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Prognostic significance of HER2, p53, Ki67, MVD-CD34, Cox2, MMP7, and vimentin in gastric cancer

Amirhossein Razavirad[1,2ǂ], Ahad Muhammadnejad[2ǂ], Saeed Soleymanjahi[1,3], Sanaz Rismanchi[2], Elnaz Saeedi[1], Amirafraz Fallah[1], Alireza Abdollahi[4], Saeid Amanpour[2], Kazem Zendehdel[1*]

1. Cancer Research Center, Cancer Institute of Iran, Tehran University of Medical Sciences, Tehran, Iran
2. Cancer Biology Research Center, Cancer Institute of Iran, Tehran University of Medical Sciences, Tehran, Iran
3. Division of Gastroenterology, Washington University in St Louis, USA
4. Department of Pathology, Imam Khomeini Hospital Complex, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran

ǂ Equally contributed to this manuscript as first co-authors.

* Corresponding author: Kazem Zendehdel

Address: Cancer Institute of Iran, Tehran University of Medical Sciences, Tehran 1419733133, Iran

Email: kzendeh@tums.ac.ir

Phone: +98 21 66581638

ABSTRACT

Background: Gastric cancer (GC) patients have a poor prognosis mainly due to late diagnosis. We aimed to study the prognostic effects of various biomarkers, including HER2, CD34, p53, Ki67, Cox2, MMP7, and vimentin in GC.
Methods: We performed immunohistochemistry (IHC) to examine the expression of potential biomarkers in 140 GC patients. CD34 protein expression was quantified to assess angiogenesis through scoring microvessel density (MVD). We used a multivariable Cox-proportional hazard model to estimate hazard ratios (HRs) representing the prognostic role of the biomarkers and the clinicopathological parameters.
Results: Patients diagnosed at the advanced tumor stage exhibited a significantly higher risk of mortality than those diagnosed at the early stages (HR = 5.96, CI: 3.73 – 9.51). We also observed higher risks of mortality in patients with high MVD-CD34 (HR = 5.35, CI: 2.36 – 12.12), HER2-positive (HR = 2.82, CI: 1.69 – 4.37), p53-positive (HR = 4.03, CI: 2.53 – 6.4), high Ki67 (HR = 4.34, CI: 2.64 – 7.13), high Cox2 (HR = 4.77, CI: 2.39 – 9.49), high MMP7 (HR = 2.75, CI: 1.53 – 4.94), and high vimentin (HR = 3.78, CI: 1.7 – 8.39) tumors compared to their corresponding reference groups. The association was statistically significant for HER2, p53, Ki67, Cox2, and MVD-CD34 among those diagnosed in an early stage.
Conclusion: Overall, evaluation of tumor biomarkers in GC patients can result in more precise estimates of prognosis, especially in early-stage tumors. These biomarkers could potentially be considered for targeted therapy of GC patients to improve their survival.

Keywords: Gastric cancer, Immunohistochemistry, Prognosis, and Tumor biomarkers.

INTRODUCTION:

Gastric cancer (GC) remains an essential challenge in oncology, with a high mortality rate worldwide, mainly due to its late diagnosis [1]. Despite ongoing advances in diagnosing and treating GC, it still has a poor prognosis [2]. GC accounts for the third-highest cancer-related disability-adjusted life years (DALYs) worldwide after lung and liver cancers [3]. Recent advances in early diagnosis could improve the prognosis. Likewise, identifying novel biomarkers can improve prognosis by enhancing risk stratification and offering targeted therapy [4,5].

Proliferation and angiogenesis play strategic roles in cancer development and progression. Thus, proteins involved in these processes could have diagnostic and prognostic significance. The first molecular biomarker available in the clinic for gastric cancer patients is the human epidermal growth factor receptor 2 (HER2), which is a proto-oncogene encoded by the ERBB2 gene [1]. HER2 is a transmembrane protein receptor that promotes cell proliferation and inhibits apoptosis, resulting in uncontrolled cell proliferation and tumorigenesis [1,6]. The prognostic significance of HER2 expression in gastric cancer is controversial [7–10]. Encoded by the human tumor protein 53 (TP53) gene, the p53 protein plays vital regulatory roles in cell cycle arrest, apoptosis, and DNA damage [11]. TP53 is the most frequently mutated gene in human tumors [11]. Mutations in the TP53 gene continue along with gastric cancer progression, leading to the accumulation of mutated p53 proteins in the nuclei of cancer cells [12]. Ki67 is a nuclear protein encoded by the MKI67 gene [12]. This protein is linked to cell cycle proliferation and is commonly quantified by immunohistochemistry as a proliferative marker [13]. Ki67 exists in the cell cycle's G1, S, G2, and mitosis phases but not in G0 [12], making it a helpful biomarker in identifying cell growth and proliferation. CD34 is a transmembrane phosphoglycoprotein encoded by the CD34 gene that plays a pivotal role in the angiogenesis associated with tumor growth and progression [14]. CD34 quantification is used to assess microvessel density (MVD), a marker for tumor angiogenesis, and is defined by the number of new blood vessels in a specified area of tumor tissue [14,15].

