

## ORIGINAL ARTICLE

# Replication Protein A (RPA1, RPA2 and RPA3) expression in gastric cancer: correlation with clinicopathologic parameters and patients' survival

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## Summary

**Purpose:** Replication Protein A (RPA) consists of three subunits (RPA1, RPA2 and RPA3) essential for all major DNA metabolic pathways. Although RPA seems to be a promising therapeutic target, its role in human cancers has not been fully elucidated. This is the first study investigating the expression of all the three RPA subunits in a series of 74 resected gastric carcinomas and analyzing the possible correlations with clinicopathologic parameters (histological type, grade, lymphovascular invasion, lymph node status and disease stage), Ki-67 proliferative index, Topoisomerase IIa expression and patients' survival.

**Methods:** Immunohistochemistry using monoclonal antibodies. Univariate and multivariate statistical analysis.

**Results:** All the three subunits showed widespread nuclear expressions in gastric carcinomas with significant associations among their expressions. RPA2 demonstrated higher expression levels in low grade carcinomas and a gradual

significant decrease from N0 to N3 and from stage I to stage IV carcinomas. All the three subunits were statistical significantly more abundant in lymph node negative and earlier stage (stage I ⊕ II) gastric carcinomas. No associations were established among RPAs and the proliferative marker Ki-67. In patients with positive lymph nodes and advanced tumor stage, RPA1 expression seemed to predict a better overall survival implying a probable predictive role.

**Conclusions:** The widespread expression of RPA(1-3) suggests one or more roles in gastric cancer. Their presence in earlier stage tumors probably offers an opportunity for early targeted therapy. Their probable predictive value in node positive and advanced stage tumors needs further investigation with respect to specific chemotherapeutic treatments.

**Key words:** gastric cancer, Replication Protein A (1-3), survival

## Introduction

Replication protein A (RPA) is the major single-strand DNA (ssDNA) binding complex in eukaryotes [1]. RPA is essential for all major DNA metabolic pathways, including DNA replication, repair, recombination, cell cycle progression, and the DNA damage response, playing a role as a sensor in multiple

DNA checkpoint pathways [1-5]. RPA is required for each of the four major DNA repair pathways: nucleotide excision repair (NER), base excision repair (BER), DNA mismatch repair (MMR), and DNA double strand break (DSB) repair [1]. Human RPA is present in cells as a heterotrimeric complex consist-

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ing of three subunits which, in order of decreasing size, have been designated as RPA1 (70kDa), RPA2 (32kDa) and RPA3 (14kDa) [6-8]. We have previously shown that RPA expression is upregulated in human tumors including colon cancer, urothelial bladder cancer, ovarian cancer and astrocytic tumors [9-12]. Other investigators have also shown RPA upregulation in esophageal, breast, nasopharyngeal and hepatocellular carcinomas [13,14].

