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Differentially localized survivin and STAT3 as markers of gastric cancer progression: Association with *Helicobacter pylori*

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Abstract

Background: Localization and differential expression of STAT3 and survivin in cancer cells are often related to distinct cellular functions. The involvement of survivin and STAT3 in gastric cancer has been reported in separate studies but without clear understanding of their kinetics in cancer progression.

Methods: We examined intracellular distribution of STAT3 and survivin in gastric adenocarcinoma and compared it with normal and precancer tissues using immunoblotting and immunohistochemistry.

Results: Analysis of a total of 156 gastric samples comprising 61 histologically normal, 30 precancerous tissues (comprising intestinal metaplasia and dysplasia), and 65 adenocarcinomas, collected as endoscopic biopsies from treatment naïve study participants, revealed a significant ($P < .001$) increase in overall protein levels. Survivin expression was detectable in both cytoplasmic (90.8%) and nuclear (87.7%) compartments in gastric adenocarcinomas lesions. Precancerous dysplastic gastric lesions exhibited a moderate survivin expression (56.7%) localized in cytoplasmic compartment. Similarly, STAT3 and pSTAT3 expression was detected at high level in gastric cancer lesions. The levels of compartmentalized expression of survivin and STAT3/pSTAT3 correlated in precancerous and adenocarcinoma lesions. Although overexpression of these proteins was found associated with the tobacco use and alcohol consumption, their expression invariably and strongly correlated with concurrent *Helicobacter pylori* infection. Receiver operating characteristic analysis of nuclear survivin, STAT3, and pSTAT3 in different study groups showed acceptable positive and negative predictive values with area under the curve above 0.8 ($P < .001$).

Conclusion: Overall, our results suggest that overall increase in survivin and STAT3 and their subcellular localization are key determinants of gastric cancer progression, which can be collectively used as potential disease biomarkers and therapeutic targets for gastric cancer.

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Abbreviations: AUC, area under the curve; DC, diffuse-type adenocarcinoma; Dys, dysplasia; IC, intestinal-type adenocarcinoma; IHC, immunohistochemistry; IM, intestinal metaplasia

KEYWORDS

dysplasia, gastric adenocarcinoma, intestinal metaplasia IV, STAT3, survivin

1 | INTRODUCTION

According to Globocan 2012, stomach cancer attributable to *Helicobacter pylori* infection is the fifth common malignancy in the world (952 000 cases, 6.8% of the total) and the third leading cause of cancer death for both sexes worldwide (723 000 deaths, 8.8% of the total).[1] In India, stomach cancer is ranked third (incidence, 43 386; mortality, 40 721) and sixth (incidence, 19 711; mortality, 18 320) in men and women, respectively.[1] Regardless of the prevailing habits of tobacco and alcohol abuse[2] and reportedly high prevalence of infection with pathogenic *H. pylori* strains[3-5] accompanied with low socioeconomic conditions, the incidence rate of gastric cancer is reported to be "low" in India.[2,6,7] However, these estimates are likely to be low because of gross underreporting and misdiagnosis of the disease, particularly in rural areas of India where *H. pylori* incidence is high, and which lacks necessary diagnostic and endoscopic facilities. Management of gastric cancer in India is alarmingly poor; age-adjusted relative 10-year survival ranges from 4% to 15% (average, 6%) in various regions in India.[2,8]

Survivin, the smallest member of the Inhibitor of Apoptosis (IAP) family, plays a critical role in apoptosis, cell division, and cell migration/metastasis.[9] In general, it is associated with poor prognosis and low patient survival.[10] Prognostic significance of survivin in gastric carcinogenesis has been investigated but with contradictory conclusions.[11-15] Earlier studies suggested that nuclear survivin is associated with cell proliferation whereas cytoplasmic and mitochondrial survivin is linked to chemoresistance. However, these results appear to be tissue specific,[16] and these discrepancies, also identified in other malignancies, were attributed to differentially localized survivin in subcellular compartments as observed in gastrointestinal carcinoma and other cancers.[15,17] Despite past studies on survivin in advanced gastric lesions, modulation of its level and subcellular distribution during the progression of gastric cancer is currently lacking.

Activation of a transcription factor STAT3 induces the survivin gene and confers resistance to apoptosis.[18] STAT3 activation is mediated by tyrosine phosphorylation at Y705, a converging point for diverse oncogenic signaling pathways.[19] In gastric cancer cells, STAT3 was found to be constitutively active, which promotes cell survival, in association with survivin.[20] *H. pylori-cagA* was subsequently shown to activate STAT3 signaling pathways, which in gastric cancer were correlated with survival, metastasis, and poor clinical outcome.[21] Interestingly, nuclear translocation of STAT3 in gastric cancer cells can be triggered by cagA-mediated clustering of Interleukin (IL) 6 and gp130 membrane receptors.[22]

In any event, while there is compelling evidence for the presence of survivin and STAT3, individually in advanced malignant gastric lesions, their levels and subcellular distribution during gastric cancer progression were poorly defined. Moreover, their association with concurrent *H. pylori* infection or other lifestyle factors like use of tobacco and alcohol is not known. As both of these proteins shuttle between the cytoplasm and nucleus, a detailed understanding of their distribution in different subcellular pools warrants investigation particularly as linked to the disease progression. In the present investigation, we have therefore assessed collectively modulation of expression of survivin and STAT3, particularly their subcellular localization in a subset of tissues representing different stages of gastric cancer. In addition, we examined the correlation between survivin expression with STAT3 and with other factors associated with gastric carcinogenesis such as the presence of *cagA*$^+$ *H. pylori* infection, tobacco usage, or alcohol abuse. Clinical utility of survivin and STAT3 as potential markers in gastric cancer progression was also evaluated.

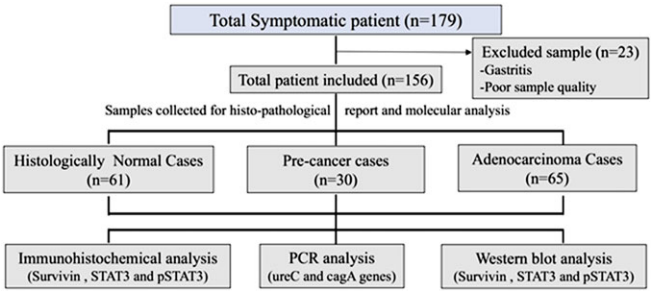
Clinical significance

Survivin and STAT3 could be suitable therapeutic targets in preventing gastric cancer progression, particularly in cases positive for concurrent *H. pylori* infection with history of tobacco smoking.