Regulatory factors of other critical cancer-promoting processes could also be useful cancer biomarkers. Inflammation can augment cancer development, and cyclooxygenase 2 (Cox2) is one of the important inflammatory mediators that is dysregulated in many tumors [16]. Cox2 is an inducible enzyme that converts arachidonic acid to prostaglandin E2 and inversely correlates with the prognosis of GC [17]. Matrix metalloproteinase 7 (MMP7) or Matrilysin is also involved in inflammation as well as tumor metastasis [18]. By degradation of other extracellular matrix proteins, higher levels of MMP7 protein link to the progression and spread of many malignancies, including gastric cancer, through promoting angiogenesis and tumor invasion [19,20]. Finally, vimentin is an intermediate filament encoded by the VIM gene with an expression predominantly restricted to mesenchymal tissues [21]. Vimentin is typically used to identify the mesenchymal cells or the cells that undergo epithelial-mesenchymal transition (EMT) during normal development and/or metastatic progression and is expectedly associated with several tumorigenesis processes [21]. Elevated tumoral vimentin expression is associated with high tumor growth and invasion and low overall survival [21,22].

Given the crucial roles of these biomarkers in cancer biology, their associations with GC have been examined separately in previous studies [7,23–27]. Nevertheless, it is unclear how the expressional profile of all these biomarkers is associated with GC prognosis. Here, we conducted a comprehensive survival study to evaluate the tumoral expression of the mentioned proteins in GC patients, which is the most common cancer among males, and their patients experience meager survival [28,29]. We used statistical modeling to adjust for clinical prognostic elements as potential confounding factors and conducted a sub-analysis to examine the exclusive prognostic relevance in early GC patients.

Material & Methods:

Patients

We established a cohort of 140 patients diagnosed with

primary gastric cancer who had undergone surgery from April 2011 to January 2016. All specimens were pathologically diagnosed with gastric cancer. We excluded patients who underwent neoadjuvant therapy. The median age at the time of surgery was 60 years, ranging from 40 to 80 years old. The fourth version of the protocol for the examination of specimens from patients with stomach adenocarcinoma approved by the College of American Pathologists (CAP), was used for tumor-node-metastasis (TNM) staging classification (taken from the 8th edition of the American Joint Committee on Cancer (AJCC)), tumor size and site, histological grade, type of histology, lymphovascular invasion, and perineural invasion (Table 1). According to the Lauren classification, 89 patients were histologically classified as intestinal type, 25 as diffuse type, and 26 as mixed type. Stages I and II

Table 1. Clinicopathological characteristics of 140 gastric cancer patients hospitalized in Cancer Institute during 2011 – 2016.

Variable	No.	Frequency (%)
Gender		
Female	**31**	**22.14**
Male	**109**	**77.86**
Age (years)		
<60	**60**	**42.86**
≥60	**80**	**57.14**
Tumor size (cm)		
<5	**68**	**48.57**
≥5	**72**	**51.43**
Tumor site		
Antrum	**11**	**7.86**
Body	**69**	**49.28**
Fundus	**60**	**42.86**
Tumor histology		
Intestinal type	**89**	**63.57**
Diffuse type	**25**	**17.86**
Mixed type	**26**	**18.57**
Vascular invasion		
Absent	**46**	**32.86**
Present	**94**	**67.14**
Perineural invasion		
Absent	**61**	**43.57**
Present	**79**	**56.43**
Histological grade		
Low	**77**	**55**
High	**63**	**45**
Tumor stage		
Early	**63**	**45**
Advanced	**77**	**55**

were grouped as early and stage III as advanced. Grades 1 and 2 were grouped as low, and grade 3 as the high histological grade. Most patients were diagnosed at an advanced stage (n=77) and were in the low histological grade (n=77). The study was approved by the Regional Research Ethics Committee (No. REC25147) and conducted under the Declaration of Helsinki. Written informed consent was obtained from all patients prior to participating in the study.

Outcome assessment

All patients were followed up until the date of death or reaching date of June 2020, which was the last termination of the study. Overall survival (OS) was calculated as the time from the surgery until death for any cause or end of the follow-up, whichever came first. In the case of patients who were lost to follow-up, we did not exclude them, and instead, we included their last survival status in our total person-time survival data. The total person-years of follow-up was 265.5, and the mean and standard deviation of overall survival were 1.9 and 0.97, respectively.