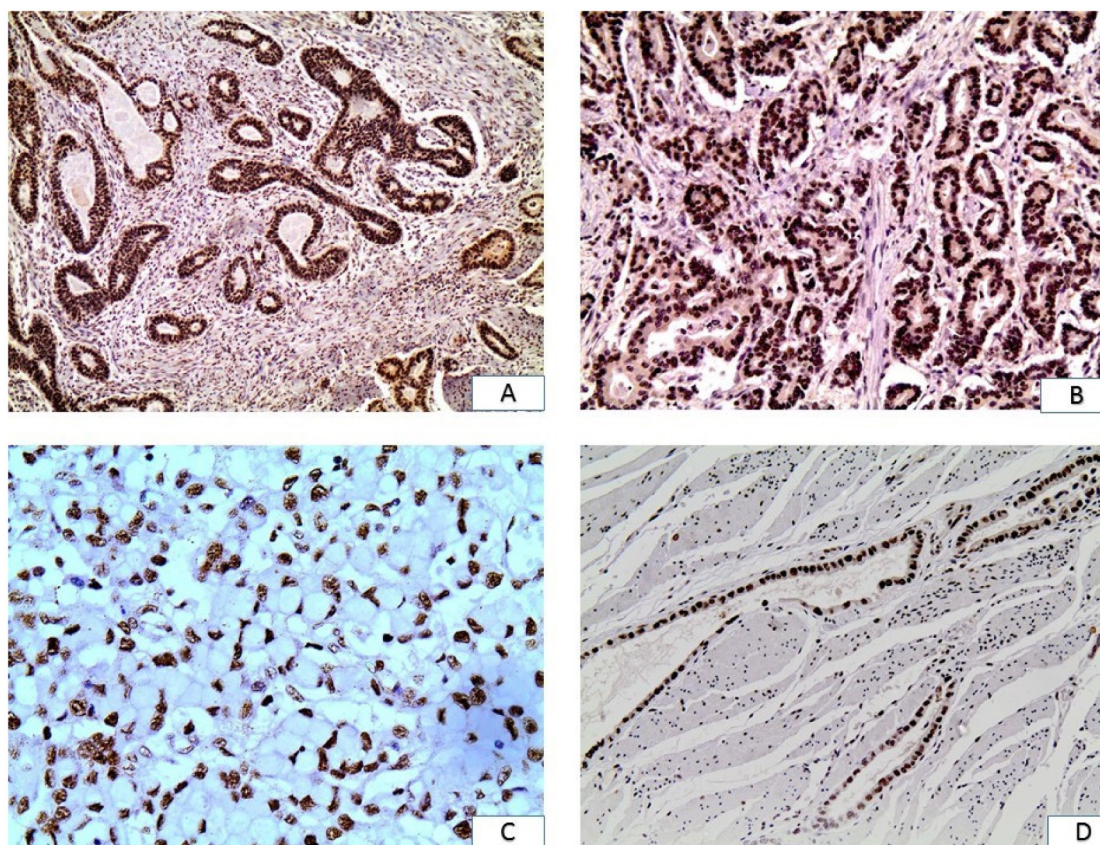
However, there is very limited data concerning gastric cancer. Recently, it has been shown that forced over-expression of miR-30a in the gastric cancer cell line SGC7901R inhibited cell proliferation by hindering the replication of DNA and inducing DNA fragmentation [15]. Interestingly, the target of miR-30a was identified as the DNA replication protein RPA1 suggesting that exploiting DNA replication through RPA might be exploited as a target for cancer therapy [15]. There is one previous study investigating the third subunit of RPA (RPA3) expression in human gastric cancer and reporting that RPA3 upregulation is involved in tumorigenic properties of gastric cancer cells [16]. However, to the best of our knowledge, no previous study has investigated all the three subunits of the RPA protein in gastric cancer.

The aim of the present study was to investigate the immunohistochemical expression of RPA1, RPA2 and RPA3 proteins in a series of 74 gastric cancer resection specimens. Moreover, RPA1, RPA2 and RPA3 expression was analyzed in relation to conventional clinicopathologic parameters (age, gender, grade, lymph node status, lymphovascular invasion and stage of the disease), Ki-67 index, TopoIIa expression and patients' survival.

## Methods

### Patients

This is a retrospective study of 74 patients who presented with primary gastric carcinoma, for whom paraffin-embedded tissue and clinical information were available. There were 51 men and 23 women with a median age of 69 years (range 35-91). None of the patients had received chemotherapy or radiation before surgery. According to the 8th edition of the TNM system of cancer staging adopted by the American Joint Committee on Cancer and the International Union Against Cancer (AJCC/IUCC), tumors were classified as stage I: 10 cases, stage II: 13 cases, stage III: 49 cases, and stage IV: 2 cases. All cases were reviewed and assigned a histologic grade according to WHO 2019 classification of gastric carcinomas: there were 21 low grade and 53 high



**Figure 1.** **A:** Diffuse RPA1 nuclear staining in a low grade gastric carcinoma (x100). **B:** Diffuse RPA2 nuclear staining in a low grade gastric carcinoma (x200). **C:** RPA3 nuclear staining in a poorly cohesive gastric carcinoma (x400). **D:** RPA3 nuclear staining in gastric carcinoma glands infiltrating muscle wall (x400).

grade carcinomas. The median follow-up period was 60 months. During this period, 10 disease-specific deaths were recorded.