2 | MATERIALS AND METHODS

2.1 | Clinical specimens and reagents

A total of 156 gastric samples comprising 61 histologically normal, 30 precancerous tissues, and 65 adenocarcinomas, collected as endoscopic biopsies, were obtained prior to any chemo/radiotherapy from the patients attending Gastroenterology Out-Patient Department of Swaroop Rani Hospital, an affiliate to Moti Lal Nehru Medical College, Allahabad, India, during the period of 2007-2012. We excluded the samples with history of prior chemotherapy. As per protocol and prevailing clinical practice, the patients were not followed. Gastric tissues with type IV intestinal metaplasia (IM, n = 15) and dysplasia (Dys, n = 15) were grouped as gastric precancerous lesions while diffuse-type adenocarcinoma (DC, n = 44) and intestinal-type adenocarcinoma (IC, n = 21) were grouped as cancer lesions. Symptomatic, clinically suspected cases found histologically normal were used as a control. We also collected gastritis cases (n = 23) as well, although these were excluded from the study to avoid any bias due to poor sample quality (study design presented as standard flow diagram). The patients taking antibiotics, with bleeding ulcers, or suffering acute hemorrhage at other sites in the upper gastrointestinal track, stomach surgery, or chemotherapy were excluded. Written informed consent was obtained from all of the subjects. The study was conducted in accordance with the principles of Helsinki Declaration. The Institutional Ethics Committees of Moti Lal Nehru Medical College, Allahabad, and Institute of Cytology and Preventive Oncology (now renamed as National Institute of Cancer Prevention and Research), Noida, India, approved this study prior



to its commencement. Clinico-epidemiological and demographic details were taken from clinical record of patients and captured on a pro forma document. The study questionnaire and the case report form included details of tobacco use (chewing or smoking) and alcohol usage. The person with these habits for 6 months or more in the past at the time of examination (at least one cigarette or bidi/tobacco chew/alcohol shot for at least 3 d/wk) was grouped as a tobacco user or alcoholic. A person with more than one habit was grouped under multiple habits.

A summary of the distribution of study subjects with respect to their clinico-epidemiological characteristics included in different disease groups is presented in Table 1. A part of the clinical sample taken for diagnostic procedures was used for research purpose. A minimum 2 punches per specimen were collected from the lesion area by endoscope-guided biopsy procedure. One punch of these tissue was immediately and invariably fixed in formalin for routine histopathological examination and for immunohistochemistry (IHC) analysis while other punch(es) were collected in chilled 1× phosphate-buffered saline for DNA and protein isolation for subsequent molecular studies. The histopathological grading of precancerous and cancerous lesions was performed following WHO/Laurén Classification, which was based on an evaluation of architectural and cytological changes. For each case, the pathologist recorded the grades and details of criteria on which the decision was based. All reagents used in the study were of analytical or molecular biology grade and procured from Sigma-Aldrich (St Louis, MO, USA) whereas primary and secondary antibodies and IHC kits were from Santa Cruz Biotechnology, Inc (Texas, USA) unless specified.

2.1.1 | *H. pylori* detection

Tissue-derived DNA was subjected to polymerase chain reaction (PCR)–based detection of *H. pylori* infection by analyzing for *ureC* and *cagA* genes using established primers listed in Table S1 (methods detailed in Methods S1).

2.2 | Immunohistochemical analysis for survivin, STAT3, and pSTAT3 levels

The immunohistochemistry staining (IHC) was performed as described previously with minor modifications.[23,24] Briefly, formalin-fixed, paraffin-embedded sections (5 μm) of the gastric tissues were collected on poly-L-lysine–coated slides and then deparaffinized in xylene. After hydration in a decreasing alcohol gradient, antigen retrieval was performed by pretreatment in a microwave oven for 10 minutes at 800 W and 5 minutes at 480 W in citrate buffer (0.01M, pH = 6.0). The sections were incubated with hydrogen peroxide (0.3% *v*/v) in methanol for 30 minutes to quench the endogenous peroxidase activity, followed by blocking with 1% bovine serum albumin to prevent

TABLE 1 Clinico-epidemiological details of the subjects enrolled in the study

Clinico-epidemiological Characteristics	Normal	Precancer	Cancer (Adenocarcinoma)
Total subjects	61	30	65
Gender			
Male	42 (68.9%)	17 (56.7%)	42 (64.6%)
Female	19 (31.1%)	13 (43.3%)	23 (35.4%)
Histopathological classification of lesions			
Intestinal metaplasia IV	…	15 (50%)	…
Dysplasia	…	15 (50%)	…
Diffuse-type adenocarcinoma	…	…	44 (67.7%)
Intestinal-type adenocarcinoma	…	…	21 (32.3%)
Age in years (median)	07-85 (40)	13-78 (41.5)	22-90 (55)
Habits[a]			
Tobacco chewers	28 (45.9%)	15 (50%)	24 (37%)
Tobacco nonchewers	33 (54.1%)	15 (50%)	41 (63%)
Tobacco smokers	22 (36.1%)	11 (36.7%)	20 (30.8%)
Tobacco nonsmokers	39 (64%)	19 (63.3%)	45 (69.2%)
Alcoholic	15 (24.6%)	8 (26.7%)	16 (24.6%)
Nonalcoholic	46 (75.4%)	22 (73.3%)	49 (75.4%)
Multiple habits	22 (36.1%)	9 (30%)	16 (24.6%)
Helicobacter pylori positive	37 (60.7%)	28 (93.3%)	34 (52.3%)
ureC positive	33 (54.1%)	27 (90%)	33 (50.7%)
cagA positive	21 (34.4%)	22 (73.3%)	17 (26.2%)

[a]Tobacco chewing habits include betel quid, areca nut, and/or pan masala use; multiple habits include 2 or more of any tobacco and/or alcohol habits.

nonspecific binding. Thereafter, the slides were incubated with mouse monoclonal antisurvivin (SC-17779), anti-STAT3 (SC-483), or anti–pSTAT3-Y705 (SC-8059) antibodies (Santa Cruz Biotechnology) for 16 hours at 4°C. The primary antibodies were detected using the streptavidin-biotin complex with the ABC kit (Santa Cruz Biotechnology). The slides were stained with diaminobenzidine chromogen for 5 minutes, rinsed in tap water, counterstained in Mayer's hematoxylin, dehydrated using increasing alcohol gradient and xylene, and mounted in DPX. Immunoreactivity was visualized in an Olympus microscope. To avoid any batch variation, we always included a positive control that was used during standardization of the protein staining and all samples were stained for a protein at the same time to avoid any bias.

