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Original Article

Differentially Localized Survivin and STAT3 as Markers of Gastric Cancer Progression: Association with *H. pylori*

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This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/cnr2.1004

Running Title: Survivin and STAT3 in gastric cancer

Keywords: Dysplasia, intestinal metaplasia-IV, gastric adenocarcinoma, survivin, STAT3

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 pre-cancer tissues using immunoblotting and immunohistochemistry. **Results:** Analysis of a total of 156 gastric samples comprising 61 histologically normal, 30 pre-cancerous tissues [comprising intestinal metaplasia (IM) and dysplasia (dys)] and 65 adenocarcinomas, collected as endoscopic biopsies from treatment naïve study participants revealed a significant ($p < 0.001$) increase in overall protein levels. Survivin expression was detectable both in cytoplasmic (90.8%) and nuclear compartments (87.7%) in gastric adenocarcinomas lesions. Pre-cancerous 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 pre-cancerous and in adenocarcinoma lesions. Though, overexpression of these proteins was found associated with the tobacco use, and alcohol consumption, their expression invariably and strongly correlated with concurrent *H. pylori* infection. ROC analysis of nuclear survivin, STAT3 and pSTAT3 in different study groups showed acceptable positive and negative predictive values with AUC above 0.8 ($p<0.001$). **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.

Abbreviation: STAT3–signal transducer and activator of transcription-3; pSTAT3-phosphorylated STAT3 (Y705); ROC – receiver operating characteristics; AUC – area under curve, Hp – *Helicobacter pylori*, IHC – immunohistochemistry; IM – intestinal metaplasia; Dys – dysplasia; DC – diffuse adenocarcinoma; IC – intestinal adenocarcinoma

Introduction

According to Globocan 2012, stomach cancer attributable to *H. pylori* infection is fifth common malignancy in the world (952,000 cases, 6.8% of the total) and 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 3rd (incidence–43,386, mortality–40,721) and 6th (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 under-reporting 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 to15% (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, cell migration/ metastasis (9). In general, it is associated with poor prognosis and low patient survival (10). Prognostic significance of survivin in gastric carcinogenesis have 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 an 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 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,

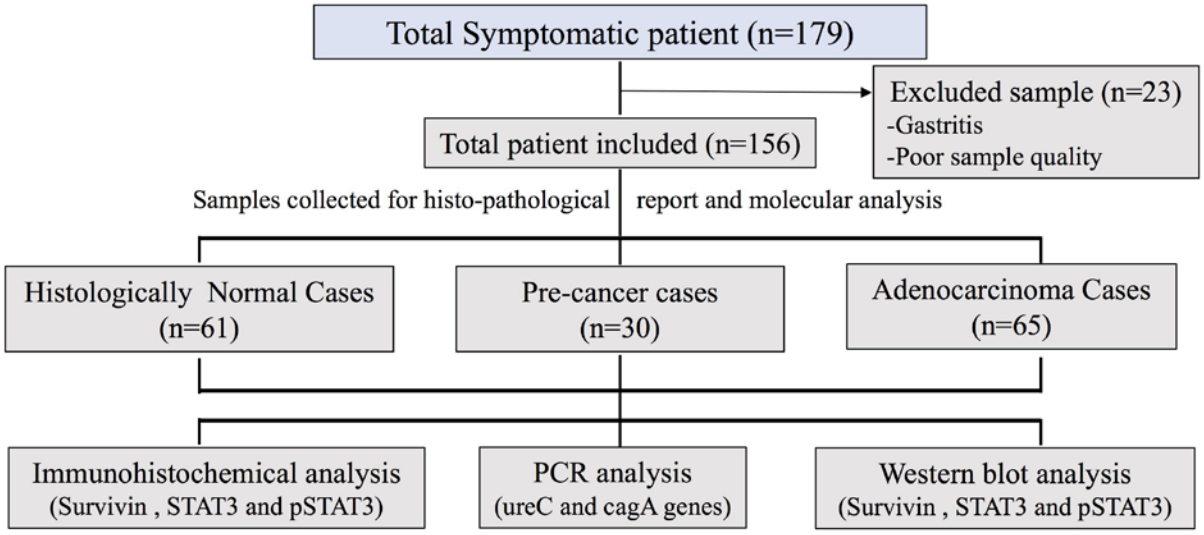
tobacco usage or alcohol abuse. Clinical utility of survivin and STAT3 as potential markers in gastric cancer progression was also evaluated.

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.

Materials and Methods

Clinical specimens and reagents

A total of 156 gastric samples comprising 61 histologically normal, 30 pre-cancerous 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 Motilal 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 pre-cancerous lesions while diffuse-type adenocarcinoma (DC, n=44) 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 stard 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 carried out 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 proforma 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 days/week) 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/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 IHC analysis while other punch(es) were collected in chilled 1xPBS for DNA and protein isolation for subsequent molecular studies. The histopathological grading of pre-cancerous 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 (Santa Cruz Biotechnology, Inc., Texas, USA) unless specified.