#### Processing of specimens and immunohistochemistry

Tissues were fixed immediately after removal in 10% buffered formalin and processed to paraffin wax. Four  $\mu\text{m}$  serial sections were cut from each specimen on superfrost plus glass slides and left to dry overnight at 37°C. Immunohistochemical detection of RPA1, RPA2 and RPA3 proteins was performed by standard streptavidin-peroxidase method using the monoclonal antibodies anti-RPA1 (P70 subunit, NA13, oncogene), anti-RPA2 (P32 subunit, NA18, oncogene) and anti-RPA3 (P14 subunit, clone 1F4, Abnova) in a dilution of 1:50 for each antibody. To enhance antigen retrieval sections underwent microwave treatment (using 1 Mm EDTA PH 8.0). For negative controls, normal goat serum was used

instead of primary antibody at 4°C overnight prior to the following staining procedure. Staining for all antibodies was assessed blindly (ie without any knowledge of the clinical data) by two observers. Whenever a difference of greater than 5% between the two assessments was observed, slides were reviewed jointly and a consensus was reached. Nuclei from about 1000 tumor cells from systematically randomized fields ( $\times 40$ ) throughout the entire section were counted and the labelling index (LI) was calculated as the percentage of labelled nuclei out of the total number of tumor cells counted. All clearly identifiable nuclear staining beyond background was recorded as positive for RPA1, RPA2 and RPA3. No lymphoid cells were included in the counts even though they expressed RPA1, RPA2 and RPA3.

#### Statistics

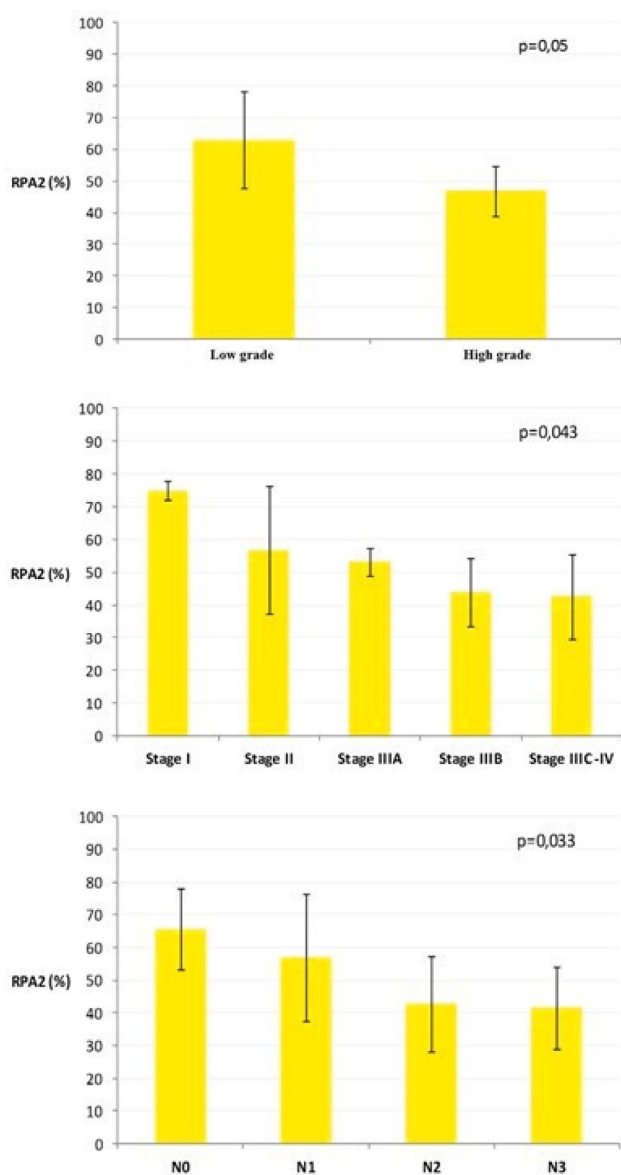
The normality of distributions was tested with the Kolmogorov-Smirnov test. Pearson's Chi-square with continuity correction and ANOVA test were used to assess possible correlations between RPA1, RPA2 & RPA3 expressions with the clinicopathologic parameters investigated. The prognostic effect of various parameters (i.e. age, gender, histologic type, grade, lymph node status, lymphovascular invasion, stage, RPA1, RPA2, RPA3, Ki-67 and TOPO IIA expression) on clinical outcome (ie death of disease) was tested by plotting survival curves according to Kaplan-Meier method and comparing groups using the log rank test, as well as by multivariate analysis using the Cox regression model. Patients dying of other causes during the follow-up period were treated as censored data. To avoid any 'data-driven' categorization, continuous variables were entered in multivariate analysis as continuous variables. Statistical analyses were performed using the SPSS for Windows Software (SPSS Inc., Chicago, IL, USA). A p value of less than or equal to 0.05 was considered indicative of a statistically significant difference.

