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Prognostic Biomarkers in Early-stage Gastric Adenocarcinoma Treated With Adjuvant Chemoradiotherapy

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Abstract. *Background/Aim: Early-stage gastric cancer has a high risk of recurrence, despite trimodality therapy with surgery, chemotherapy and radiation. To improve patient selection for adjuvant chemoradiotherapy, we evaluated the prognostic significance of immunohistochemical and genetic biomarkers in patients with resected gastric adenocarcinoma. Patients and Methods: Tumors from 119 patients were subjected to immunohistochemistry for 12 protein biomarkers, as well as next-generation sequencing. Clinical and biomarker data were available for 91 patients. Results: EBV-positive tumors and tumors with mutations had higher intratumoral CD8 tumor-infiltrating lymphocyte density (p=0.009 and p=0.017, respectively). PIK3CA mutations were correlated with VEGFA overexpression (p=0.042), while KRAS mutations and HER2 expression were mutually exclusive (p=0.036). PTEN expression univariately confirmed longer overall survival (HR=0.27; p=0.046), while there was a trend between the presence of KRAS mutations and inferior disease-free and overall survival. Conclusion: PTEN protein expression and KRAS mutations may predict disease outcome in early-stage gastric cancer. These results need to be further validated in larger cohorts.*

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Gastric cancer is the fifth most common cancer and the third leading cause of cancer-related mortality worldwide (1). Patients with stage I disease treated with gastrectomy have a 5-year survival rate of approximately 65%, whereas patients with more advanced disease have poorer outcomes with a 5-year survival rate of 30% (2, 3). Complete surgical resection is required to cure gastric cancer (4). However, given the high recurrence rate after surgery, additional therapeutic approaches have been explored. These therapeutic strategies include

adjuvant concurrent chemotherapy and radiation, perioperative chemotherapy and adjuvant chemotherapy (5-9). Several clinical trials have clearly demonstrated the benefit of these adjunctive approaches compared to surgery alone. According to the National Comprehensive Cancer Network and the European Society for Medical Oncology guidelines, for patients with ≥stage IB gastric cancer who have undergone potentially curative surgical resection, postoperative 5-fluorouracil-based chemoradiotherapy is recommended. Despite the proven survival benefit of this approach over surgery alone (5), approximately half of the patients will still relapse and die of their cancer.

Currently, there are no established prognostic or predictive biomarkers for patients with gastric adenocarcinoma who receive adjuvant chemoradiotherapy after potentially curative surgical resection (10). The aim of our current study was to evaluate the prognostic significance of a host of genetic and protein biomarkers in an effort to improve patient selection for adjuvant chemoradiotherapy.

Patients and Methods

Patient cohort. We performed a retrospective analysis in patients with histologically confirmed primary gastric adenocarcinoma treated at cancer centers affiliated with the Hellenic Cooperative Oncology Group (HeCOG). This translational study was approved by the Bioethics Committee of "Attikon" Hospital, Athens, Greece (8/24-09-09). All patients included in the study provided their written informed consent for the use of their biological material for future research. A total of 119 patients who underwent gastrectomy, adjuvant chemotherapy and radiation therapy for gastric cancer from 2005 to 2013 were included in the study. Of the 119 evaluable patients, 26 did not have complete clinical data, including treatment information and follow-up, and were excluded from further analysis.

Tumor blocks from the 93 patients with complete clinical data were retrieved from the HeCOG tumor repository. Upon histological review, tumors were transferred to tissue microarrays (TMA, 2×1.5 mm cores per tumor) that were constructed with a manual arrayer (Model I, Beecher Instruments, San Prairie, WI, USA), as previously described (11, 12). Upon review of hematoxylin-eosin stained sections from the TMA blocks, 2 tumors had insufficient tissue for biomarker analysis. The REMARK diagram for the study is shown in Figure 1.

Immunohistochemistry. Immunohistochemical staining was performed according to standard protocols on serial 2.5-μm thick sections from the TMA blocks. To assure optimal reactivity, immunostaining was applied 7-10 days after sectioning at the Laboratory of Molecular Oncology of the Hellenic Foundation for Cancer Research, Aristotle University of Thessaloniki School of Medicine. The staining procedures for EGFR, HER2, IGF1R, PTEN, pAKT^{308}, MTOR, VEGFA, MLH1, MSH2, MSH6, PMS2 are shown in Table I. Tumors were also evaluated for Epstein-Barr virus (EBV) status using Epstein-Barr encoding region (EBER) in situ hybridization (ISH), as previously described (13).

Interpretation of IHC results. The evaluation of all IHC staining was performed by two experienced pathologists, blinded to the patients' clinical characteristics and survival data. For each tumor core, the intensity of staining (0=no staining, 1=weakly positive, 2=moderately positive, 3=strongly positive), the percentage of tumor cells staining positive and the localization of the stain (nuclear, cytoplasmic or membranous) were assessed. Based on these results, protein expression of all markers was summarized using the H-score (or "histo" score), a semiquantitative approach that scores samples based on the percentage of cells at each staining intensity level, with the final score ranging from 0 to 300, as previously described (14). If one of the tissue cores was lost or damaged the overall score was determined from the remaining one. Cut-offs for protein markers were selected based on previously published studies (15-21). Evaluation of CD8 as intratumoral and stromal infiltrates was performed as previously described (22, 23).

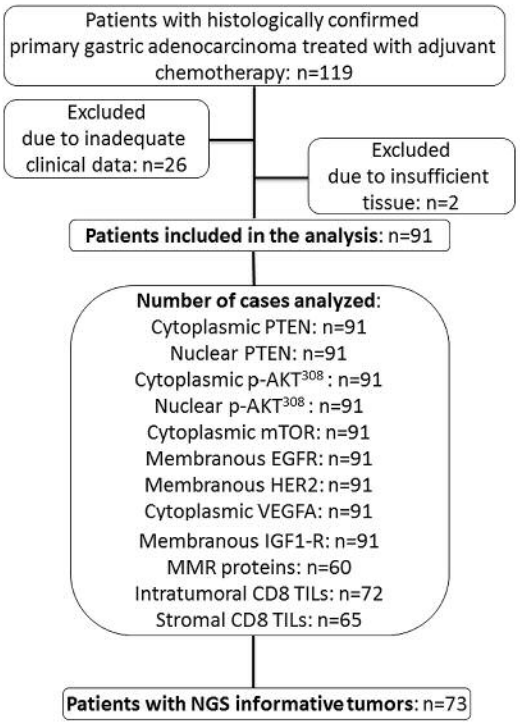

Figure 1. *REMARK diagram detailing study cohort. PTEN: Phosphatase and tensin homolog; mTOR: mammalian target of rapamycin; EGFR: epidermal growth factor receptor; HER2: human epidermal growth factor receptor 2; VEGFA: vascular endothelial growth factor A; IGF1-R: insulin-like growth factor 1 receptor; MMR: mismatch repair; TILs: tumor-infiltrating lymphocytes; NGS: next-generation sequencing.*