2.3 | Evaluation of immunohistochemical staining

Each slide was evaluated for survivin, STAT3, and pSTAT3 immunostaining using a semiquantitative scoring system for both staining intensity and the percentage of positive epithelial cells.[24] The sections were scored independently by 2 investigators (A.P. and S.C.T.). The tissue sections were scored on the scale of 0 to 7 based on the percentage of immunostained cells as <10% = 0; 10-30% = 1; 30-50% = 2; 50-70% = 3; and 70-100% = 4. Sections were also scored semiquantitatively on the basis of staining intensity as no-stain = 0; mild = 1; moderate = 2; and intense = 3. Finally, a total score was obtained by adding the scores of percent positivity and intensity. For intracellular localization of proteins in various compartments, the nuclear and cytoplasmic staining were scored independently. Less than 5% cases showed the interobserver discrepancy. In the cases with score discrepancy between the 2 observers, a third independent review was performed (V.M.) and a consensus on the final result was reached. The sections were considered positive if cells showed immunopositivity in the nucleus or cytoplasm and attained a total score of 2 or more for survivin and 3 or more for STAT3 and pSTAT3.

2.4 | Total protein isolation and immunoblotting

Total proteins from biopsies and cell lines were isolated and examined by the method described previously.[23,25] Detailed procedures are provided in Methods S1.

2.5 | Statistical analysis

The IHC data were subjected to statistical analyses using the SPSS statistics 19.0 software (IBM, New York, USA). Sensitivity and specificity were calculated and quantified using receiver operating characteristic analyses. The predictive value was calculated to describe the proportion of correctly classified cases. Based on sensitivity and specificity values, a cutoff was defined independently for both cytoplasmic and nuclear immunopositivity for statistical analyses. The systematic and rigorous assessment of positive and negative predictive values for markers was conducted as described earlier.[26] The relationships between proteins expression (western blotting and IHC), PCR analysis, and clinicopathological parameters were tested using chi-square and the Fischer exact test. Two-sided *P* values were calculated, and *P* value of .05 or less was considered significant. The correlation between positivity for *ureC*, *cagA*, survivin, STAT3, and pSTAT3 was evaluated using Pearson correlation method whereas nonparametric correlation in the total sample was checked by the Spearman ρ and Pearson correlation method since the data obtained by the 2 methods were similar only Pearson correlation is indicated.

3 | RESULTS

3.1 | Expression and intracellular localization of survivin in gastric lesions

Increased level of survivin was detected by immunoblotting in both DC and IC tissues as compared with the normal tissues (Figure 1A). Precancerous lesions showed a moderate or low level of survivin. The cumulative densitometric analysis revealed that these differences in the level of survivin were statistically significant in different disease groups (Figure 1B).

To assess intracellular variation and heterogeneity in expression of survivin within the cells and tissues of the lesion area, corresponding formalin-fixed gastric tissues were subjected to IHC analysis for survivin. The box plot analysis of scores for survivin immunostaining in cytoplasmic and nuclear compartments shows the interquartile range of distribution and the outliers in respective category (Figures 1C,D and S1). Histologically normal tissues invariably lacked survivin expression, both in epithelial cells of gastric glands and in stromal cells (Figure 1D and Table S2). However, there was sporadic survivin immunopositivity in a proportion of histologically normal tissues (10/61). During the analysis, we found that survivin-positive normal tissues were from individuals who had the habit of tobacco chewing and simultaneously carried *cagA*$^+$ *H. pylori* infection (Table S2). Intestinal metaplasia and dysplastic lesions showed significantly increased survivin expression ($P < .001$) in epithelial cells of gastric glands compared with normal lesions. Interestingly, a low to moderate diffused expression of survivin was also observed in the stromal cells in these dysplastic lesions but not in IM. Cytoplasmic and nuclear immunopositivity of survivin in precancer lesions was associated with the multiple habits of the patients (irrespective of the type of habits). Interestingly, cytoplasmic survivin positivity in precancer was also strongly associated with *cagA*$^+$ *H. pylori* infection (Table S3).

A significant increase in survivin expression was observed in DC and IC compared to the precancerous lesions. Interestingly, survivin was diffusely expressed in both cytoplasmic (90.8%) and nuclear (87.7%) regions of the advanced adenocarcinoma cells compared to predominant cytoplasmic distribution of survivin in precancerous lesions. Tobacco usage in any form was found to be invariably associated with survivin overexpression in both cytoplasmic and nuclear regions (Table S4). Similarly, all *H. pylori*-positive adenocarcinoma lesions, irrespective of *ureC* or *cagA* positivity, were found to be immunopositive for cytoplasmic and nuclear survivin.

3.2 | Expression and activation of STAT3 in various grades of gastric biopsies

Next, we examined the levels of total STAT3 and its active form (pSTAT3-Y705 or pSTAT3) in different grades of gastric lesions. Increased level of STAT3 was present in adenocarcinoma tissues