## Results

RPA1, RPA2 and RPA3 nuclear immunoreactivity was observed in all carcinomas examined. RPA1, RPA2 and RPA3 LIs ranged from 2 to 85% for RPA1 and RPA3 and from 2 to 90% for RPA2, with a median of 40%, 50% and 40% for RPA1, RPA2 and RPA3, respectively. The pattern of staining was mostly nuclear, although a faint cytoplasmic staining was seen in a few cases, which was disregarded as non-specific (Figure 1). Nuclear staining was strong to moderate in almost all cases investigated.

Moreover, RPA1, RPA2 and RPA3 nuclear immunoreactivity was also seen in normally-appearing gastric mucosa adjacent to carcinomas, in all examined cases. In normal mucosa, positive cells were evenly distributed and did not occur in clusters.

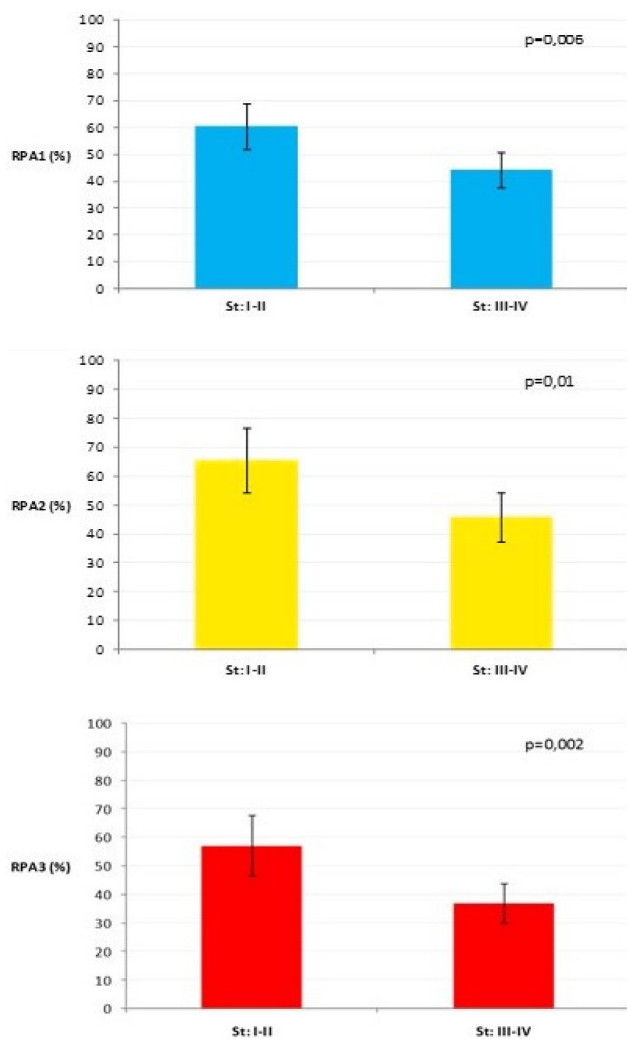
In carcinomas, immunostaining for Ki-67 and Topoisomerase IIA were also found in all carcino-



