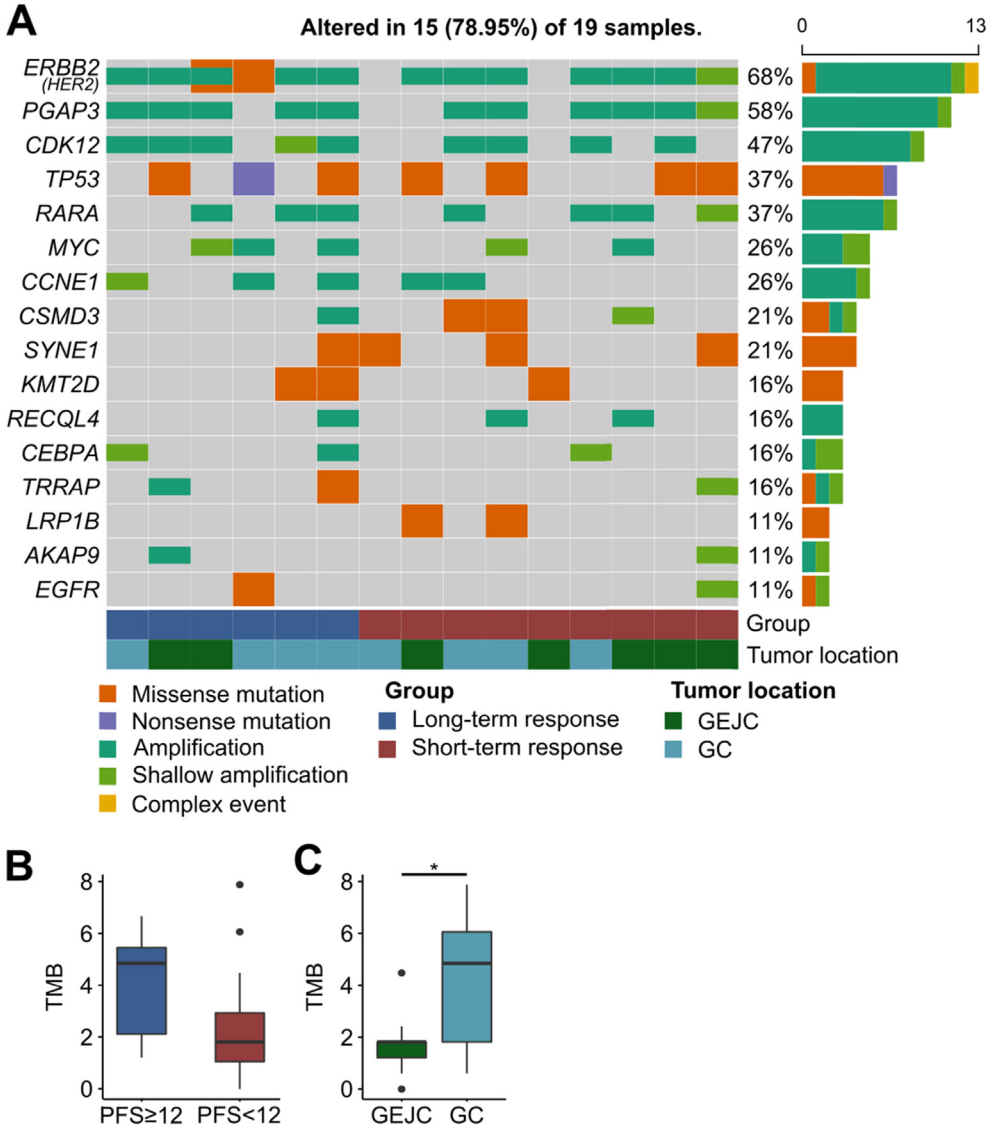

Fig. 5. Distribution of molecular alterations and TMB in HER2-positive mGC/mGEJC patients A) Oncoplot summarising the single nucleotide and copy number variants. C) Tumour mutational burden (TMB) calculated for both patient groups D) TMB association with tumour location.

amplified with *PGAP3*, *RARA* and *CDK12*, which are in spatial proximity to *ERBB2* on chr17. Furthermore, *CCNE1* and *MYC* amplifications were both found in 26% of the patients. The copy numbers of these co-amplified genes did not differ significantly between responder groups (Supplementary figure 4).

In the next step, the TMB was calculated based on the SNV data. A non-significant trend of higher TMB in long-term responding patients could be observed ($p = 0.141$, Fig. 5B), but the TMB was significantly different depending on tumour location ($p = 0.049$, Fig. 5C). The median TMB was 1.82 and 4.85 in tumours located in the GEJC and GC, respectively.

4. Discussion

In this retrospective, multicenter study, we screened for prognostic biomarkers of long-term response to trastuzumab in HER2-positive mGC/mGEJC and found increased PD-L1 expression in the patient group with superior response. In addition, we detected that CD4+ memory T-cells were upregulated in PD-L1-positive patients. A significant prognostic value of *ERBB2* CN, TMB and genomic alterations associated with HER2 signaling could not be confirmed in this cohort.

PD-L1 expression has been found to co-occur with HER2-positivity in 25–85% of GC/GEJ patients [21–23,32]. With 47.3% of HER2+/PD-L1+ patients, our result falls within the reported range. The prognostic role of PD-L1 expression in context of HER2-positive GC is controversial in literature. In the study by Lian *et al.*, combined HER2 and PD-L1 positivity was associated with improved overall survival in resectable GC [22]. In contrast, Lv *et al.* showed that HER2+/PD-L1+ GC patients had inferior survival compared with HER2-/PD-L1- patients [32]. The authors speculated that immunotherapy would be less suitable for HER2-positive GC, which is controversial since the first interim analysis of the KEYNOTE-811 demonstrated an increased response rate of HER2-positive mGC/mGEJC patients to trastuzumab and pembrolizumab combined with chemotherapy [21]. We found improved PFS in HER2+/PD-L1+ mGC/mGEJC patients, which is in accordance with the Lian *et al.* [22]. Due to the design of the study, it could not be concluded if PD-L1 expression is prognostic or predictive in the context of trastuzumab treatment. Taking into account that even in the context of immune checkpoint inhibitors PD-L1 has limitations as predictive biomarker due to variability in evaluated cell type and thresholds for positivity [33], clarifying this issue is reserved for biomarker analysis within large clinical trials, like the KEYNOTE-811 [21].