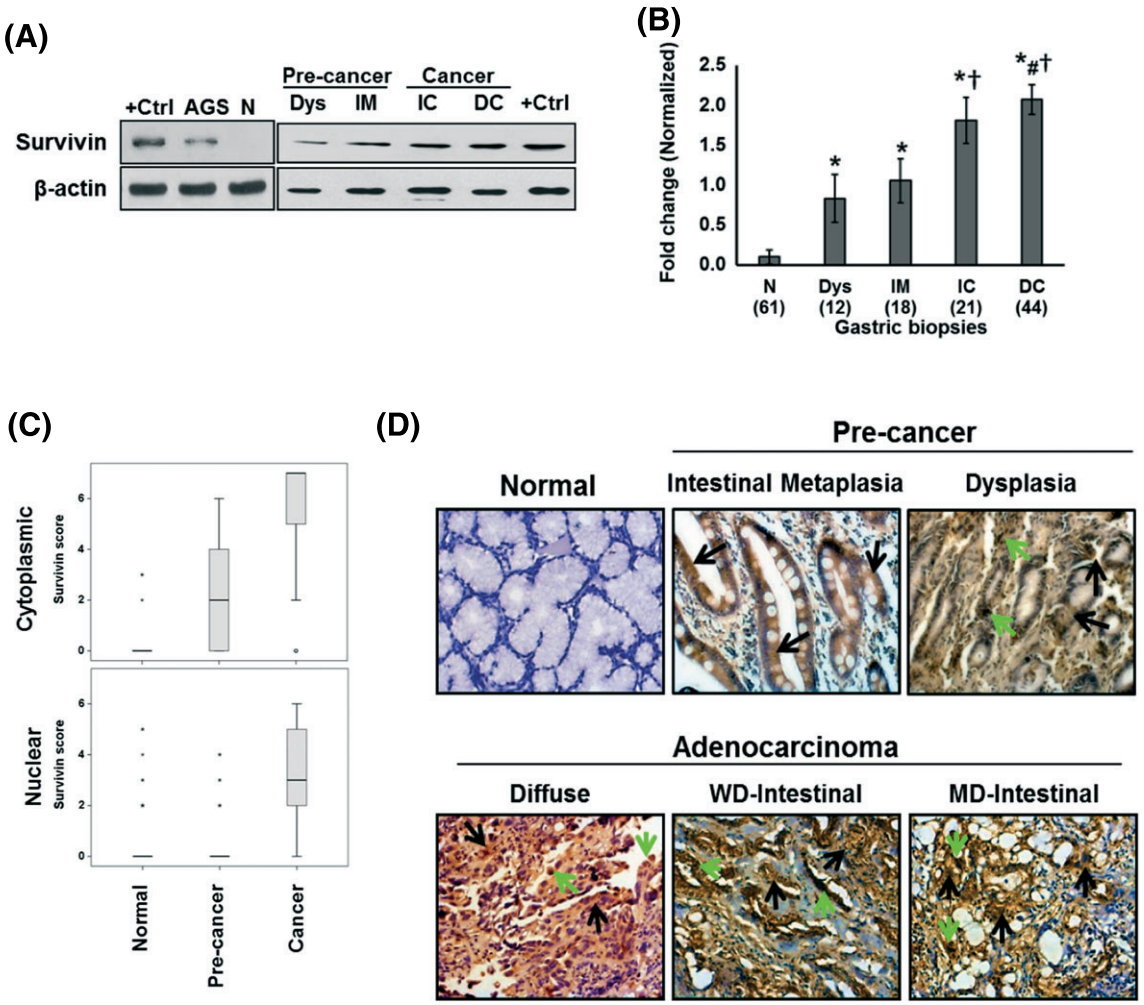


FIGURE 1 Expression and subcellular localization of survivin in human gastric biopsies. A, Representative immunoblots showing level of survivin. Positive control (Ctrl; HeLa cells), gastric adenocarcinoma cells (AGS) and freshly collected normal (N), intestinal metaplasia (IM), dysplasia (Dys), diffuse-type adenocarcinoma (DC), and intestinal-type adenocarcinoma (IC) tissues. Blots were reprobed for β-actin, which was used as a loading control (50 μg of total cellular proteins/lane). B, Mean fold difference in the integrated densitometric values of bands in the immunoblots was calculated as described below after normalizing the values to β-actin. Error bars indicate standard deviation. $^{*}P$ value ≤ .001 vs normal, $^{\#}P$ value = .004 vs intestinal metaplasia, and $^{\dagger}P$ value < .05 vs dysplasia. C, Box plots showing distribution of nuclear and cytoplasmic survivin total scores based on immunohistochemical analysis. Normal, Precancer, and Cancer. Scoring was performed on the scale of 0 to 7 on percent positivity and intensity of staining as described in Section 2. The outlier samples with their individual score represented as asterisk/dot. D, Representative immunohistochemical photomicrographs showing expression of survivin. Well-differentiated IC (WD-IC), moderately differentiated IC (MD-IC). Black arrows = cytoplasmic expression; green arrows = nuclear expression (original magnification, ×200)

irrespective of their type compared with the normal tissues. A small proportion (30%) of precancer tissues also showed moderate STAT3 positivity (Figure 2A). Similarly, we examined pSTAT3 level to evaluate the fraction of active STAT3 pool. The pSTAT3 level in lesions of different disease groups correlated pretty well with the STAT3 level (Figure 2A,B). Immunohistochemistry and box plot analysis revealed the distribution of differentially expressed STAT3 pools in various cellular compartments in gastric tissues (Figure 2C,D). In agreement with our immunoblotting data, we did not find STAT3 or pSTAT3 positivity in either epithelial or stromal cell in the histologically normal tissues; however, weak nuclear STAT3 and pSTAT3 positivities were noticed in the tissues with *H. pylori* infection (Table S5). On the other hand, significantly (P < .001) higher STAT3 expression was observed in precancerous lesions compared with the normal tissues. Intestinal metaplasia lesions showed moderate expression of STAT3, diffusely localized in the cytoplasm of gastric gland cells, while in Dys lesions, similar moderate STAT3 expression was observed, but STAT3 was localized in both the nucleus and the cytoplasm. In Dys, STAT3 expression was also detected in stromal cells (Figure 2C,D and Table S6).

The pSTAT3 immunostaining correlated well with the STAT3 localization within cellular compartments as well as among different types of cells in respective tissues. Significantly (P < .001) higher STAT3 and pSTAT3 immunopositivities were invariably detected in both nucleus and cytoplasm of adenocarcinoma cells as compared to precancerous and normal lesions (Figure 2C,D and Table S7).

The habit of tobacco chewing/smoking/alcoholism was significantly associated with increased nuclear and cytoplasmic STAT3/pSTAT3 positivity (Tables S6 and S7). However, both STAT3 and pSTAT3 positivities (cytoplasmic and nuclear) invariably showed a stronger correlation (P < .001) with *H. pylori* positivity not only in adenocarcinoma lesions (Table S7) but also in all other grades of tissues (Tables S5 and S6).