Next-generation sequencing (NGS). We used a custom Ampliseq panel targeting coding regions in genes previously reported (24) as frequently mutated in gastric cancer. The interrogated genes (number of amplicons in parentheses) were: *APC* (4), *AURKA* (1), *BRCA1* (9), *CDH1* (5), *CTNNBB1* (4), *FAT4* (10), *FGFR1* (1), *FGFR2* (2), *FGFR3* (1), *FGFR4* (3), *KRAS* (2), *MLH1* (1), *PIK3CA* (5), *PTCH1* (1), *SMAD4* (2), *TP53* (8). *FFPE* DNA was extracted from TMA cores with >30% tumor cell content; samples were assessed for quality and processed for semiconductor sequencing as previously described (25). Variants were called with Variant Caller,

Table I. *Staining procedures for EGFR, HER2, IGF1R, PTEN, pAKT308, MTOR, VEGFA, MLH1, MSH2, MSH6, PMS2.*

IHC stains	Ab info	Manufacturer	Staining protocol
PTEN	Clone 6H2.1, code M 3627, mouse monoclonal	DAKO, Glostrup, DK	20'ER2, 1:300-1h, Bond polymers
p-mTOR (Ser 2448)	Clone 49F9, code 2976, rabbit monoclonal	Cell Signaling Technology, Inc, Danvers, MA	20'ER1, 1:30-20', Bond polymers
EGFR	Clone 31G7, code 28-005, mouse monoclonal	Invitrogen Corporation, Camarillo, CA	enz2-8', 1:50-20', Bond polymers
HER2 (c-erbB-2)	Code A 0485	DAKO, Glostrup, DK	20'ER1, 1:500-30', Bond polymers
VEFG A	Clone VG1, code M7273	DAKO, Glostrup, DK	20'ER2, 1:75-1h, Bond polymer
MSH-6	Clone EP49, M3646	DAKO, Glostrup, DK	20'ER2, 1:70-30', Bond polymers
MSH-2	Clone 25D12, Code NCL-MSH2	Novocastra, Leica Biosystems, Newcastle, Upon Tyne, UKs	20'ER1, 1:40-30', Bond polymer
MLH 1	Clone ES05	MONOSAN	20'ER1, 1:70-20', Bond polymers
PMS-2	Clone M0R4G	Novocastra, Leica Biosystems, Newcastle, Upon Tyne, UK	20'ER2, 1:50-20', Bond polymers
IGF-IRa	Clone 24-31, code MS-641-P, mouse monoclonal	Thermo Scientific	enz2-8', 1:50-1h, Bond polymers
pAKT308	Code sc-16646-R	Santa Cruz, Santa Cruz, CA	20'ER1, 1:1000 O/N, Bond polymer
CD8	Clone C8-144B, code M7103	DAKO, Glostrup, DK	20'ER2, 1:80-20', Bond polymer Enz2=proteinase ER1=citric acid ER2=EDTA

extensively filtered for quality, initially annotated with Ion Reporter (Thermo-Fisher) and further with COSMIC based on provided fathmm scores for pathogenicity. We processed amino acid and splice site changing variants as mutations for minor allele frequencies <0.01% (NCBI dbSNP, 5000Exomes, ExAC) that were read at least ×40 for positions read at least ×100.

With the applied panel of 59 informative amplicons, we considered >7,500 mapped reads and >125 mean depth for sample eligibility. The 73 informative tumors had average and median mean depth of 975.4 and 645, respectively; an average and median number of 12.4 and 11 variants, respectively (range=2-72); and, an average of 1.6 mutations (range=0-28).

Statistical analysis. The cut-offs previously described in the "Patients and Methods" section were used to categorize tumors into positive and negative protein-expressing. In addition, the respective distributions of the calculated H-score of the immunohistochemical markers was plotted (Figure 2) to detect potential cut-offs by examining the quartiles of the distributions. Selected cut-offs for mTOR, p-AKT^{308} and intratumoral and stromal CD8 TILs were the 50th percentiles (median value) of the respective distributions, while the 25th percentile was selected for IGF1-R.

The Chi-square or Fisher's exact (where appropriate) test was used for group comparisons of categorical data, while the Wilcoxon rank-sum test was performed to detect differences between categorical and continuous variables.

Disease-free survival (DFS) was defined as the time (in months) from surgery to first documented progression, death from gastric cancer or last contact (whichever occurred first). Overall survival (OS) was defined as the time from surgery to death from gastric cancer, with alive patients being censored at the date of last contact. Survival curves were estimated using the Kaplan-Meier product limit method and compared across groups with the log-rank test.

The prognostic significance of the examined immunohistochemical biomarkers and genes was evaluated by hazard ratios (HRs) estimated with univariate and multivariate Cox proportional hazard regression models with Firth's correction (where appropriate). All parameters were tested for proportionality using time-dependent covariates. In multivariate analysis, each of the markers or genes that showed significance in the univariate analyses was adjusted for age at diagnosis, histological grade (reference category: grade I-II), number of positive nodes (reference category: 0-3) and performance status (reference category: 0).

Associations of gene mutational status with immunohistochemical markers, clinicopathological characteristics and outcome were performed only for *TP53*, *PIK3CA* and *KRAS*, as the mutation frequency of the other genes was too low to allow for meaningful interpretation.

All analyses were performed in the entire cohort. All tests were two-sided at an alpha 5% level of significance. Analyses were conducted using the SAS software (version 9.3, SAS Institute Inc., Cary, NC, USA).

Results

Patient characteristics. As shown in the REMARK diagram (Figure 1), among 119 patients initially included in the analysis, 91 had complete clinical and biomarker data. Basic clinical and pathological characteristics are presented in Table II. The median age at diagnosis was 64 years and the majority of patients were males (73.6%) with intestinal type