**Figure 2.** RPA2 association with histological grade, stage of disease and lymph node status.

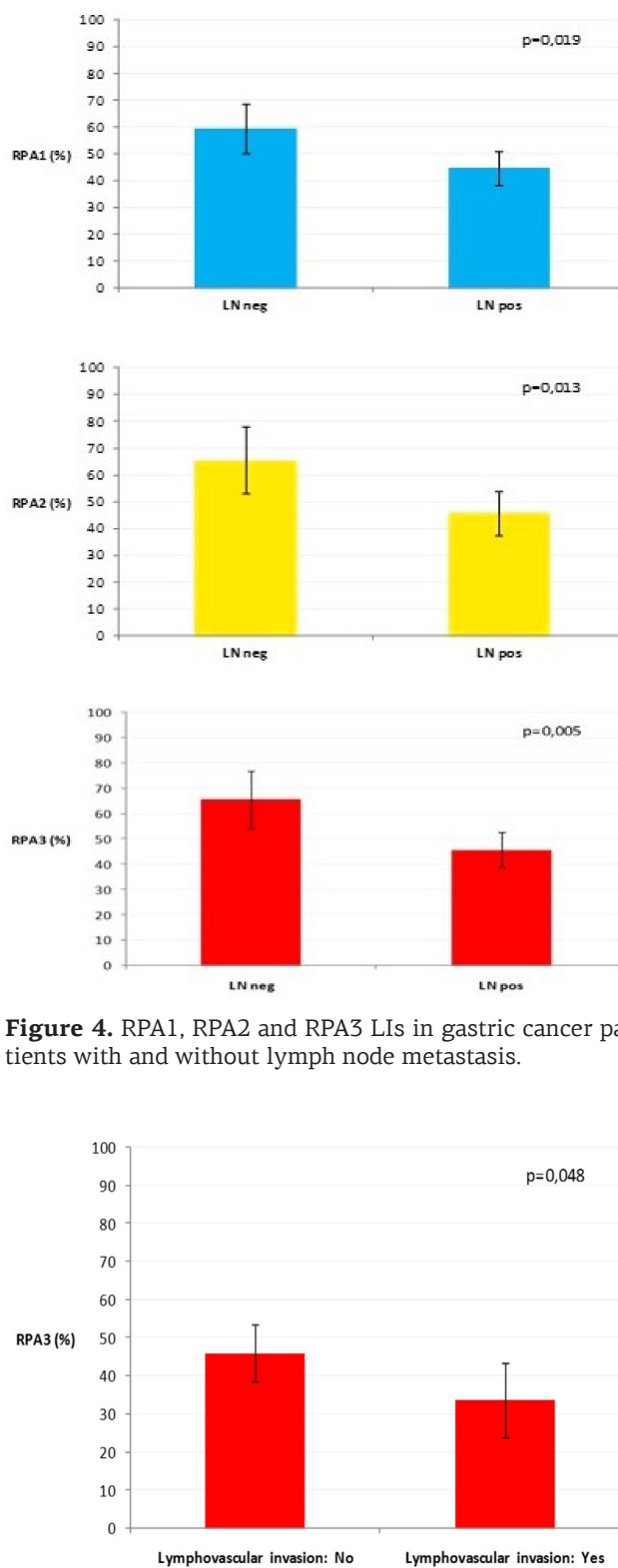
mas ranging from 5 to 90% for each protein (median: 45% and 40%, respectively). Ki-67 and Topoisomerase IIA expressions, were significantly lower in the adjacent normal epithelium compared to cancers.

Strong positive correlations emerged, using the Pearson's correlation test, between RPA1 and RPA2 ( $p=0.002$ ), RPA1 and RPA3 ( $p<0.0001$ ), RPA2 and RPA3 ( $p<0.0001$ ) expressions. A significant correlation was also found between Ki-67 and Topoisomerase IIA expressions ( $p<0.0001$ ). No significant associations were found between RPAs and either Ki-67 or Topoisomerase IIA expression, when analyzed in the whole cohort. However, when investigating these associations stage by stage, significant correlations emerged in stage I carcinomas between RPA2 protein and Topoisomerase IIA expressions ( $p=0.05$ ) and between RPA3 protein and Topoisomerase IIA expressions ( $p=0.04$ ) as well. No significant correlations were established between RPA1 or RPA3 and Topoisomerase IIA nor among RPAs and Ki-67 expressions.



**Figure 3.** RPA1, RPA2 and RPA3 LIs in association with stage of disease.

When analyzing the relationship between RPA1, RPA2 or RPA3 expression and the various clinicopathologic parameters, using ANOVA test, a statistical significant inverse correlation was established between RPA2 labeling index and histologi-



**Figure 4.** RPA1, RPA2 and RPA3 LIs in gastric cancer patients with and without lymph node metastasis.

**Figure 5.** RPA3 LI in association with lymphovascular invasion.

cal grade ( $p=0.05$ ) in that high grade carcinomas demonstrated lower RPA2 expression than low grade carcinomas (Figure 2). No significant correlation emerged between each protein and histologic type (according Lauren's or WHO classification).