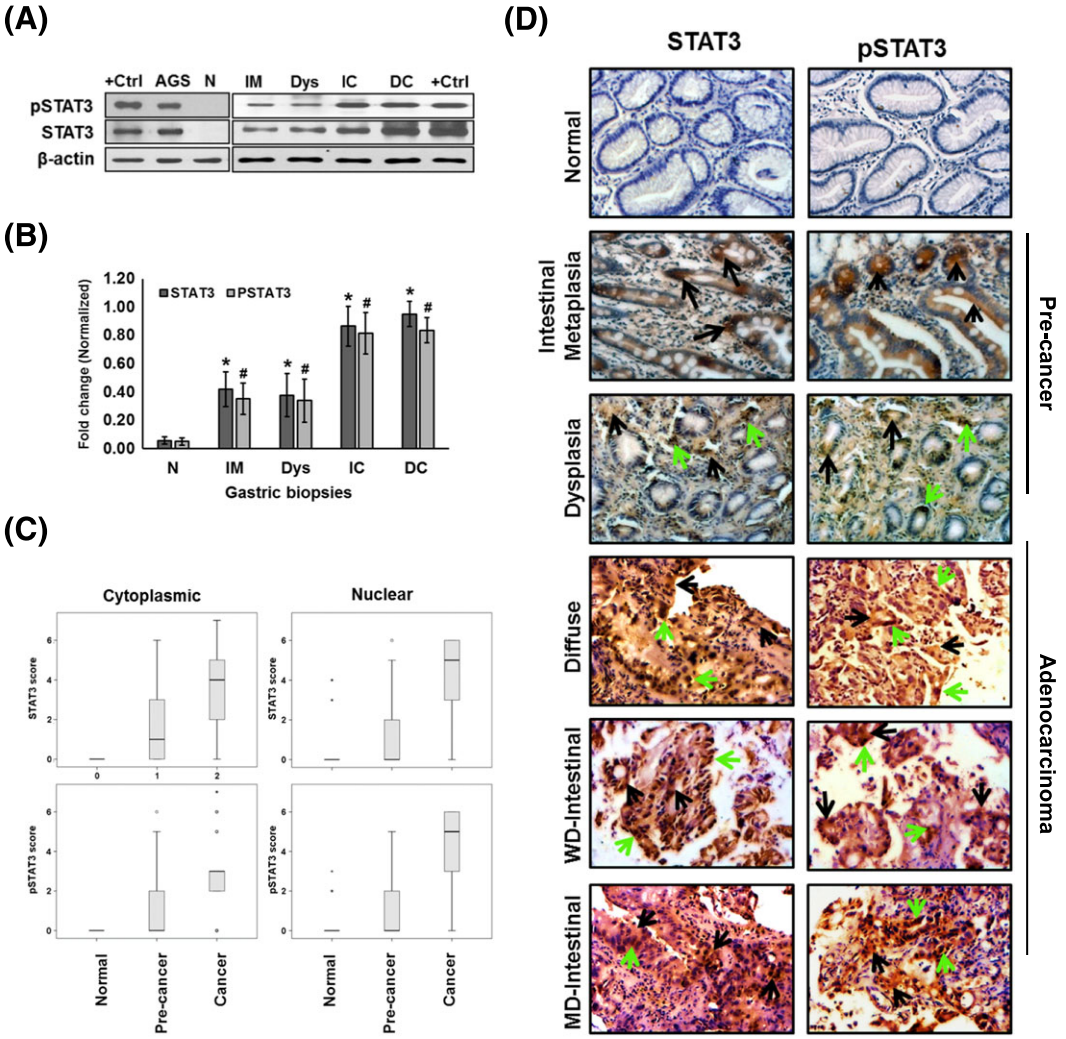


FIGURE 2 Expression of active STAT3 in different grades of gastric biopsies. A, Immunoblot analysis of STAT3 and pSTAT3 (Y705). Positive control (Ctrl; HeLa cells), gastric adenocarcinoma cells (AGS) and freshly collected normal (N), intestinal metaplasia (IM), dysplasia (Dys), diffuse-type adenocarcinoma (DC), and intestinal-type adenocarcinoma (IC). Blots were reprobed for β-actin, which was used as an internal control. B, Mean fold difference in the integrated densitometric values of bands in the immunoblots was calculated as described below after normalizing the values to β-actin. Error bars indicate standard deviation. $^{*}P$ value ≤ .001 vs normal for STAT3, $^{\#}P$ value ≤ .01 vs normal for pSTAT3. C, Box plots showing distribution of nuclear and cytoplasmic survivin total scores based on immunohistochemical analysis. Normal, Precancer, and Cancer. Scoring was performed on the scale of 0 to 7 on percent positivity and intensity of staining as described in Section 2. The outlier samples with their individual score represented as asterisk/dot. D, Representative photomicrograph of STAT3 and pSTAT3 immunohistochemical analysis. Well-differentiated IC (WD-IC), moderately differentiated IC (MD-IC). Black arrows = cytoplasmic expression; green arrows = nuclear expression (original magnification, ×200)

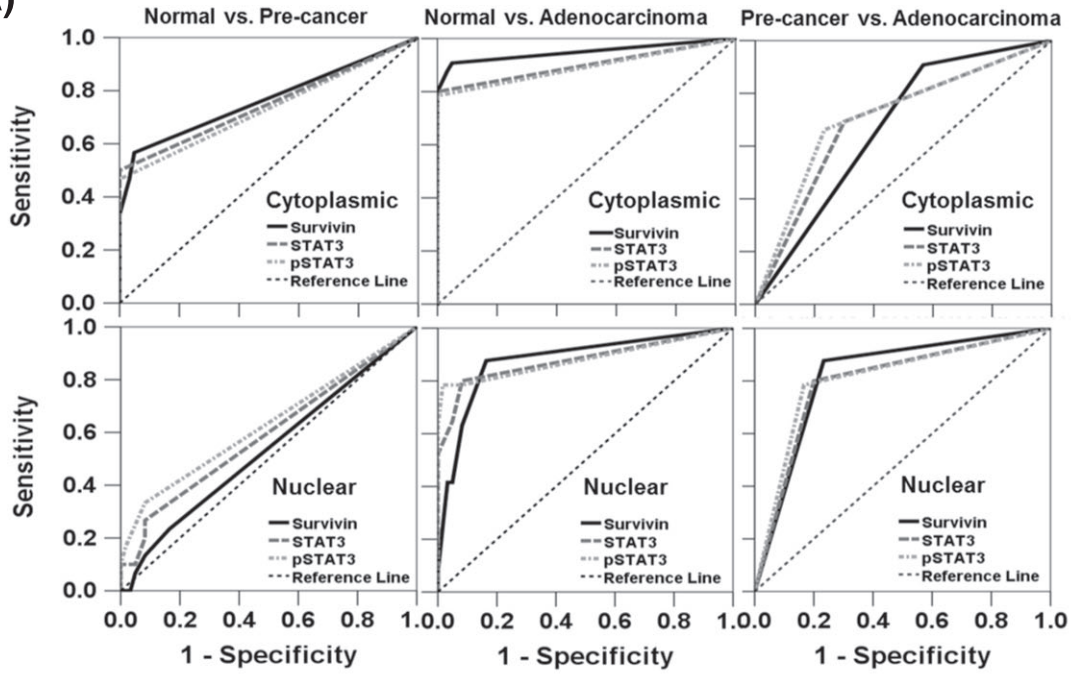
3.3 | Evaluation of survivin, STAT3, and pSTAT3 as diagnostic markers for precancerous states and adenocarcinoma in gastric lesions