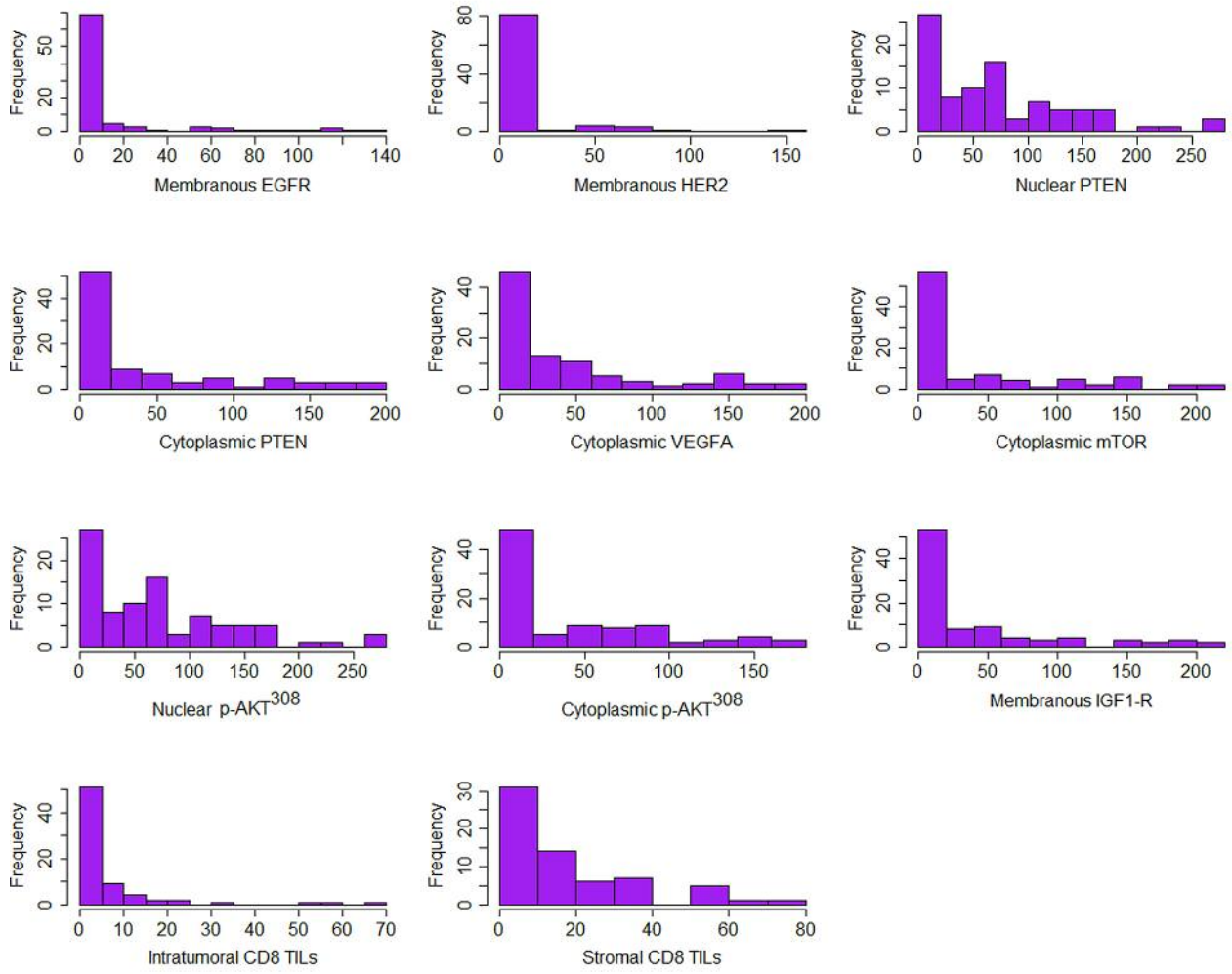

Figure 2. *Distribution of calculated H-scores of immunohistochemical markers across the cohort. Histograms represent the distribution of expression of membranous EGFR, membranous HER2, nuclear PTEN, cytoplasmic PTEN, cytoplasmic VEGFA, cytoplasmic mTOR, nuclear p-AKT^{308}, cytoplasmic p-AKT^{308}, membranous IGF1-R, intratumoral CD8 TILs and stromal CD8 TILs.*

tumors (56%), of T3 stage (60.4%) involving the distal stomach (43.7%). All patients received adjuvant chemotherapy and 93.3% of patients received adjuvant radiotherapy. In our cohort, 4 patients (4.4%) had confirmed EBV-positive disease, based on EBER ISH.

Frequency distribution of immunohistochemical markers and clinically relevant mutations in the cohort. Among 91 patients, 9 (9.9%) had HER2-positive tumors, while 14 (15.4%) had EGFR-positive disease. MMR status was informative for 60 patients (65.9%) and 11 of them (18.3%) had MMR deficient (dMMR) tumors.

Data on intratumoral and stromal CD8 TILs were available for 72 (79.1%) and 65 (71.4%) patients, respectively. More than half of the patients with available information had tumors with high intratumoral CD8 TILs (40 patients; 55.6%) and 34 patients (52.3%) had high stromal CD8 TILs expression, while 24 patients (36.9%) had high expression of both intratumoral and stromal CD8 TILs. The frequency distribution of all examined immunohistochemical markers is presented in Table III.

Regarding mutations in clinically relevant genes, NGS revealed 115 mutations (median: 1; range=0-28) distributed in 44 of the 73 informative tumors (60.3%). The most frequently mutated genes were *TP53* in 19 tumors (43.2% of mutated; 26.0% of all informative tumors), *PIK3CA* in 9 tumors (20.5% of mutated; 12.3% of all informative tumors) and *KRAS* in 24 tumors (54.5% of mutated; 32.9% of all informative tumors) (Figure 3). Less frequent mutations occurred in the following genes: *CDH1* (11.0%), *FAT4* (6.8%), *BRCA1* (4.1%), *CTNNB1* (2.7%), *AURKA* (2.7%), *FGFR4* (2.7%), *SMAD4* (1.4%), *APC* (1.4%) and *FGFR2* (1.4%).

Table II. *Basic patient and tumor characteristics*.

	Total (N=91)
Age	64.0 (37.0,78.0)
≤64	46 (50.5)
>64	45 (49.5)
Gender	
Male	
Female	67 (73.6)
24 (26.4)	
N of positive nodes	
0-3	47 (51.6)
≥4	44 (48.4)
Histological grade*	
I-II	38 (42.7)
III	51 (57.3)
Histology	
Adenosquamous	1 (1.1)
Diffuse	25 (27.5)
Intestinal	51 (56.0)
Mixed	10 (11.0)
Mucinous	4 (4.4)
T stage	
1	2 (2.2)
2	21 (23.1)
3	55 (60.4)
4	13 (14.3)
Primary site*	
Proximal	24 (27.6)
Distal	38 (43.7)
Neither	25 (28.7)
Performance status*	
0	77 (86.5)
1-2	12 (13.5)
Adjuvant radiotherapy*	
No	6 (6.7)
Yes**	84 (93.3)
Type of lymphadenectomy	
D0	2 (2.2)
D1	66 (72.5)
D2	23 (25.3)
Helicobacter pylori infection*	
No	79 (91.9)
Yes	7 (8.1)
EBV status	
Negative	87 (95.6)
Positive	4 (4.4)
Smoking*	
No	40 (47.6)
Yes	44 (52.4)
Alcohol abuse*	
No	71 (85.5)
Yes	12 (14.5)

N: Number. *Data not available for all subjects Missing values: Histological grade=2, Primary site=4, Performance status=2, Adjuvant radiotherapy=1, *Helicobacter pylori* infection=5, Smoking=7, Alcohol abuse=8. **37 patients underwent radiotherapy with concurrent adjuvant chemotherapy. Values are presented as Median (min, max) or N (column %).

Table III. *Frequency distribution of the immunohistochemical markers of interest*.