We observed an increased CD4+ memory T-cells score in PD-L1-positive patients which is in accordance with literature as PD-L1 expression in GC was associated with increased numbers of tumour infiltrating lymphocytes (TIL), such as CD4+ and CD8+ T-cells [34,35]. However, the studies are controversial about the effect of TIL on survival. The presence of CD8+ T-cells in combination with PD-L1 expression was associated with improved and impaired OS in HER2 negative GC [36,37]. To our knowledge, increased CD4+ memory T-cells in PD-L1 and HER2 positive mGC/mGEJC versus PD-L1 negative and HER2 positive mGC/mGEJC have not been reported yet.

To date, positive HER2 status by IHC or ISH is the prerequisite for the administration of trastuzumab in mGC/mGEJC patients. Besides that, a higher amplification level of *ERBB2* was tended to be associated with improved survival under trastuzumab treatment [9–12]. NGS is an emerging technology with the potential to detect the absolute *ERBB2* copy number. A cut-off defining high *ERBB2* amplification in sequencing data has not been established yet. Studies have used the median *ERBB2* CN or calculation of the receiver operating characteristic curve to set a threshold for identification of patients with long-term benefit. However, there is no consensus about the best threshold defining a high *ERBB2* amplification in NGS data [10,12]. In our cohort, the anticipated *ERBB2* amplification could only be confirmed by using NGS in 68% of the patient. Heterogeneity as well as low tumour purity could explain this discrepancy between detected HER2 status by IHC/ISH and *ERBB2* CN assessed by NGS [38,39].

ERBB2 mutations rarely occur in HER2-positive mGC/mGEJC but are mainly associated with resistance to trastuzumab [10,40,41]. In our study, we detected two *ERBB2* missense mutations (p.V777L and p.P489L) in two long-term responding patients. The p.V777L missense mutation was located in the tyrosine kinase domain and was reported once in a HER2-positive mGC patient with fast-progressing disease under trastuzumab [10]. One may speculate that in our case the mutational surrounding complementing the *ERBB2* mutation might have changed the anticipated effect of this mutation. The other *ERBB2* mutation, p.P489L, was located in the extracellular domain of HER2. It has not been reported in context of trastuzumab response in mGC/mGEJC, yet but it was associated with increased sensitivity to trastuzumab in acute myeloid leukaemia [42].

Mutations or amplification in HER2 signaling associated genes like *EGFR*, *KRAS*, *MET* or *PIK3CA* occurring in addition to *ERBB2* alterations were found to be predictors for impaired response or resistance to trastuzumab treatment [10,15]. Of the above-mentioned genes, only *EGFR* alterations were detected in our study but no association to the patient groups was obvious. In general, the detected genetic alterations were not associated with PFS in our study.

Our work has several limitations. First, our retrospective cohort has a small sample size. In addition, the

cohorts are heterogeneous with respect to tumour location (GEJC vs. GC). All analysed samples were collected prior to trastuzumab treatment, thus acquired response mechanisms have not been investigated. We perform targeted panel sequencing which restricts the analysis of genomic alterations to 409 genes. Alterations in genomic regions outside the targeted genes might have been missed in this study. A stringent AF (>0.1) and DP (>250) thresholds for SNV calling to exclude sequencing artifacts could have resulted in missing low frequency mutations.

5. Conclusions

To conclude, in our study *ERBB2* CN, TMB, co-amplifications or mutations in HER2 pathway associated genes did not exhibit prognostic relevance for detecting trastuzumab long-term responding patients. However, the data highlight the clinical relevance of PD-L1 IHC testing also in the context of trastuzumab treatment and offers a biological rational by demonstrating elevated CD4+ memory T-cells in the PD-L1-positive group.

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Author contributions

Isabel Porth: Investigation, Visualisation, Writing – Original Draft, Software. **Daniela Hirsch**: Investigation, Writing – Review and Editing. **Yonca Ceribas**: Investigation. **Philip Weidner**: Resources. **Wilko Weichert**: Resources. **Thorsten Oliver Götze**: Resources. **Sven Perner**: Resources. **Kim Luley**: Resources. **Christian Moritz Heyer**: Software. **Carolina de la Torre**: Investigation, Data Curation. **Ralf-Dieter Hofheinz**: Resources, Writing – Review and Editing. **Sylvie Lorenzen**: Resources, Writing – Review and Editing. **Timo Gaiser**: Conceptualisation, Writing – Original Draft, Supervision.

Conflict of interest statement

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Wilko Weichert has attended and given talks at Advisory Boards, gave advice to and served as speaker on national and international conferences for MSD, BMS, AstraZeneca, Roche, Pfizer, Merck, Lilly, Boehringer, Novartis, Takeda, Bayer, Amgen, Astellas, Eisai, Johnson & Johnson, Janssen, Illumina, Siemens, Agilent, ADC, GSK and Molecular Health. Wilko Weichert receives research funding from Roche, MSD, BMS and AstraZeneca; Timo Gaiser received honoraria from MSD, Roche, AstraZeneca and BMS. All remaining authors have declared no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejca.2023.01.022.

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