Receiver operating characteristic analysis was used to determine the potential of survivin, STAT3, and pSTAT3 expressions to distinguish precancerous and cancer lesions from normal gastric tissues. We observed that cytoplasmic as well as nuclear expression of survivin, STAT3, and pSTAT3 revealed significant differences between normal, precancer, and gastric adenocarcinoma samples with increased area under the curve (AUC) values (Figure 3A). Interestingly, the nuclear survivin, STAT3, and pSTAT3 showed AUC values of 0.822, 0.800, and 0.809 (P < .001) with a positive predictive value of 89.06, 89.66, and 91.07, respectively, to discriminate between gastric precancer and adenocarcinoma lesions (Figure 3B).

3.4 | *H. pylori* infection is correlated with survivin, STAT3, and pSTAT3 expressions in precancerous lesions and adenocarcinomas

Subsequent assessment of subcellular localization of survivin, STAT3, and active STAT3 in study samples with respect to their *H. pylori* status revealed that survivin and active STAT3 were chiefly associated with *H. pylori* positivity in both malignant and nonmalignant gastric tissues (Table 2). The Pearson correlation was used to analyze the variability of expressions of survivin, STAT3, and pSTAT3 by IHC and PCR-based detection of *H. pylori* infection in gastric tissues. The expressions of *ureC* and *cagA* are congruent (r = 0.490, P < .001) in all tissue samples (r = 0.516, P < .001). High concordance was observed between the 2 PCR experiments in adenocarcinoma tissues (Table S8).

(A)



(B)

IHC Staining	Cut off value	Sensitivity	Specificity	Correctly Classified	PPV	NPV	AUC	p-value
Survivin Cytoplasmic								
1) Normal vs. Pre-cancer	>= 2	56.67%	95.08%	82.42%	85.00	81.69	0.766	<0.001
2) Normal vs. Adenocarcinoma	>= 2	90.77%	95.08%	92.86%	95.16	90.63	0.949	<0.001
3) Pre-cancer vs. Adenocarcinoma	>= 3	87.69%	53.33%	76.84%	77.63	68.42	0.671	0.008
Survivin Nuclear								
1) Normal vs. Pre-cancer	>= 2	23.33%	83.61%	63.74%	41.18	68.92	0.534	0.065
2) Normal vs. Adenocarcinoma	>= 2	87.69%	83.61%	85.71%	85.07	86.44	0.878	<0.001
3) Pre-cancer vs. Adenocarcinoma	>= 2	87.69%	76.67%	84.21%	89.06	74.19	0.822	<0.001
STAT3 Cytoplasmic								
1) Normal vs. Pre-cancer	>= 3	50%	100%	83.52%	100.0	74.39	0.750	<0.001
2) Normal vs. Adenocarcinoma	>= 3	80%	100%	89.68%	100.0	75.31	0.900	<0.001
3) Pre-cancer vs. Adenocarcinoma	>= 2	80%	50%	70.53%	83.33	51.22	0.696	0.002
STAT3 Nuclear								
1) Normal vs. Pre-cancer	>= 2	26.67%	91.80%	70.33%	54.55	70.00	0.591	0.159
2) Normal vs. Adenocarcinoma	>= 3	80%	91.80%	85.71%	91.23	81.16	0.879	<0.001
3) Pre-cancer vs. Adenocarcinoma	>= 3	80%	80%	80%	89.66	64.86	0.800	<0.001
pSTAT3 Cytoplasmic								
1) Normal vs. Pre-cancer	>= 4	46.67%	100%	82.42%	100.0	72.62	0.733	<0.001
2) Normal vs. Adenocarcinoma	>= 3	78.46%	100%	88.89%	100.0	73.49	0.892	<0.001
3) Pre-cancer vs. Adenocarcinoma	>= 2	78.46%	53.33%	70.53%	86.00	51.11	0.714	0.001
pSTAT3 Nuclear								
1) Normal vs. Pre-cancer	>= 2	33.33%	91.80%	72.53%	83.33	70.59	0.631	0.044
2) Normal vs. Adenocarcinoma	>= 3	78.46%	98.36%	88.10%	98.08	81.08	0.882	<0.001
3) Pre-cancer vs. Adenocarcinoma	>= 3	78.46%	83.33%	80.00%	91.07	64.10	0.809	<0.001

FIGURE 3 The biomarker analysis of survivin, STAT3, and pSTAT3 in gastric precancer and cancer (adenocarcinoma) cases. A, The receiver operating characteristic curves analysis of survivin, STAT3, and pSTAT3 protein expression. The reference line (small dotted line) showed 0.5 values of sensitivity and specificity. B, The biomarker analysis of survivin, STAT3, and pSTAT3 in different gastric pathologies. Values indicate optimal sensitivity, specificity, positive predictive value (PPV), negative predictive values (NPV), and area under the curve (AUC) of the test at the specified cutoff

The *ureC* expression was correlated with cytoplasmic survivin particularly in precancer and adenocarcinoma cases (r = 0.381, P = .038 and r = 0.324, P < .01) (Table S8). The *ureC* positivity also showed a strong correlation with STAT3 and pSTAT3 irrespective of their subcellular localization in adenocarcinoma lesions. Similarly, *cagA* positivity was correlated with cytoplasmic localization of survivin and STAT3 in precancerous lesions. Interestingly, *cagA* positivity was only strongly associated with STAT3 and pSTAT3 positivities in adenocarcinomas. Interestingly, survivin expression was found strongly associated with STAT3 and pSTAT3 levels irrespective of their subcellular localization (Table S8).