	Total (N=91)
CD8 intratumoral TILs*	5.0 (0.00,70.0)
High	40 (55.6)
Low	32 (44.4)
CD8 stromal TILs*	15.0 (1.00,80.0)
High	34 (52.3)
Low	31 (47.7)
Membranous EGFR	
Negative	77 (84.6)
Positive	14 (15.4)
Membranous HER2	
Negative	82 (90.1)
Positive	9 (9.9)
Nuclear PTEN	
Negative	78 (85.7)
Positive	13 (14.3)
Cytoplasmic PTEN	
Negative	41 (45.1)
Positive	50 (54.9)
Cytoplasmic VEGFA	
Negative	42 (46.2)
Positive	49 (53.8)
Cytoplasmic mTOR	
Negative	46 (50.5)
Positive	45 (49.5)
Nuclear p-AKT308	
Negative	46 (50.5)
Positive	45 (49.5)
Cytoplasmic p-AKT308	
Negative	48 (52.7)
Positive	43 (47.3)
Membranous IGF1-R	
Negative	31 (34.1)
Positive	60 (65.9)
MMR status*	
Deficient	11 (18.3)
Proficient	49 (81.7)

*Data not available for all subjects. Missing values: CD8 intratumoral TILs=19, CD8 stromal TILs=26, MMR=31. Values are presented as Median (min, max) or N (column %).

Association of immunohistochemical markers with TP53, PIK3CA and KRAS mutations. The associations between the examined IHC markers and the mutational status of the three top mutated genes (*KRAS*, *TP53*, *PIK3CA*) are presented in Table IV. Tumors without *TP53* mutations had more frequent cytoplasmic VEGFA protein expression (chi-square p=0.034, Figure 4A). Notably, all 24 tumors carrying *KRAS* mutation were HER2-negative by IHC (Fisher's p=0.036, Figure 4B), while 8 of the 9 tumors with *PIK3CA* mutation were also positive for VEGFA protein expression (Fisher's p=0.042, Figure 4C). Tumors with mutations had more frequently negative nuclear PTEN protein expression (Fisher's p=0.035,

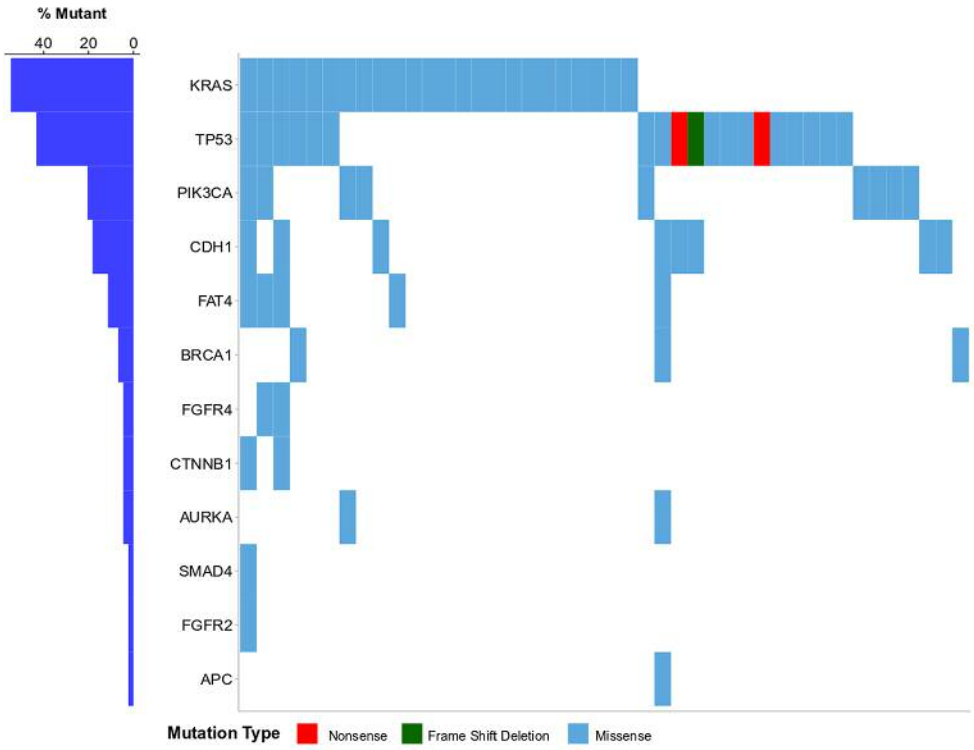

Figure 3. *Map of pathogenic mutations in 73 gastric cancers. Using a targeted panel of 16 frequently mutated genes, we detected mutations in 44 of 73 tumors (60.3%). The most frequently mutated genes were TP53 in 19 tumors (43.2% of mutated; 26.0% of all informative tumors), PIK3CA in 9 tumors (20.5% of mutated; 12.3% of all informative tumors) and KRAS in 24 tumors (54.5% of mutated; 32.9% of all informative tumors).*

Figure 4D) and higher density of intratumoral CD8 TILs (Wilcoxon rank-sum p=0.017, Figure 4E) as compared to those without. No further significant associations were observed.

Association of immunohistochemical markers with clinicopathologic parameters. Patients with tumors of positive HER2 and VEGFA protein expression were of older age at the time of diagnosis (median age: 73 *vs*. 63.5, Wilcoxon rank-sum *p=0.011* and median age: 68 *vs*. 62.5, p=0.011, respectively). In addition, tumors with negative VEGFA protein expression were more frequently of higher grade (III) compared to those with positive expression of VEGFA (70% *vs*. 46.9%, chi-square p=0.029). Positive cytoplasmic expression of PTEN was associated with advanced nodal stage (p=0.042). In addition, EBV-positive tumors were characterized by higher intratumoral CD8 TIL density as compared to EBV-negative (median 60 *vs*. 5, Wilcoxon rank-sum p=0.009), while there was no association between stromal CD8 TILs and EBV status (p=0.54).

The differences in the distribution of the number of mutations per tumor based on the expression of the examined immunohistochemical markers were also examined. The number of mutations was higher in tumors of negative HER2 protein expression compared to tumors of positive HER2 expression (median 1 *vs*. 0, Wilcoxon rank-sum p=0.030). The number of mutations per tumor did not differ between tumors with high and low intratumoral or stromal CD8 TILs (p=0.11 and p=0.46, respectively) but the presence of any number of mutations was associated with high intratumoral CD8 infiltrates (Table IV).

Association of TP53, PIK3CA and KRAS mutations with clinicopathologic characteristics. *PIK3CA*-mutant tumors were more likely to be well or moderately differentiated (grade I or II) (Fisher's *p=0.025*), distal (*p=0.026*) and more frequently observed in non-smokers (p=0.031) compared to *PIK3CA* wild-type tumors, while no further significant associations were observed between the mutational status of *TP53*, *PIK3CA* or *KRAS* and the selected clinicopathological parameters. The presence of mutation (s) was more frequent in male patients (p=0.034).