Sample preparation and immunohistochemistry (IHC)

All primary tumor specimens were fixed in formalin, embedded in paraffin, and stored in the pathology department. Specimens were collected from the archives based on identification numbers and clinical information. The specimens were then sliced and processed in 3 ?M thicknesses for IHC. The tissue sections were first placed in the oven for 40 minutes, immediately immersed in xylene to remove the residual paraffin, and then hydrated by gradient alcohol. The slides were boiled in citrate buffer (pH=6.0) with 10 to 20% reduced power to retrieve the antigens for 20 minutes and incubated for 30 minutes at room temperature. After exhausting endogenous peroxidase using H2O2 in methanol for 15 minutes, sections were rinsed three times with phosphate-buffered saline (PBS) and then blocked with 5% bovine serum albumin (BSA) at room temperature for one hour. The sections were rinsed in PBS three times, incubated with specific antibodies at room temperature for one hour, and rinsed thrice with PBS. The sections were incubated in horseradish peroxidase (HRP) as per instructions, rinsed thrice with PBS, and counterstained by the Mayer Hematoxylin method. Antibodies against HER2 (mouse monoclonal, SKU: 076), CD34 (mouse monoclonal, SKU: 084), p53 (mouse monoclonal, SKU: 042), Ki67 (rabbit monoclonal, SKU: 325), Cox2 (rabbit monoclonal, SKU: 306), and vimentin (mouse monoclonal, SKU: 048) were purchased from Biocare Medical (CA, USA). The anti-MMP7 antibody (rabbit polyclonal, AA 165-210) was purchased from antibodies-online GmbH (Aachen, Germany).

Immunoreactivity scoring

As shown in Figures 1 and 2, all Formalin-Fixed Paraffin-Embedded (FFPE) tissue sections were examined by two expert pathologists. HER2 as a transmembrane protein was quantified as 0 and/or +1 as negative, +2 as equivocal, and +3 as positive, according to the College of American Pathologists (CAP) protocol [30]. In addition, we computed microvessel density (MVD) as a measure to evaluate CD34 expression. For this, the total scores of four hotspots were measured with high power fields (HPFs), where a score of 1-19 was considered low, 20-39 as moderate, and ?40 as high. Additionally, p53 ranged from 0 to 42 (in percentage) among our data set and was quantified by scoring the nuclear staining intensity, where a score ?10% was considered negative and ?10% as positive. Also, Ki67 ranged from 5 to 33 (in percentage) in our data set. Since there was no specific protocol for Ki67 in gastric cancer and different studies applied various cut-points, we used the breast cancer protocol to score the nuclear staining intensity, where a score ?15% was considered as low and ?15% as high [31]. To prevent any bias caused by cut-points, we set different cut-points for Ki67, as presented in supplementary table 1. Moreover, for Cox2, MMP7, and vimentin, we used the modified Allred score, which was a combination of the percentage of positive cells and the intensity of the reaction product, where a score ?2 was considered as negative, 2-5 as low, and 6-8 as high expression.

Statistical analysis

The Cox proportional hazard model was applied to the univariate and multivariable survival analysis. Univariate analyses were individually applied for all variables,