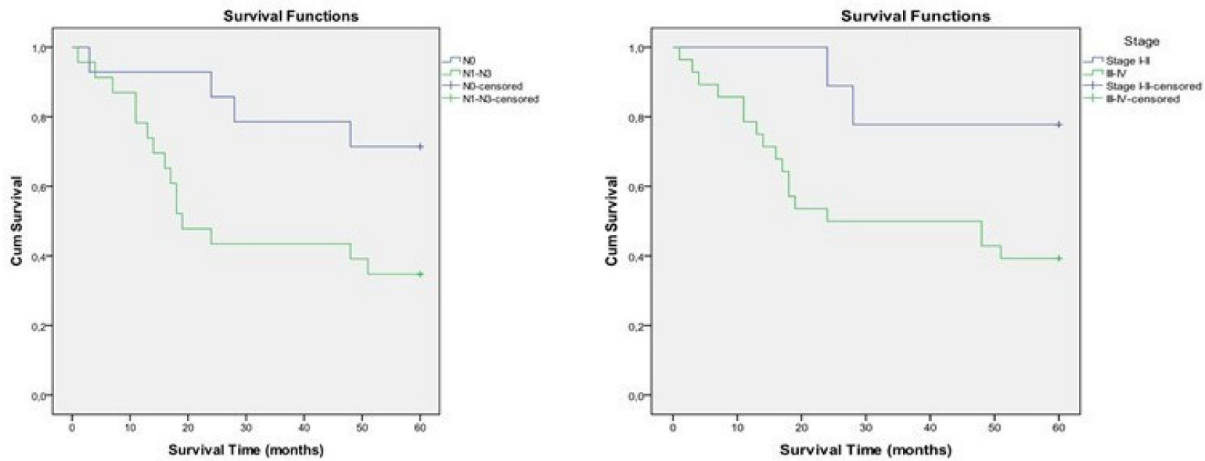
A gradual decrease in RPA2 labeling index was observed from stage I to stage IV carcinomas and this association was of statistical significance ( $p=0.043$ ) (Figure 2).

RPA1, RPA2 and RPA3 labeling indices were significantly higher in stage I and II carcinomas compared to stage III and IV carcinomas ( $p= 0.006$ ,  $p= 0.01$  and  $p=0.002$  respectively) (Figure 3).

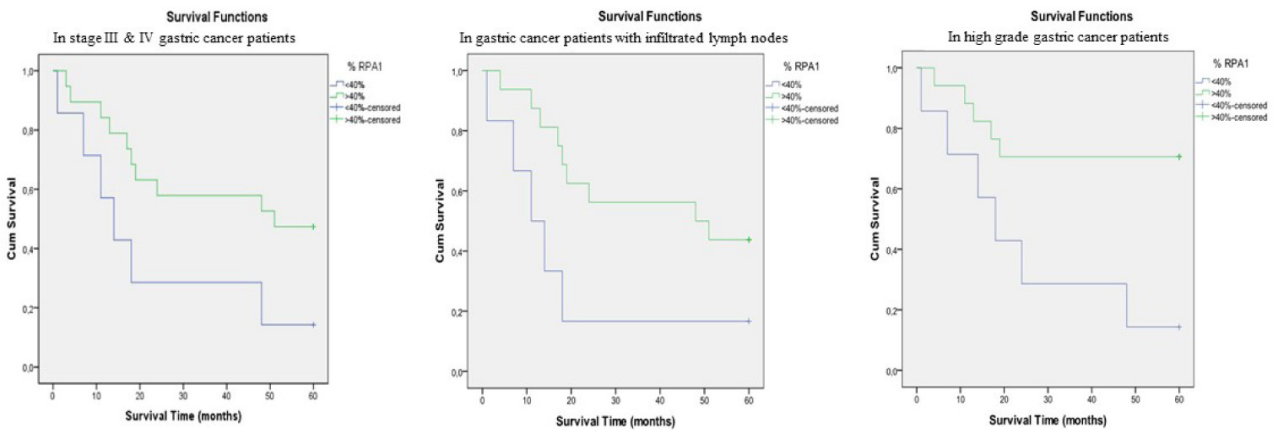
When analyzing the associations among RPAs and staging parameters (T and N), we found that RPA1, RPA2 and RPA3 expressions were significantly higher in patients without lymph node metastasis (N0) compared to patients with infiltrated lymph nodes (N1, N2 and N3) ( $p= 0.019$ ,  $p=0.013$  and  $p=0.005$ , respectively, Figure 4). Moreover, a gradual decrease of RPA2 labeling index from N0

to N3 lymph node status was also noted ( $p=0.033$ , Figure 2). A significantly lower RPA3 labeling index was recorded in cases with lymphovascular invasion ( $p=0.048$ , Figure 5). No association with T-category was established.