Prognostic value of immunohistochemical biomarkers and mutational status. At a median follow-up of 98.1 months (95% CI=87.7-109.3), a total of 49 events of progression or death (DFS events) had been reported. Fifty-four patients

Table IV. *Associations of the examined immunohistochemical markers with TP53, KRAS, PIK3CA and the presence of mutations.*

	TP53 mutations			KRAS mutations			PIK3CA mutations			Presence of mutations		
	No	Yes	*p*-Value	No	Yes	*p*-Value	No	Yes	*p*-Value	No	Yes	*p*-Value
CD8 intratumoral TILs	5.0 (0.00,70.0)	5.0 (0.00,60.0)	0.21	5.0 (0.00,55.0)	6.0 (0.00,70.0)	0.10	5.0 (0.00,70.0)	5.5 (0.00,55.0)	0.45	1.0 (0.00,25.0)	5.0 (0.00,70.0)	0.017
CD8 stromal TILs	10.0 (1.00,70.0)	20.0 (5.0,80.0)	0.14	15.0 (1.00,70.0)	10.0 (1.00,80.0)	0.38	12.5 (1.00,80.0)	5.0 (1.00,60.0)	0.54	10.0 (1.00,70.0)	15.0 (1.00,80.0)	0.74
Membranous EGFR			0.93			0.53			0.65			0.88
Negative	45 (83.3)	16 (84.2)		40 (81.6)	21 (87.5)		53 (82.8)	8 (88.9)		24 (82.8)	37 (84.1)	
Positive	9 (16.7)	3 (15.8)		9 (18.4)	3 (12.5)		11 (17.2)	1 (11.1)		5 (17.2)	7 (15.9)	
Membranous HER2			0.36			0.036			0.26			0.052
Negative	47 (87.0)	18 (94.7)		41 (83.7)	24 (100.0)		56 (87.5)	9 (100.0)		23 (79.3)	42 (95.5)	
Positive	7 (13.0)	1 (5.3)		8 (16.3)	0 (0.0)		8 (12.5)	0 (0.0)		6 (20.7)	2 (4.5)	
Nuclear PTEN			0.64			0.097			0.20			0.035
Negative	46 (85.2)	17 (89.5)		40 (81.6)	23 (95.8)		54 (84.4)	9 (100.0)		22 (75.9)	41 (93.2)	
Positive	8 (14.8)	2 (10.5)		9 (18.4)	1 (4.2)		10 (15.6)	0 (0.0)		7 (24.1)	3 (6.8)	
Cytoplasmic PTEN			0.97			0.68			0.40			0.88
Negative	23 (42.6)	8 (42.1)		20 (40.8)	11 (45.8)		26 (40.6)	5 (55.6)		12 (41.4)	19 (43.2)	
Positive	31 (57.4)	11 (57.9)		29 (59.2)	13 (54.2)		38 (59.4)	4 (44.4)		17 (58.6)	25 (56.8)	
Cytoplasmic VEGFA			0.034			0.55			0.042			0.74
Negative	19 (35.2)	12 (63.2)		22 (44.9)	9 (37.5)		30 (46.9)	1 (11.1)		13 (44.8)	18 (40.9)	
Positive	35 (64.8)	7 (36.8)		27 (55.1)	15 (62.5)		34 (53.1)	8 (88.9)		16 (55.2)	26 (59.1)	
Cytoplasmic mTOR			0.32			0.56			0.99			0.094
Negative	27 (50.0)	7 (36.8)		24 (49.0)	10 (41.7)		30 (46.9)	4 (44.4)		17 (58.6)	17 (38.6)	
Positive	27 (50.0)	12 (63.2)		25 (51.0)	14 (58.3)		34 (53.1)	5 (55.6)		12 (41.4)	27 (61.4)	
Nuclear p-AKT^{308}			0.55			0.21			0.48			0.14
Negative	27 (50.0)	11 (57.9)		23 (46.9)	15 (62.5)		32 (50.0)	6 (66.7)		12 (41.4)	26 (59.1)	
Positive	27 (50.0)	8 (42.1)		26 (53.1)	9 (37.5)		32 (50.0)	3 (33.3)		17 (58.6)	18 (40.9)	
Cytoplasmic p-AKT^{308}			0.21			0.79			0.16			0.27
Negative	28 (51.9)	13 (68.4)		27 (55.1)	14 (58.3)		34 (53.1)	7 (77.8)		14 (48.3)	27 (61.4)	
Positive	26 (48.1)	6 (31.6)		22 (44.9)	10 (41.7)		30 (46.9)	2 (22.2)		15 (51.7)	17 (38.6)	
Membranous IGF1-R			0.37			0.25			0.21			0.86
Negative	14 (25.9)	7 (36.8)		12 (24.5)	9 (37.5)		20 (31.3)	1 (11.1)		8 (27.6)	13 (29.5)	
Positive	40 (74.1)	12 (63.2)		37 (75.5)	15 (62.5)		44 (68.8)	8 (88.9)		21 (72.4)	31 (70.5)	
MMR status			0.20			0.20			0.34			0.39
Deficient	8 (22.9)	1 (7.1)		8 (22.9)	1 (7.1)		8 (17.0)	1 (50.0)		5 (23.8)	4 (14.3)	
Proficient	27 (77.1)	13 (92.9)		27 (77.1)	13 (92.9)		39 (83.0)	1 (50.0)		16 (76.2)	24 (85.7)	
CD8 intratumoral TILs			0.50			0.45			0.45			0.048
High	25 (56.8)	10 (66.7)		23 (56.1)	12 (66.7)		29 (56.9)	6 (75.0)		10 (43.5)	25 (69.4)	
Low	19 (43.2)	5 (33.3)		18 (43.9)	6 (33.3)		22 (43.1)	2 (25.0)		13 (56.5)	11 (30.6)	
CD8 stromal TILs			0.094			0.59			0.99			0.48
High	17 (42.5)	9 (69.2)		20 (51.3)	6 (42.9)		23 (50.0)	3 (42.9)		10 (43.5)	16 (53.3)	
Low	23 (57.5)	4 (30.8)		19(48.7)	8 (57.1)		23 (50.0)	4 (57.1)		13 (56.5)	14 (46.7)	

(59.3%) had died, 42 from their disease. The median DFS and OS was 49.9 months and 91.9 months, respectively.

None of the examined immunohistochemical markers showed prognostic significance with respect to DFS (Table V). Similarly, mutations in *TP53* and *PIK3CA* genes were not found to be prognostic for DFS. In terms of OS, only positive expression of nuclear PTEN was associated with marginally longer survival (HR=0.27; Wald's p=0.046) (Figure 5A). It should be noted, however, that there were only 2 events of death among patients with nuclear PTEN expression. The power of this result is, therefore, limited by the small number of patients with events and should be interpreted with caution until further validated in larger cohorts. A trend associated with shorter DFS and OS was observed for *KRAS* mutations (Figure 5B and 5C, respectively).