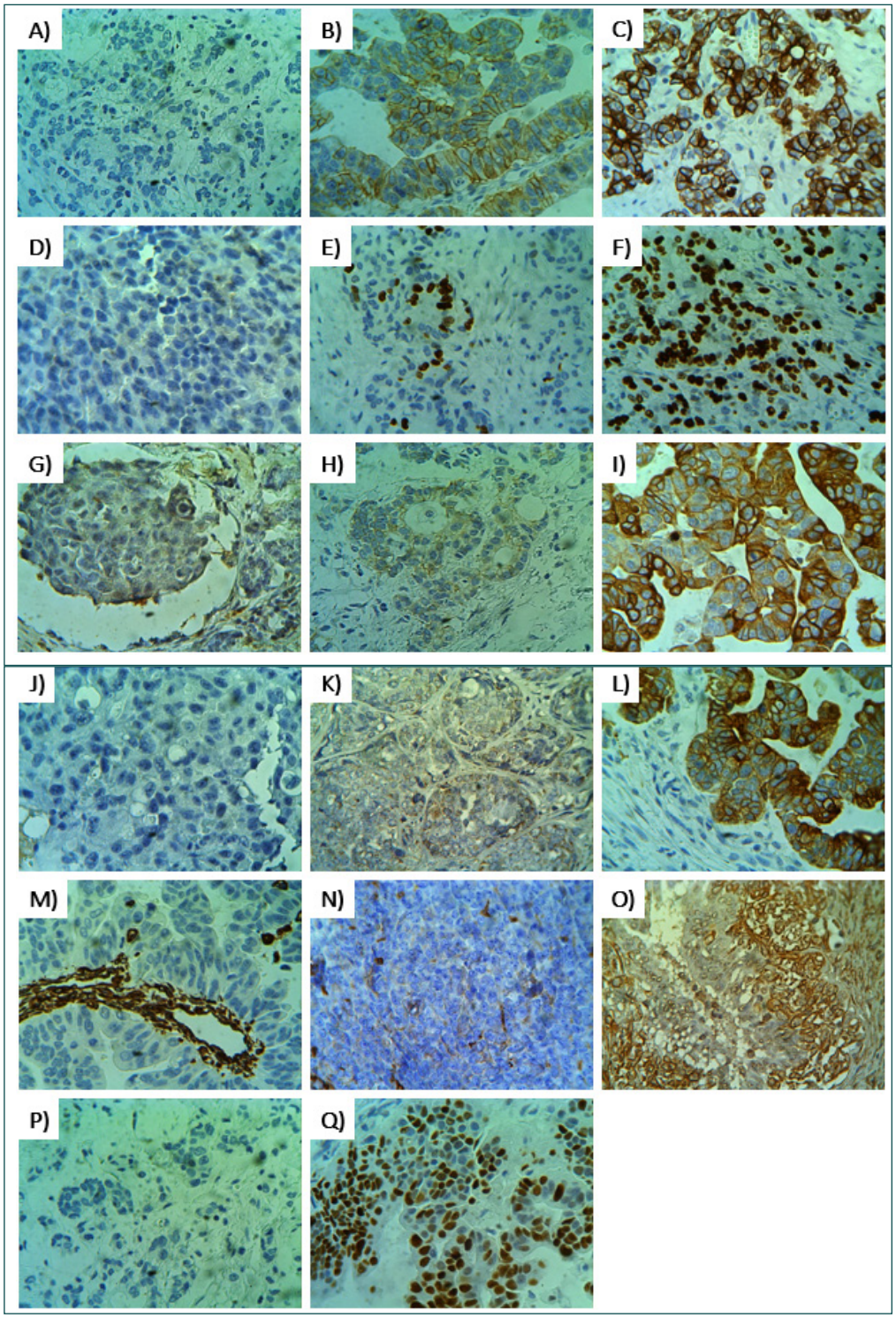

Figure 1. Representative images of HER2, Ki67, Cox2, MMP7, vimentin, and p53 staining in gastric cancer by immunohistochemistry (IHC). Gastric adenocarcinomas with HER2-negative (A), equivocal HER2 (B), HER2-positive (C), low Ki67 (D and E), high Ki67 (F), Cox2-negative (G), low Cox2 (H), high Cox2 (I), MMP7-negative (J), low MMP7 (K), high MMP7 (L), vimentin-negative (M), low vimentin (N), high vimentin (O), p53-negative (P), and p53-positive (Q) staining at x400.

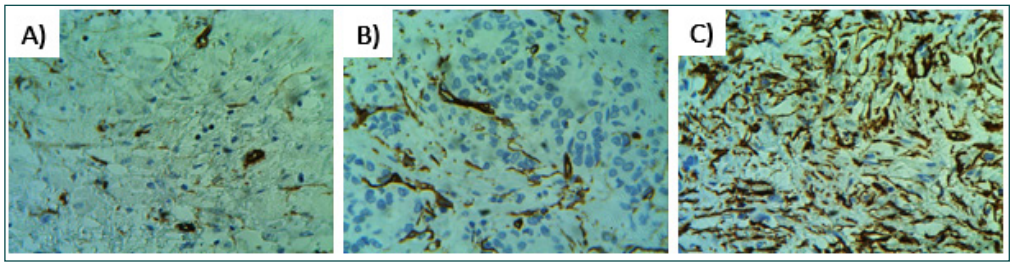

Figure 2. Representative images of MVD-CD34 staining as a marker for angiogenesis in gastric cancer by immunohistochemistry (IHC). Gastric adenocarcinomas with low MVD-CD34 (A), moderate MVD-CD34 (B), and high MVD-CD34 (C) staining at x400. CD34 protein expression is used to assess microvessel density (MVD).

and multivariable analyses were adjusted for personal and clinical factors, including gender, age, vascular invasion, perineural invasion, histological grade, and TNM stage. Survival curves were created by the Kaplan-Meier method, and the p values were computed by the log-rank test. We considered $p < .05$ as statistically significant for all analyses. In sub-analyses, we repeated our analyses among patients who were diagnosed in early and advanced stages, as well as among patients with different histologic tumor subtypes. We performed all statistical analyses using RStudio statistical software version 1.2.5033.

Results:

As shown in Figure 3, survival curves generated by Kaplan-Meier plots and relevant log-rank p values exhibited significantly poor overall survival rates for advanced TNM stage, HER2-positive, p53-positive, high Ki67, high MVD-CD34, high Cox2, high MMP7, and high vimentin subgroups compared with their relevant references ($p < .001$ for all).