In univariate survival analysis, lymph node status [Log Rank (Mantel-Cox):  $p=0.026$ , chi-square: 4.962, df:1] and stage of disease [Log Rank (Mantel-Cox):  $p=0.047$ , chi-square: 3.934, df:1] showed statistically significant correlations with patients' survival (Figure 6). In the group of patients with stage III & IV carcinomas, in the group of patients with infiltrated lymph nodes (N1-N3) and in the group of patients with high grade carcinomas, we found significant associations between RPA1 labeling index greater than 40% and a better overall survival [Log Rank (Mantel-Cox)  $p=0.05$  and  $p=0.05$  and  $p=0.014$  respectively, Figure 7]. In multivariate Cox's regression analysis of the whole cohort, significant associations with survival were found for lymph node status and RPA1 ( $p=0.025$ ; Table 1).



**Figure 6.** Univariate survival analysis showing significant correlations between lymph node status, stage of disease and patients' survival.



**Figure 7.** RPA1 impact on survival of patients with infiltrated lymph nodes (N1-N3), stage III & IV carcinomas, and in the group of patients with high grade carcinomas.

**Table 1.** COX regression analysis. Variables in the Equation

	B	SE	Wald	df	Sig.	HR	95.0% CI for HR	
							Lower	Upper
Step 2 rpa1.100	-.036	.016	5.001	1	.025	.965	.934	.996
LN	.358	.189	3.570	1	.059	1.430	.987	2.074

## Discussion

This is the first study investigating the immunohistochemical expression of all the three subunits of RPA protein in gastric cancer. According to our results, RPA1, RPA2 and RPA3 proteins showed a widespread nuclear expression in gastric carcinomas, in keeping with our previous results in other types of human cancer such as colon, bladder, ovarian and astrocytic tumors [9-12]. Moreover, all the three subunits were expressed in the adjacent, apparently normal gastric mucosa, in keeping with our results concerning RPA1 and RPA2 in normal colon mucosa adjacent to carcinomas. The widespread expression of RPAs in normal appearing mucosa, reflects the role of these proteins under physiological conditions, in the regulation of DNA replication or as a part of the reparatory genomic machinery. In view of the above, the widespread presence of RPAs in cancer cells suggests that their expression is not lost during malignant transformation. Moreover, it seems that RPA proteins have a role to play in malignant cells, although probably not the same to their role in normal cells.

One of the many differences between normal cells and cancer cells is the amount of replication stress that occurs during replication. Cancer cells with activated oncogenes generate increased levels of replication stress [17]. Replicative stress seems to be unique to cancer cells since it is rarely observed in normal cells even when they proliferate rapidly [17]. It has been shown that RPA inhibition increases the replication stress and suppresses tumor growth [18]. This might designate a role of RPAs in cancer cells and explain their widespread expression in gastric and other carcinomas. In other words, it seems logical that RPAs facilitate tumor growth helping cancer cells to withstand the replicative stress.

In view of the increasing levels of replication stress in cancer cells and the reported role of RPAs in decreasing this stress, it would be probably expected an increasing need for RPAs in carcinomas with higher proliferation rates. However, in our study, no significant associations were established between RPAs and the proliferation marker Ki-67. This finding is in keeping with the results reported in ovarian carcinomas [11]. In bladder cancer we have seen a positive association between RPA1 and

RPA2 and cyclin D1 expression [10]. Recently, it has been shown that in hepatocellular carcinoma cells (HCC), RPA1 influences cell cycle through CDK4/Cyclin-D pathway [19]. CDK-4/cyclin-D expression helps cell overcome the restriction between G1 and S phase. So RPA1 promotes HCC proliferation with up-regulation of CDK-4/cyclin-D, indicating that RPA1 functions to drive more HCC cells into S and G2 phases [19]. It has been shown that in gastric carcinomas, the D-type cyclins reach maximum levels of expression and form functional complexes with CDK4 during the mid-G1 phase [20]. On the other hand, it has been recently suggested that Ki67 is a graded rather than a binary marker both for cell-cycle progression and time since entry into quiescence [21]. Miller et al reported that Ki-67 accumulation occurs only during S, G2, and M phases and that it is degraded continuously in G1 and G0 phases, regardless of the cause of entry into G0/quiescence. Consequently, the level of Ki67 during G0 and G1 in individual cells is highly heterogeneous and depends on how long an individual cell has spent in G0 [21]. The above observations might probably explain the absence of significant correlations between RPAs and Ki-67 expressions.