Upon multivariate analysis with respect to DFS, adjusting for selected clinicopathological parameters (see Methods Statistical Analysis), mutated *KRAS* did not show prognostic

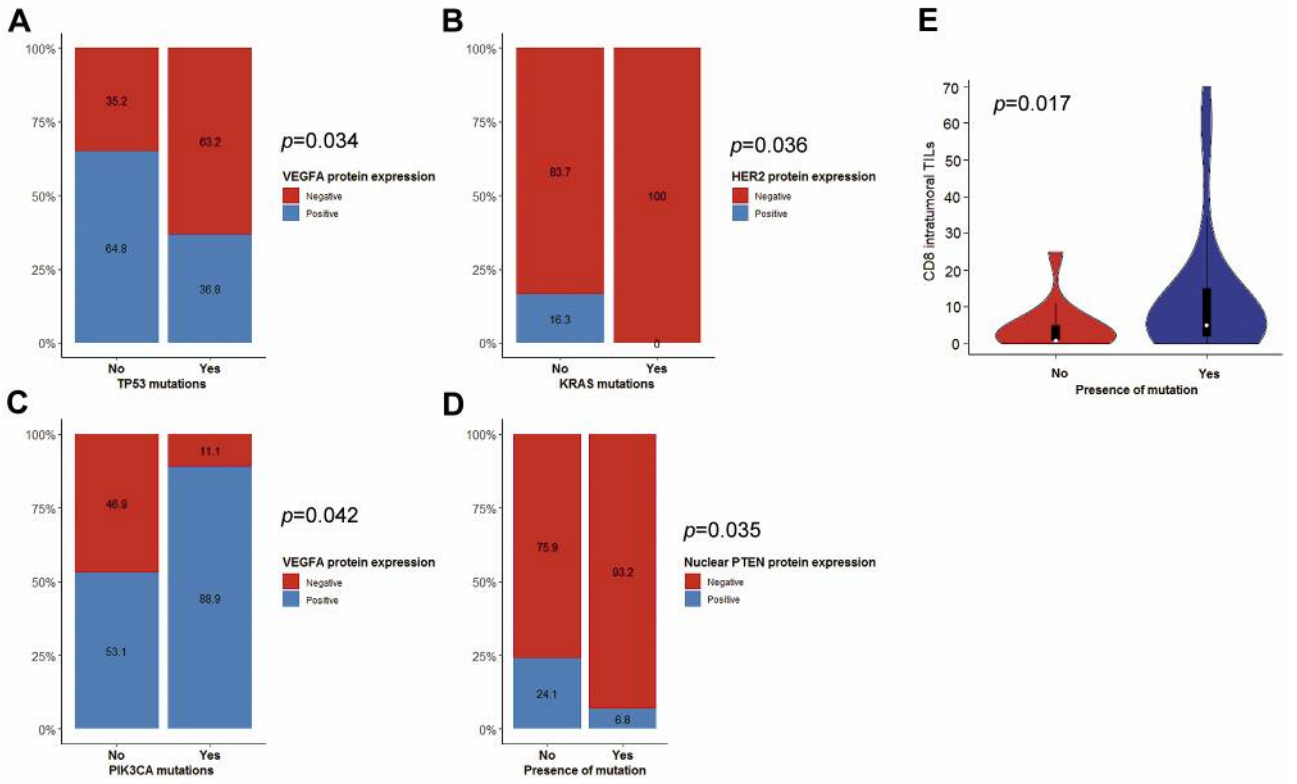

Figure 4. *Associations of immunohistochemical markers with TP53, PIK3CA and KRAS mutations. A. Tumors without TP53 mutations had more frequent cytoplasmic VEGFA protein expression (chi-square p=0.034). B. All 24 tumors harboring KRAS mutation were HER2-negative (Fisher's p=0.036). C. Eight of 9 tumors with PIK3CA mutation were positive for VEGFA protein expression (Fisher's p=0.042). D. Tumors with mutations had more frequently negative nuclear PTEN protein expression (Fisher's p=0.035). E. Tumors with mutations had a higher density of intratumoral CD8 TILs (Wilcoxon rank-sum p=0.017).*

significance for DFS (HR=1.60; p=0.15) (Table VI). Regarding OS, both mutated *KRAS* and positive nuclear PTEN protein expression were not prognostic for OS upon adjustment for clinicopathological parameters.

Discussion

In the present study, we sought to determine the prognostic significance of genetic and protein biomarkers in patients with potentially curable gastric adenocarcinoma, who underwent surgical resection and adjuvant chemoradiotherapy.

Of the evaluable patients in our cohort, we identified 4.4% (4/91) of patients with EBV-positive tumors and 18.3% (11/60) of patients with dMMR tumors. Our findings are in line with the frequency of EBV-positivity and microsatellite instability (MSI) in The Cancer Genome Atlas (TCGA) cohort (24). EBV-positive and dMMR or MSI-high gastric cancer has been associated with high intratumoral CD8 TIL density (26-28). In our study, we indeed found a significant correlation between EBV-positivity and intratumoral CD8 TILs. Given this marked immune infiltration, metastatic EBV-positive gastric cancer has exhibited sensitivity to pembrolizumab in a single-arm phase II clinical trial (29). While this clinical activity needs to be confirmed in larger clinical trials, it also begs the question whether immune checkpoint inhibition is efficacious in the adjuvant treatment of EBV-positive gastric cancer. In contrast to previous reports, there was no statistically significant association between MMR deficiency and CD8 lymphocyte infiltration in our cohort. As shown by Cho and colleagues (26), CD8 TIL infiltration is more pronounced in EBV-positive tumors, compared to MSI-high tumors. It is, therefore, likely that in our study, we did not have sufficient statistical power to detect a significant association between MMR deficiency and CD8 TIL density due to our small sample size. Interestingly, we did not find an association between MMR status and survival, as has been shown in early-stage colorectal cancer (30). A recent analysis of MSI/MMR status in the MAGIC trial cohort showed that patients with MSI-high/dMMR tumors who were treated with surgery alone without perioperative chemotherapy had superior survival compared to patients with MSI-high/dMMR

Table V. *Results of Cox univariate regression for immunohistochemical markers and TP53, KRAS and PIK3CA with respect to DFS and OS.*