We observed a significant association between tumor stage and overall survival, where patients with advanced stage had about 6-fold higher mortality risk compared to the patients who were diagnosed in an early stage (HR = 5.96 (3.73 – 9.51)) (Table 2). Also, tumors with high MVD-CD34 expression resulted in significantly lower overall survivals compared to the tumors with low MVD-CD34 (HR = 5.35, CI: 2.36 – 12.12). We also found worse survivals among patients with HER2- positive (HR = 2.82, CI: 1.69 – 4.37), p53-positive (HR = 4.03, CI: 2.53 – 6.4), high Ki67 (HR = 4.34, CI: 2.64 – 7.13), high Cox2 (HR = 4.77, CI: 2.39 – 9.49), high MMP7 (HR = 2.75, CI: 1.53 – 4.94), and high vimentin (HR = 3.78, CI: 1.7 – 8.39) tumors in comparison with their relevant reference groups (Table 2).

We found that most patients in the early stage of their tumor were placed in negative or low expression subgroups for almost all biomarkers, and patients in the advanced stage were shifted towards high expression subgroups (Table 3). In addition, patients with high expression of HER2, p53, Ki67, CD34-MVD, and Cox2 exhibited lower survival rates than their corresponding reference groups, in early and advanced stages. However, mortality risk in high MMP7 (HR = 3.56, CI: 1.28 – 9.97) and vimentin (HR = 8.81, CI: 1.02 – 76.22) groups were higher in the early-stage subgroup.

Discussion:

This study evaluated the expression levels of some of the most critical proteins affecting gastric cancer prognosis, including HER2, CD34, p53, Ki67, Cox2, MMP7, and vimentin, and found that these biomarkers exert a significant prognostic role in gastric cancer patients. The prognostic role was significant in the early and advanced stages for all biomarkers except for MMP7. Previous studies have mainly examined these biomarkers individually. The overlapping mechanisms underlying their prognostic role can introduce confounding relationships between these biomarkers. Hence, exploring the independent prognostic role of each of these biomarkers can be imperative. Using comprehensive statistical modeling, this is the first study that reports the independent prognostic significance of these biomarkers after adjusting for other clinical factors and biomarkers. This work further signifies the clinical im-