Another interesting finding of this study was the correlations between RPAs expressions and clinicopathologic parameters such as grade, lymphovascular invasion, lymph node status and the stage of disease. Similar associations have been previously recorded in bladder carcinomas, suggesting that the proper function of the RPA machinery in these tumors, may be preserved in the better differentiated and less advanced tumors [10]. However, this is an issue that requires more studies to be clarified since these associations may be organ or cancer type-dependent, as, for instance, our study in colon carcinomas revealed parallel associations with tumor grade and/or stage. Our survival analysis suggested that higher RPA1 expression was significantly associated with better overall survival in the group of patients with lymph node metastasis or advanced disease stage. This finding could imply a possible predictive value of RPA1 with respect to chemotherapy given in lymph node-positive and advanced stage patients. In multivariate survival analysis RPA1 retained its significance along with lymph node status.

A limitation of our study, as it happens with most immunohistochemical studies, is the inability to discern the functional status of the immunohistochemically expressed proteins. It is not certain that the immunohistochemically detected protein is functionally intact. RPA is hyperphosphorylated upon DNA damage or replication stress by checkpoint kinases including ATM (ataxia telangiectasia mutated), ATR (ATM and Rad3-related) and DNA-PK (DNA-dependent protein kinase) [22]. The hyperphosphorylation may change the functions of RPA and, thus, the activities of individual pathways in which it is involved [22]. There is growing evidence that hyperphosphorylation alters RPA-DNA and RPA-protein interactions [22]. Apart from hyperphosphorylation, extensive genetic analysis of the yeast homologue of RPA1 (RFA1) has shown that viable mutations in this gene exhibit defects in DNA repair, recombination, and elevated chromosome rearrangements and mutation rates [23]. In mammals, the L221P RPA1 mutation causes a defect in ssDNA binding and a nonfunctional protein complex, causing an increase in the levels of DNA damage and in the incidence of cancer [24]. Whether the observed in our study proteins are hyperphosphorylated, mutated, functional or not, and if the reduced expression levels in the less differentiated and more advanced tumors is a consequence of accumulating mutations or other cancer progression related events, needs further investigation.

On the other hand, the widespread presence of RPAs in gastric cancer offers a potential target for therapeutic intervention. It has been shown that RPA proteins are involved in the ATR/Chk1 pathway which is a critical surveillance network that maintains genomic integrity during DNA replication by stabilizing the replication forks during normal replication to avoid replication stress [18]. This creates an increased dependency on the ATR/Chk1 pathway in cancer cells [18]. Glanzer et al have identified a novel protein termed HAMNO which inhibits RPA through selectively binding to the N-terminal domain of RPA 70 and by inhibiting both ATR autophosphorylation and phosphorylation of RPA 32Ser33 by ATR [18]. According to Glanzer et al, HAMNO treatment creates DNA

replication stress in cancer cells that are already experiencing replication stress, but not in normal cells, and it acts synergistically with etoposide to kill cancer cells *in vitro* and slow tumor growth *in vivo* [18]. Thus, HAMNO illustrates how RPA inhibitors represent candidate therapeutics for cancer treatment, providing disease selectivity in cancer cells by targeting their differential response to replication stress [18]. One could postulate that targeted inhibition of the RPAs might open up an opportunity to preferentially kill cancer cells by inhibiting this pathway. Moreover, in a recent study exploring the role of RPA in gastric cancer cells, silencing of RPA1 induced cell cycle arrest at the G1 phase and promoted cell apoptosis by regulating the protein level of Caspase 3 [25]. The observed in our study RPA expression upregulation in gastric cancer might thus provide an opportunity for targeted intervention.

In conclusion, our study has demonstrated for the first time the widespread expression of all the three RPA subunits in gastric cancer. This expression seems to be more abundant in low grade tumors without lymph node metastasis and in the earlier stages of disease. Further studies are required to clarify the functional status of RPA in gastric carcinomas inasmuch as it has been suggested that RPA influences chemoresistance [26,27]. More specifically, it has been reported that in ovarian tumors, cisplatin cancer cell killing is potentiated by mutations that cripple RPA binding to DNA and that modulation of RPA protein levels/dynamics is a critical determinant of chemoresistance via multiple mechanisms (27,28). Under these observations, and as it happens with other markers associated with favorable prognosticators which act simultaneously as predictive factors (for instance hormonal receptors in breast cancer) the predictive value of RPA needs further investigation as RPA expression could offer an opportunity for targeted inhibition.

### Conflict of interests

There are no conflicts of interest that could be perceived to bias this work, nor financial support or any other personal connections.

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