				DFS				*OS*		
Parameter	Categories	No. of patients	No. of events	HR	95% CI	*p*-Value	No. of events	HR	95% CI	*p*-Value
Cytoplasmic PTEN	Positive *vs*. Negative	50 *vs*. 41	25 *vs*. 24	0.73	0.41-1.28	0.27	22 *vs*. 20	0.86	0.47-1.58	0.63
Nuclear PTEN	Positive *vs*. Negative	13 *vs*. 78	4 *vs*. 45	0.44	0.17-1.18	0.10*	2 *vs*. 40	0.27	0.07-0.98	0.046*
Cytoplasmic VEGFA	Positive *vs*. Negative	49 *vs*. 42	23 *vs*. 26	0.73	0.41-1.27	0.26	19 *vs*. 23	0.65	0.35-1.19	0.16
Cytoplasmic mTOR	Positive *vs*. Negative	45 *vs*. 46	21 *vs*. 28	0.69	0.39-1.22	0.20	20 *vs*. 22	0.87	0.48-1.60	0.66
Cytoplasmic p-AKT^{308}	Positive *vs*. Negative	43 *vs*. 48	22 *vs*. 27	0.98	0.56-1.71	0.93	21 *vs*. 21	1.24	0.68-2.27	0.49
Nuclear p-AKT^{308}	Positive *vs*. Negative	45 *vs*. 46	21 *vs*. 28	0.62	0.35-1.10	0.10	19 *vs*. 23	0.71	0.39-1.30	0.27
Membranous EGFR	Positive *vs*. Negative	14 *vs*. 77	8 *vs*. 41	0.95	0.45-2.03	0.90	8 *vs*. 34	1.22	0.57-2.64	0.61
Membranous HER2	Positive *vs*. Negative	9 *vs*. 82	3 *vs*. 46	1.13	0.38-3.40	0.83*	3 *vs*. 39	1.49	0.49-4.51	0.49*
Membranous IFG1-R	Positive *vs*. Negative	60 *vs*. 31	29 *vs*. 20	0.66	0.37-1.17	0.16	26 *vs*. 16	0.78	0.42-1.45	0.42
MMR status	Proficient *vs*. Deficient	49 *vs*. 11	28 *vs*. 5	1.45	0.57-3.66	0.44*	26 *vs*. 3	2.18	0.70-6.77	0.18*
Intratumoral CD8 TILs	Low *vs*. High	32 *vs*. 40	20 *vs*. 21	1.44	0.78-2.66	0.24	18 *vs*. 17	1.38	0.71-2.68	0.34
Stromal CD8 TILs	Low *vs*. High	31 *vs*. 34	20 *vs*. 16	1.68	0.87-3.24	0.12	17 *vs*. 14	1.43	0.70-2.90	0.32
No. of mutations per tumor^				0.98	0.90-1.07	0.63		0.99	0.91-1.08	0.83
KRAS mutation	Yes *vs*. No	24 *vs*. 49	18 *vs*. 23	1.79	0. 97-3.34	0.064	15 *vs*. 20	1. 79	0.92-3.51	0.089
PIK3CA mutation	Yes *vs*. No	9 *vs*. 64	3 *vs*. 38	0.44	0.15-1.35	0.15*	2 *vs*. 33	0. 40	0.11-1.47	0.17*
TP53 mutation	Yes *vs*. No	19 *vs*. 54	10 *vs*. 31	0.70	0.34-1.43	0.33	10 *vs*. 25	0.94	0.45-1.96	0.87

No.: Number, HR: hazard ratio, CI: confidence interval. *Firth's correction was applied due to rare events. ^continuous variable.

Table VI. *Results of Cox multivariate regression analysis with respect to DFS and OS.*

Parameter	Categories	No. of patients	No. of events	HR	95% CI	*p*-Value
DFS						
KRAS mutation	Yes *vs*. No	23 *vs*. 47	17 *vs*. 23	1.60	0.84-3.04	0.15
Histological grade	III *vs*. I-II	39 *vs*. 31	23 *vs*. 17	1.00	0.53-1.91	0.99
No. of positive nodes	≥4 *vs*. 0-3	32 *vs*. 38	18 *vs*. 22	1.15	0.59-2.21	0.69
Performance status	1-2 *vs*. 0	9 *vs*. 61	7 *vs*. 33	2.33	1.01-5.39	0.048
Age^				1.00	0.97-1.04	0.97
OS						
KRAS mutation	Yes *vs*. no	23 *vs*. 47	14 *vs*. 20	1.41	0.70-2.85	0.34
Nuclear PTEN	Positive *vs*. Negative	8 *vs*. 62	2 *vs*. 32	0.50	0.12-1.99	0.32*
Histological grade	III *vs*. I-II	39 *vs*. 31	19 *vs*. 15	1.05	0.51-2.17	0.87
No. of positive nodes	≥4 *vs*. 0-3	32 *vs*. 38	18 *vs*. 16	1.70	0.84-3.47	0.14
Performance status	1-2 *vs*. 0	9 *vs*. 61	6 *vs*. 28	2.68	1.10-6.52	0.030
Age^				1.01	0.97-1.05	0.56

No.: Number, HR: hazard ratio, CI: confidence interval. ^continuous variable. *Firth's correction was applied for rare events.

tumors who received perioperative chemotherapy. In contrast, patients with MMR-proficient (pMMR) tumors benefited from perioperative chemotherapy (31). A limitation of our study is that all patients received adjuvant therapy post-surgery, and therefore the lack of difference in survival between the dMMR and pMMR groups may be due to the detrimental effect of adjuvant therapy in the dMMR patients and its beneficial effect in the pMMR patients. Separate from and in addition to microsatellite instability, high tumor mutational burden is emerging as a potential predictive biomarker of response to immune checkpoint inhibition (32). Mutations can give rise to neoantigens that can be recognized and targeted by the immune system. Treatment with immune checkpoint inhibitors can further enhance the immune response against hypermutated tumors. Although we evaluated mutations in a limited panel of genes, we found a significant correlation between the presence of mutations and the intratumoral CD8 TIL density, suggesting that immune checkpoint blockade may be an effective adjuvant treatment for patients with resected gastric cancer with high mutational burden.