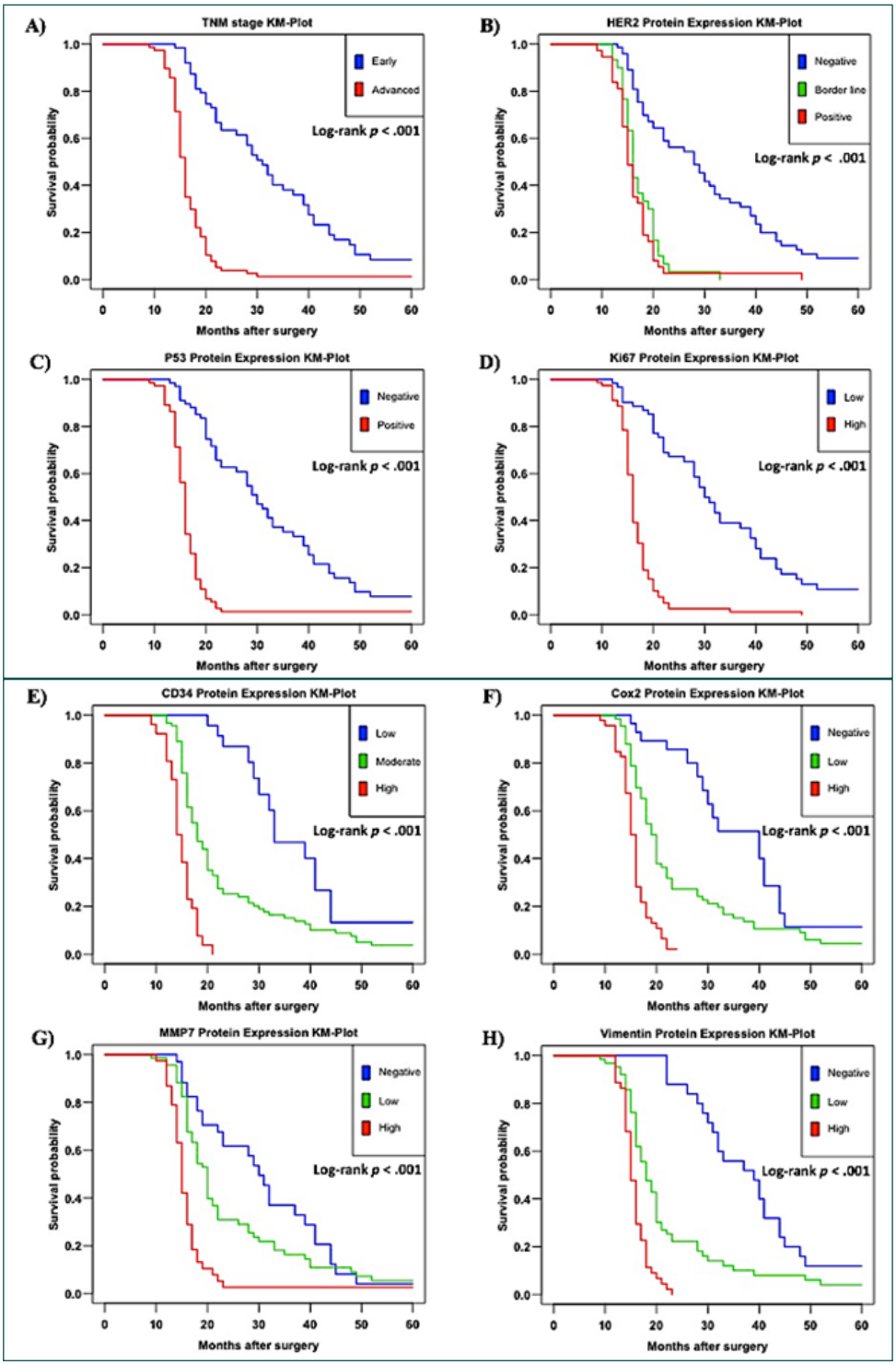

Figure 3. Kaplan-Meier curves indicate the statistically significant differences in overall survival among 140 gastric cancer patients. Patients with tumors having advanced stage (A) and expressing HER2-positive (B), p53-positive (C), high Ki67 (D), high MVD-CD34 (E), high Cox2 (F), high MMP7 (G), and high vimentin (H) significantly demonstrate poor overall survival. For each survival plot, a corresponding log-rank p-value is presented.

Table 2. Univariate and multivariable analyses identifying the prognostic significance of available clinicopathological variables in 140 gastric cancer patients.

Variable	Number			Univariate	Multivariable
	Patients (n=140)	Deaths (n=125)	Person-years	Crude HR (95% CI)	Adjusted HR (95% CI)
Gender					
Female	31	25	2.14	1	1
Male	109	100	6.58	1.3 (0.83 – 2.04)	1.31 (0.8 – 2.14)
Age (years)					
<60	60	49	3.87	1	1
≥60	80	76	4.85	1.33 (0.93 – 1.91)	1.18 (0.8 – 1.77)
Histological grade					
Low	77	62	5.24	1	1
High	63	63	3.48	1.82 (1.27 – 2.6)	1.37 (0.94 – 2)
Tumor stage					
Early	63	49	5.17	1	1
Advanced	77	76	3.55	5.25 (3.51 – 7.84)	5.96 (3.73 – 9.51)
Vascular invasion					
Absent	46	42	3.05	1	1
Present	94	83	5.67	1.15 (0.79 – 1.67)	0.7 (0.45 – 1.08)
Perineural invasion					
Absent	61	49	4.36	1	1
Present	79	76	4.37	1.95 (1.35 – 2.83)	1.36 (0.9 – 2.06)
HER2 status					
Negative	73	58	5.62	1	1
Equivocal	30	30	1.42	3.73 (2.32 – 5.99)	1.79 (1.02 – 3.15)
Positive	37	37	1.68	4.35 (2.8 – 6.76)	2.82 (1.69 – 4.37)
P53 status					
Negative	67	53	5.43	1	1
Positive	73	72	3.29	5.79 (3.85 – 8.71)	4.03 (2.53 – 6.4)
Ki67 status					
Low	61	46	5.08	1	1
High	79	79	3.65	5.66 (3.75 – 8.56)	4.34 (2.64 – 7.13)
MVD-CD34 status					
Low	23	14	2.04	1	1
Moderate	91	85	5.62	2.99 (1.69 – 5.28)	1.56 (0.82 – 2.96)
High	26	26	1.05	11.74 (5.84 – 23.59)	5.35 (2.36 – 12.12)
Cox2 status					
Negative	28	17	2.4	1	1
Low	66	63	4.34	2.58 (1.5 – 4.42)	1.73 (0.98 – 3.06)
High	46	45	1.98	8.66 (4.72 – 15.9)	4.77 (2.39 – 9.49)
MMP7 status					
Negative	34	27	2.63	1	1
Low	68	61	4.37	1.58 (1 – 2.49)	1.02 (0.62 – 1.7)
High	38	37	1.72	4.31 (2.57 – 7.23)	2.75 (1.53 – 4.94)

HR: Hazard ratio; CI: Confidence interval; Adjusted HR: Adjusted for personal and clinical factors, including gender, age, vascular invasion, perineural invasion, histological grade, and tumor stage.

Table 3. Multivariable analyses identifying the prognostic significance of available biomarkers in early and advanced stage subgroups of 140 gastric cancer patients.