TABLE 2 Distribution of survivin, STAT3, and pSTAT3 in different gastric tissue types with respect to the status of *Helicobacter pylori* infection

		Normal (n = 61)			Precancer (n = 30)			Cancer (n = 65)			Total Cases (n = 156)		
		H. pylori Positive (%)	*H. pylori* Negative (%)	*P* Value	*H. pylori* Positive (%)	*H. pylori* Negative (%)	*P* Value	*H. pylori* Positive (%)	*H. pylori* Negative (%)	*P* Value	*H. pylori* Positive (%)	*H. pylori* Negative (%)	*P* Value
	Cases (%)	37 (60.7%)	24 (39.3%)		28 (93.3%)	2 (0.7%)		34 (52.3%)	31 (47.7%)		99 (63.5%)	57 (36.5%)	
Markers[a]	Subcellular localization												
Survivin	Cytoplasmic (A)	3 (8.1)	0 (0)	.153	17 (60.7)	0 (0)	.094	34 (100)	25 (80.6)	**.007**	54 (54.5)	25 (43.9)	.199
	Nuclear (B)	10 (27)	0 (0)	**.005**	7 (25)	0 (0)	.419	34 (100)	23 (74.2)	**.002**	51 (51.5)	23 (40.4)	.179
	Both (A∩B)	3 (8.1)	0 (0)	.153	7 (25)	0 (0)	.419	34 (100)	23 (74.2)	**.002**	44 (44.4)	23 (40.4)	.619
STAT3	Cytoplasmic (A)	0 (0)	0 (0)	...	9 (32.1)	0 (0)	.338	33 (97.1)	12 (38.7)	**≤.001**	42 (42.4)	12 (21.1)	**.007**
	Nuclear (B)	5 (13.5)	0 (0)	.060	6 (21.4)	0 (0)	.464	34 (100)	18 (58.1)	**≤.001**	45 (45.5)	18 (31.6)	.089
	Both (A∩B)	0 (0)	0 (0)	...	5 (17.9)	0 (0)	.513	33 (97.1)	12 (38.7)	**≤.001**	38 (38.4)	24 (21.1)	**.026**
pSTAT3	Cytoplasmic (A)	0 (0)	0 (0)	...	7 (25)	0 (0)	.419	33 (97.1)	10 (32.3)	**≤.001**	40 (40.4)	10 (17.5)	**.003**
	Nuclear (B)	0 (0)	1 (2.7)	.417	5 (17.9)	0 (0)	.513	34 (100)	17 (54.8)	**≤.001**	40 (40.4)	17 (29.8)	.186
	Both (A∩B)	0 (0)	0 (0)	...	4 (14.3)	0 (0)	.566	33 (97.1)	10 (32.3)	**≤.001**	37 (37.4)	10 (17.5)	**.009**

Bold emphasize statistically significant.

[a]The tissue sections were scored on the scale of 0 to 7 based on the percentage of immunostained cells as <10% = 0; 10-30% = 1; 30-50% = 2; 50-70% = 3; and 70-100% = 4. Sections were also scored semiquantitatively on the basis of staining intensity as no-stain = 0; mild = 1; moderate = 2; and intense = 3. Sections were considered positive when total score was ≥2 for survivin and ≥3 for STAT3 and pSTAT3.

4 | DISCUSSION

We observed overexpression of survivin and STAT3 proteins during gastric tumor progression, specifically cytoplasmic survivin along with nuclear pSTAT3 in gastric precancerous lesions. A strong correlation was observed between *H. pylori* positivity as measured by PCR-based detection of its 2 key genes, cagA and ureC, with survivin and STAT3 expression present in different subcellular compartments.

Our results revealed the absence of survivin in normal tissues and significant survivin expression in both premalignant and cancerous lesions. Previous studies established survivin as an apoptosis-associated marker in different gastric tissue types.[11,13,14,27] Interestingly, these studies showed complete lack of survivin expression in normal gastric tissues. In contrast, we observed a low to moderate expression of survivin in normal gastric tissues. These discrepancies can be explained due to likely region-specific variation in *H. pylori* strains as well as genetic and epigenetic variation in the patient population. Our observations imply the potential involvement of survivin in gastric cancer progression. The IHC analysis revealed differential expression of survivin in gastric lesions. The precancerous lesions expressed survivin predominantly in the cytoplasmic compartment, compared with advanced malignant lesions with nuclear and cytoplasmic expression. Subcellular distribution of survivin thus may be a dynamic process that may function as a switch cells between proliferative and prosurvival phase, possibly reflecting survivin's distinct functions in different cellular compartments.[16] Based on its antiapoptotic role in the cytoplasm, it is likely that cytoplasmic survivin dominantly seen in precancer lesions assists in preventing cell death and imparting chemoresistance in cells in these lesions, whereas nuclear survivin in adenocarcinomas may help stimulate cell division frequently observed in advanced malignant lesions. These assumptions are supported by observation indicating that nuclear localization of survivin reduces its stability and is not cytoprotective,[28,29] which makes tumor cell more sensitive to antitumor drugs.[30] A similar context, survivin expression in tumor cell nuclei has been proposed to be the predictor of favorable prognosis[11] that strongly suggests contrasting functional outcome of differentially localized survivin.

It is interesting to note in this context that tobacco usage is strongly associated with nuclear survivin in all tissues of test subjects indicating a potential contribution of tobacco use habit to tumor cell proliferation as observed in hepatocellular carcinoma.[31] However, this effect may not be limited to nuclear survivin as the cytoplasmic pool was also found significantly upregulated in tumor tissues of tobacco users suggesting an overall increase of survivin. Studies on normal human bronchial epithelial cells revealed a specific upregulation of survivin due to tobacco exposure.[32]

We observed an association between *cagA* DNA positivity and cytoplasmic survivin particularly in histopathologically normal and in precancerous tissues. *CagA* activates survivin expression via activation of the STAT3 pathway,[21] which in concert with survivin promotes gastric cell survival.[20] Interestingly, cells incapable of overexpressing survivin underwent apoptotic death at a very early stage[33] through γ-glutamyl transpeptidase activity that leads to proteasome-mediated degradation of survivin.[34] These observations suggest a strong prosurvival axis mediated by *cagA* that is manifested through active

STAT3 and survivin, which, if located in the cytoplasm, may impart cytoprotection to precancerous cells during the early phase of gastric carcinogenesis.