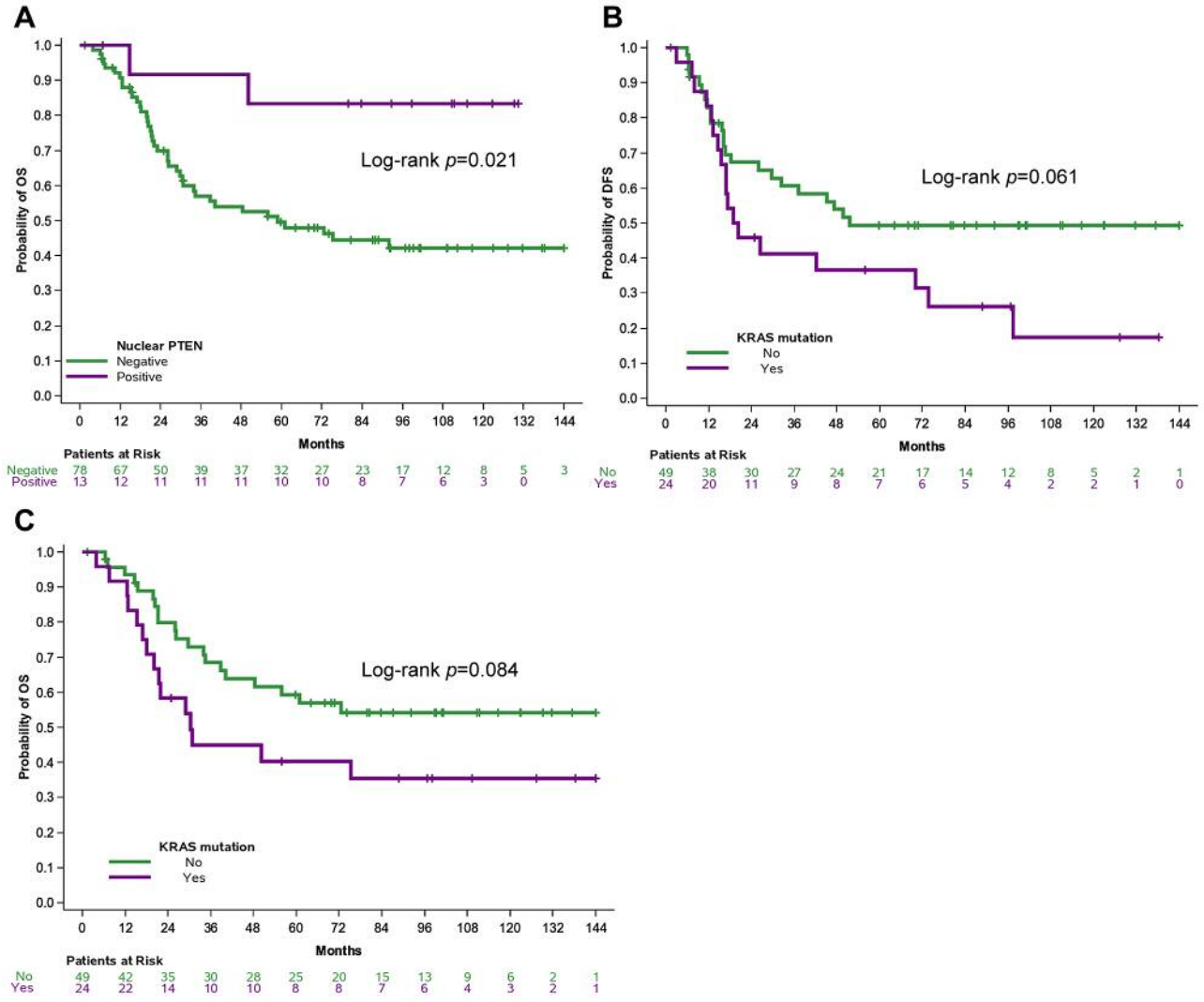

Figure 5. *Kaplan-Meier plots of overall survival (OS) by PTEN expression, and disease-free survival (DFS) and overall survival (OS) by KRAS mutation status. A. PTEN-positive tumors were associated with longer OS (HR=0.27; Wald's p=0.046). B. For patients with KRAS mutant tumors, we observed a trend associated with shorter DFS. C. A similar trend associated with shorter OS was also observed for patients with KRAS mutant tumors, compared to KRAS wild-type tumors.*

We further showed that none of the HER2-positive tumors in our cohort harbored a *KRAS* mutation. This finding is in keeping with TCGA data that showed that a mutual exclusivity between ERBB2 amplification and KRAS mutation (24). As both *KRAS* mutations and HER2 overexpression activate the MAPK pathway, they subsequently promote cancer cell growth, invasion and metastasis (33, 34). In contrast, in colon cancer, EGFR expression co-occurs with *KRAS* mutation (35), and mutations in *KRAS* predict for resistance to anti-EGFR therapy (36). Based on the results of our study and others (24, 37, 38), it seems that *KRAS* mutations do not play a role in the resistance to HER2-directed therapy in gastric cancer, since these features seem to be mutually exclusive. Additionally, we found a significant association between the presence of *PIK3CA* mutations and VEGFA protein expression. Studies have demonstrated that the activation of the PI3K pathway results in increased expression of the hypoxia-inducible factor-1α (HIF-1α) protein, HIF-1α transcriptional activity and expression of VEGF (39-41). Further, inhibition of the PI3K pathway with the pan-PI3K small molecule inhibitor LY294002 suppresses VEGF transcription and protein expression, and therefore inhibits angiogenesis and tumor growth (41, 42). These findings suggest that combined inhibition of PI3K and angiogenesis may be synergistic and warrants further evaluation in the treatment of *PIK3CA* mutant gastric cancer.

Lastly, we investigated the potential prognostic significance of these genetic and protein biomarkers in operable gastric adenocarcinoma. We found that the association between *KRAS* mutations and inferior clinical outcomes, both DFS and OS, trended toward significance. Although *KRAS* mutations have been shown to confer poor prognosis in colorectal cancer (43-45), its role in gastric cancer is less clear. Matsusaka and colleagues showed that *KRAS* mutations were predictors of inferior survival in patients with metastatic gastric cancer treated with platinum-based therapy (46), while Warneke and colleagues showed similar results in a cohort of patients with stage I-IV gastric cancer (47). However, other studies have demonstrated disparate results, ranging from lack of association between *KRAS* mutations and survival in resected gastric cancer (48) to a high prevalence of *KRAS* mutations in a group of patients with favorable prognosis (49). Even though KRAS, for the most part, remains undruggable, several treatment strategies targeting the MAPK pathway, including MEK, ERK and SHP2 inhibitors, are currently being investigated in KRAS mutant tumors. While our results need further validation, it is possible that these patients may benefit from novel adjuvant treatment approaches targeting the MAPK pathway, rather than traditional fluorouracil-based adjuvant chemotherapy.

Additionally, of all immunohistochemical biomarkers we studied, only loss of nuclear PTEN expression was associated with inferior OS. Indeed, loss of PTEN expression has been shown to be a poor prognostic marker in several malignancies, including breast, prostate and non-small cell lung cancer (50-52). Furthermore, cytoplasmic PTEN loss has also been associated with worse outcomes in gastric cancer (20, 53, 54). However, to our knowledge, this is the first study to demonstrate a correlation between nuclear PTEN loss and inferior OS in operable gastric cancer. While the role of cytoplasmic PTEN as a negative regulator of the PI3K/AKT pathway has been well described, its nuclear function needs further elucidation. However, there are emerging data that nuclear PTEN downregulates the MAPK pathway and decreases cyclin D1 expression inducing G0-G1 arrest (55), but also enhances DNA repair and thus maintains chromosomal stability (56) and enhances apoptosis (57). Despite the limitations of our small sample size, we showed that patients whose tumors exhibited loss of nuclear PTEN expression experienced shorter OS compared to patients with intact PTEN expression in their tumors. Given the poor outcomes of these patients despite adjuvant chemoradiotherapy, it would be reasonable to consider intensification of adjuvant therapy and testing of novel agents that target the MAPK pathway, block the cell cycle or induce apoptosis.

Our study has certain limitations. A major limitation is its small sample size, which may have obscured the prognostic significance of certain genetic or protein biomarkers. Additional limitations include the retrospective nature of our study and the lack of a control group of patients treated with surgery alone, that would allow the identification of biomarkers predictive of response or resistance to adjuvant chemoradiotherapy.

In summary, we evaluated a large panel of genetic and protein biomarkers in a cohort of patients with gastric cancer who underwent surgery and adjuvant chemoradiotherapy. We demonstrated that EBV-positive tumors and tumors with mutations in cancer-associated genes had increased CD8 TIL density. We further showed that *PIK3CA* mutations are associated with increased VEGFA protein expression, an association that may inform treatment strategies for gastric cancer patients. Lastly, we found that the presence of KRAS mutations and loss of nuclear PTEN expression are associated with inferior OS. This finding, if validated, may have significant implications for the prognostic classification of operable gastric cancer and could identify patients who would benefit from intensification of adjuvant therapy or novel treatment strategies to prevent recurrence.

Conflicts of Interest

The Authors declare no conflicts of interest in regard to this study.

Authors' Contributions

Conceptualization: EP, IC, VK, DP, GF. Methodology: VK, KP, EG, MB, SC. Formal Analysis: GAK. Investigation: KP, EG, MB, AF. Resources: VGG, MB, CP, DP, GF. Supervision: EP, VK, GF. Writing/editing: EP, IC, VK, GAK, KP, MB, SC. Writing/review and editing: All Authors.

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