Variable	Early stage				Advanced stage			
	Patients (n=63)	Deaths (n=49)	Person-years	Adjusted HR (95% CI)	Patients (n=77)	Deaths (n=76)	Person-years	Adjusted HR (95% CI)
HER2 status								
Negative	52	38	4.5	1	21	20	1.12	1
Equivocal	6	6	0.32	7.16 (2.1 – 24.48)	24	24	1.11	1.08 (0.56 – 2.09)
Positive	5	5	0.36	4 (1.31 – 12.24)	32	32	1.32	2.45 (1.29 – 4.63)
P53 status								
Negative	48	35	4.31	1	19	18	1.11	1
Positive	15	14	0.86	4.11 (1.99 – 8.47)	58	58	2.44	3.28 (1.78 – 6.07)
Ki67 status								
Low	46	32	4.18	1	15	14	0.89	1
High	17	17	0.99	7.06 (3.33 – 14.99)	62	62	2.66	2.8 (1.42 – 5.51)
MVD-CD34 status								
Low	23	14	2.04	1	0	0	0	-
Moderate	38	33	3.02	1.73 (0.87 – 3.4)	53	52	2.6	1
High	2	2	0.11	12.1 (2.07 – 70.46)	24	26	0.95	3.91 (2.1 – 7.29)
Cox2 status								
Negative	24	13	2.21	1	4	4	0.19	1
Low	31	29	2.55	1.8 (0.91 – 3.55)	35	34	1.79	1.51 (0.5 – 4.56)
High	8	7	0.41	9.78 (3.32 – 28.78)	38	38	1.57	3.58 (1.15 – 11.12)
MMP7 status								
Negative	25	18	2.22	1	9	9	0.4	1
Low	29	23	2.41	1.34 (0.65 – 2.75)	39	38	1.97	0.72 (0.33 – 1.6)
High	9	8	0.54	3.56 (1.28 – 9.97)	29	29	1.18	1.72 (0.79 – 3.74)
Vimentin status								
Negative	33	23	3.13	1	0	0	0	-
Low	29	25	1.99	2.05 (1.1 – 3.79)	34	33	1.7	1
High	1	1	0.05	8.81 (1.02 – 76.22)	43	43	1.85	1.51 (0.89 – 2.56)

HR: Hazard ratio; CI: Confidence interval; Adjusted HR: Adjusted for personal and clinical factors, including gender, age, vascular invasion, perineural invasion, and histological grade.

plications of our findings.

Among all biomarkers examined in this study, HER2 could have the highest significance due to prognostic importance and therapeutic implications, as anti-HER2 medication such as trastuzumab is widely used in GC management [10]. Our observations suggested a significantly poor prognostic role of HER2 in GC. Some studies reported a similar prognostic role for HER2 [7,8], while others indicated no prognostic significance [9] or even reported an association of HER2 with higher median overall survival [10]. This controversy can be partly due to intratumoral heterogeneity in HER2 expression that occurs in 69 – 75% of cases [32]. Therefore, 3-4 slides from different parts of tumor tissues could be examined.

Microvessel density (MVD) is a substitute marker for angiogenesis and is graded based on CD34 protein expression. In agreement with previous studies, we found

a significant poor prognostic role of MVD-CD34 in GC [15,33], which links angiogenesis to poor prognosis in GC. TP53 is a tumor suppressor gene widely inactivated in many malignancies, including GC [11]. The nuclear staining intensity of p53 can be due to the accumulation of degradation-resistant mutant proteins [34]. Intratumoral protein expression of p53 ranges from 13-54% in GC [35]. This range was 0%-42% in the current study. We found that GC patients with p53-positive tumors have worse overall survival, and p53 protein level is an independent poor prognostic factor. These findings are concordant with previous reports of more aggressive tumor behavior and worse overall survival in GC patients with p53-positive tumors [27,35,36]. Cox2 is constitutively expressed in specific organs such as the brain, kidney, and eye under normal conditions [37–39]. After replication, Cox2 expression can be considerably up-regulated with downstream induction of pro-inflammatory cytokines, growth factors, and tumor initiators [40]. Expectedly, Cox2 is upregulated in many cancers, including GC [16], and its upregulation is linked to angiogenesis, tumor invasion, and poor prognosis [41–43]. Similarly, we found that Cox2 upregulation is associated with poor overall survival. Given the availability of elective Cox2 inhibitors such as Celecoxib, Cox2-targeted therapy combined with radiotherapy and chemotherapy can improve the prognosis of GC patients [43,44].