Based on above evidence, we investigated corresponding levels of the pSTAT3 (Y705) that represents the active state of the transcription factor in gastric lesions along with survivin. Interestingly, increased expression of STAT3 and pSTAT3 was significantly associated with the severity of the lesions. The result of IHC using STAT3 or pSTAT3 with respect to overall expression or subcellular distribution resembled but was not concordant in each case. The discordance could be primarily due to the differential affinity of antigen-antibody interaction but also due to the possibility of the noncanonical presence of unphosphorylated STAT3 in the nuclei.[35] It is also possible that pSTAT3 is present in the cytoplasm as well.[36,37] Nevertheless, a strong concordance of STAT3 with survivin confirms its transcriptional regulatory role in upregulation of survivin in gastric carcinogenesis, as proposed earlier.[20]

Interestingly, stromal cells in dysplastic lesions also showed moderate positivity for active STAT3 and survivin, which was absent in IM or normal gastric mucosa. Appearance of active STAT3 and consequently the expression of survivin in stromal cells indicate towards establishment of a cooperative tumor-promoting interaction of gastric tumor cells with its microenvironment through autocrine/paracrine stimulation. Gastric cancer cells have been shown to secrete PGE2 and tumor necrosis factor α that stimulate production of IL-6 via stromal cells,[38] which by itself activates STAT3 in stromal fibroblasts.[39]

Earlier studies established the presence of active STAT3 in human gastric precancerous and cancer tissues,[40] in an animal model,[21,41] and also in cancer cell lines.[20] Active STAT3 has been associated with lymph node metastasis[42] and poor prognosis in gastric cancer patients.[43] Aberrant activation of STAT3 is shown to be mediated by IL-6/IL-11–dependent[44] or IL-6–independent mechanism by the direct action of *cagA* on ligand-independent gp130 activation,[44] loss of its feedback inhibitors SOCS-2/SOCS-3, or through direct activation by EFGR.[45] Incidentally, we observed a strong correlation between *H. pylori* positivity and STAT3 or pSTAT3 irrespective of their subcellular location. Although *H. pylori* infection may not be essential in advanced gastric lesions as seen in the present study, our observations suggest that the presence of concurrent *H. pylori* infection may assist tumor cells in maintenance of high level of STAT3/pSTAT3. This assumption was further supported by the presence of nuclear STAT3 in *H. pylori*-positive normal tissues.

Apart from *H. pylori* infection, the habits of tobacco or alcohol abuse were also found associated with STAT3 localized to the nuclei of precancer and cancer tissues. Activation of STAT3 has been demonstrated in tobacco chewing–mediated oral carcinogenesis earlier[46] whereas tobacco smoke induces IL-6/STAT3 pathways in lung tissues.[47] However, there is no evidence to date that suggests any relation of tobacco habit with STAT3 levels in gastric cancer. Similarly, habit of alcohol abuse also correlated with high levels of STAT3. Alcohol consumption and smoking are considered risk factor for gastric cancer.[48] However, link of alcohol use habit with active STAT3 in our study and its association with gastric carcinogenesis depicted in previous studies are indicative of an association that needs future investigation.

Some studies that have coevaluated overall survivin and STAT3 in gastric tissues[49-51] or in experimental model[20] lacked the detailed analysis in regard to the subcellular distribution of survivin. It was interesting to note that correlation coefficients were significantly lower in precancer lesions indicating a subset of tissues with preferential localization of survivin in one compartment. Despite strong correlation with each other, survivin and STAT3/pSTAT3 showed differential expression and variable subcellular localization in different gastric lesions. Therefore, to understand the disease grade-specific expression characteristics in different cellular pools, we reexamined our IHC data for marker analysis. Receiver operating characteristic curves for survivin, STAT3, and pSTAT3 in the different subcellular pool, which demonstrated high AUC values, and low cutoff value distinguished the level of expression among different tissue types. To the best of our knowledge, this is the first attempt to evaluate the subcellular content of survivin and STAT3 as a marker in gastric carcinogenesis. These observations along with reports from other investigators[52-55] suggest the role of survivin and active nuclear STAT3 in gastric cancer progression.

5 | CONCLUSIONS

Despite a small sample size and a cross-sectional study, present investigation demonstrates overexpression of survivin and STAT3 in gastric cancer that correlated with increasing severity of the gastric lesions. The specific increase in cytoplasmic survivin together with enhanced nuclear pSTAT3 in gastric precancerous cells and diffused overexpression of survivin and STAT3 in cancer lesions was observed. Survivin and STAT3 levels were also highly correlated with concurrent *H. pylori* infection. Survivin and STAT3 are key players in promoting carcinogenesis and mediating the intrinsic/primary chemoresistance.[56,57] Hence, these proteins could be suitable therapeutic targets in preventing gastric cancer progression. However, further follow-up studies are needed to validate clinical utility of survivin and STAT3 as prognostic markers.

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CONFLICT OF INTEREST

The authors declare that there is no competing interest.

AUTHOR CONTRIBUTION

Conceptualization, ACB, AP; Methodology, AP, SCT, SS; Investigation, AP, SCT, SS, SM, KV Formal Analysis, AP, SCT, ACB; Resources, AP ACB; Writing - Original Draft, AP, ACB, SCT. DM, SPM, VM; Writing

- Review & Editing, AP, ACB, SCT. DM, SPM, VM; Visualization, AP, ACB; Supervision, ACB, MD, SPM, VM SM.; Funding Acquisition, ACB, AP.

ETHICAL STANDARDS

Written informed consent was obtained from all of the subjects. The study was conducted in accordance with the principles of Helsinki Declaration. The Institutional Ethics Committees of Moti Lal Nehru Medical College, Allahabad, and Institute of Cytology and Preventive Oncology (now National Institute of Cancer Prevention and Research), Noida, India, approved this study prior to its commencement. Clinico-epidemiological and demographic details were taken from clinical record of patients and captured on a pro forma document.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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