The clinical importance of Ki67 has been previously suggested in many cancers, such as GC [12,45]. In a meta-analysis of the data from 53 studies containing 7078 patients, Xiong et al. concluded the predictive and prognostic values for Ki67 expression in GC [46]. In addition, they found associations between Ki67 overexpression and advanced TNM stage, poor tumor differentiation, and poor overall survival [46]. In support, we found that Ki67 overexpression was independently associated with advanced stage and poor overall survival. It is essential to mention that cut-point selection is critical in analyzing Ki67 expression, as different cut-points could produce diverse results and interpretations (Supplementary Table 1). MMPs are well-known for their involvement in tumor invasion, metastasis, and progression. MMP7 is mainly overexpressed in gastric carcinomas, and its expression is associated with deeper in-situ, lymphatic, and vascular invasion of the tumor [18]. A meta-analysis also concluded a poor prognostic role of tumoral MMP7 and its association with aggressive tumor features in GC patients that was more emphasized in Asian ethnicity [26]. Likewise, we found the poor prognostic significance of MMP7 expression in GC in this study. Vimentin is an intermediate filament predominantly expressed in mesenchymal tissues but not in epithelial tissues [21]. Its expression is considered a canonical marker of epithelial-to-mesenchymal transition (EMT) [21]. Vimentin is overexpressed in various tumors, including GC, and is associated with vascular and perineural invasions and poor prognosis [21,22]. Similarly, we observed a significantly poor prognostic role of vimentin protein expression in GC patients regarding stage and overall survival.

There are limitations in this study, such as the unavailability of FISH experiments for samples with equivocal HER2 status and the need for molecular mechanistic investigations. Although we had a relatively large sample size, it did not provide sufficient power to examine the interaction of biomarkers with each other and clinical information, especially in the early-stage group. Hence, a larger population, utilizing the FISH experiment in determining the equivocal HER2 status, and deeper mechanistic exploration are suggested for future studies. The combination of molecular biomarkers and clinical data can serve as more efficient criteria for risk stratification and individualized management of the patients.

Overall, the molecular biomarkers examined in this study could be potentially reliable and predictive prognostic factors, especially in the early stage of GC. These findings help generate a risk assessment tool with high precision to delineate high-risk patients who may benefit from targeted therapy and active surveillance during the treatment. Ultimately, these biomarkers can help develop new targeted drugs and improve the survival of gastric cancer patients. Further studies with a larg-

er sample size are required to examine biomarker-biomarker and biomarker-clinical factor interactions in their prognostic impact on GC. Such studies should prioritize early-stage patients who can benefit from tailored management using targeted therapies.

Abbreviations:

Gastric cancer: GC; Microvessel density: MVD; Immunohistochemistry: IHC; Confidence interval: CI; Human epidermal growth factor receptor 2: HER2; Cluster of differentiation: CD; Tumor protein 53: TP53; Cyclooxygenase 2: Cox2; Matrix metalloproteinase 2: MMP2; Matrix metalloproteinase 9: MMP9; Centimeter: cm; Epithelial-mesenchymal transition: EMT; College of American Pathologists: CAP; Tumor Node Metastasis: TNM; American Joint committee on Cancer: AJCC; Phosphate-buffered saline: PBS; Bovine serum albumin: BSA; Horseradish peroxidase: HRP; Fluorescence in situ hybridization: FISH; Overall survival: OS; Micrometer: ?M; Kaplan-Meier Plot: KM-Plot; Epithermal growth factor receptor: EGFR; Figure: Fig.

Author Contributions:

All authors contributed to the study's conception and design. Amirhossein Razavirad, Ahad Muhammadnejad, Saeed Soleymanjahi, Sanaz Rismanchi, and Kazem Zendehdel performed material preparation, data collection, and analysis. Amirhossein Razavirad wrote the first draft of the manuscript, and all authors commented on previous versions. All authors read and approved the final manuscript.

Competing interests:

The authors declare that they have no competing interests.

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Supplementary Data

Supplementary Table 1. Univariate and multivariable analyses identifying the prognostic significance of Ki67 protein expression with different cut-points in 140 gastric cancer patients.

Variable	Number			Univariate	Multivariable
	Patients (n=140)	Deaths (n=125)	Person-years	Crude HR (95% CI)	Adjusted HR (95% CI)
Ki67 status					
Low (<10%)	**20**	**15**	**1.88**	**1**	**1**
High (>10%)	**120**	**110**	**6.84**	**2.87 (1.66 – 4.96)**	**1.48 (0.78 – 2.8)**
Ki67 status					
Low (<15%)	**61**	**46**	**5.08**	**1**	**1**
High (>15%)	**79**	**79**	**3.65**	**5.66 (3.75 – 8.56)**	**4.34 (2.64 – 7.13)**
Ki67 status					
Low (<20%)	**102**	**87**	**7.12**	**1**	**1**
High (>20%)	**38**	**38**	**1.6**	**4.72 (3.05 – 7.32)**	**2.97 (1.87 – 4.71)**
Ki67 status					
Low (<10%)	**20**	**15**	**1.88**	**1**	**1**
Moderate (10 – 20%)	**93**	**83**	**5.75**	**2.47 (1.41 – 4.32)**	**1.44 (0.7 – 2.74)**
High (>20%)	**27**	**27**	**1.09**	**11.65 (5.86 – 23.15)**	**6.12 (2.7 – 13.84)**

HR: Hazard ratio; CI: Confidence interval; Adjusted HR: Adjusted for personal and clinical factors, including gender, age, vascular invasion, perineural invasion, histological grade, and tumor stage.