***H. pylori* detection:**

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

Immunohistochemical analysis for survivin, STAT3, and pSTAT3 levels

The immunohistochemical staining (IHC) was performed as described previously with minor modifications (23, 24). Briefly, formalin-fixed, paraffin-embedded sections (5μm) of the gastric tissues were collected on poly-L lysine-coated slides and then de-paraffinized in xylene. After hydration in a decreasing alcohol gradient, and antigen retrieval was performed by pre-treatment in a microwave oven for 10 min at 800 W and 5 min at 480 W in citrate buffer (0.01 M, pH-6.0). The sections were incubated with hydrogen peroxide (0.3% v/v) in methanol for 30 min to quench the endogenous peroxidase activity, followed by blocking with 1% bovine serum albumin to prevent non-specific binding. Thereafter, the slides were incubated with either mouse monoclonal anti-survivin (SC-17779), anti-STAT3 (SC-483) or anti-pSTAT3-Y705 (SC-8059) antibodies (Santa Cruz Biotechnology) for 16 h at 4ºC. 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 min, 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. In order 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.

Evaluation of immunohistochemical staining

Each slide was evaluated for survivin, STAT3 and pSTAT3 immunostaining using a semi-quantitative scoring system for both staining intensity and the percentage of positive epithelial cells (24). The sections were scored independently by two investigators (AP and SCT). The tissue sections were scored on the scale of 0-7 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 semi-quantitatively on the basis of staining intensity as no-stain = 0; mild = 1; moderate = 2; 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 inter-observer discrepancy. In the cases with score discrepancy between the two observers, a third independent review was performed (VM) 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.

Total protein isolation & immunoblotting

Total proteins from biopsies and cell lines were isolated and examined by the method described previously (23, 25). Detailed procedures are provided in **Supplementary Methods.**

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 (ROC) analyses. The predictive value (PV) 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 (PPV and NPV respectively) for markers was carried out as described earlier (26). The relationships between proteins expression (western blotting and IHC), PCR analysis and clinicopathological parameters were tested using Chi-Square and Fischer's exact test. Two-sided *p*-values were calculated and *p*-value of 0.05 or less was considered significant. The correlation between positivity for *ureC*, *cagA*, survivin, STAT3 and pSTAT3 was evaluated using Pearson's correlation method whereas nonparametric correlation in the total sample was checked by Spearman's rho and Pearson's correlation method since the data obtained by the two methods was similar only Pearson's correlation is indicated.

Results

Expression and intracellular localization of survivin in gastric lesions

Increased level of survivin was detected by immunoblotting in both diffuse and intestinal type adenocarcinoma tissues as compared to the normal tissues (**Fig. 1A**). Pre-cancerous 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 (**Fig. 1B**).

In order 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 (IQR) of distribution and the outliers in respective category (**Fig. 1C, 1D and Suppl. Fig 1**). Histologically-normal tissues invariably lacked survivin expression, both in epithelial cells of gastric glands as well as in stromal cells (**Fig. 1D** and **Table S2)**. However, there was sporadic survivin immuno-positivity 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 ($p < 0.001$) in epithelial cells of gastric glands compared to 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 intestinal metaplasia. Cytoplasmic and nuclear immuno-positivity of survivin in pre-cancer lesions was associated with the multiple habits of the patients (irrespective of the type of habits). Interestingly, cytoplasmic survivin positivity in pre-cancer was also strongly associated with *cagA*$^+$ *H. pylori* infection (**Table S3**).

A significant increase in survivin expression was observed in diffuse and intestinal type adenocarcinoma compared to the precancerous lesions. Interestingly, survivin was diffusely expressed both in cytoplasmic (90.8%) and nuclear (87.7%) region of the advanced adenocarcinoma cells compared to predominant cytoplasmic distribution of survivin in pre-cancerous 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 immuno-positive for cytoplasmic and nuclear survivin.

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 adenocarcinomas tissues irrespective of their type compared to the normal tissues. A small proportion (30%) of pre-cancer tissues also showed moderate STAT3 positivity (**Fig. 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 (**Fig. 2A & 2B**). IHC and box plot analysis revealed the distribution of differentially expressed STAT3 pools in various cellular compartments in gastric tissues (**Fig. 2C and 2D**). In agreement with our immunoblotting data, we did not find STAT3 or pSTAT3 positivity in either epithelial

or stromal cells in the histologically normal tissues; however, weak nuclear STAT3 and pSTAT3 positivity were noticed in the tissues with *H. pylori* infection (**Table S5)**. On the other hand, significantly ($p<0.001$) higher STAT3 expression was observed in precancerous lesions compared to the normal tissues. Intestinal metaplasia lesions showed moderate expression of STAT3, diffusely localized in the cytoplasm of gastric gland cells while in dysplasia lesions, similar moderate STAT3 expression was observed, but STAT3 was localized in both the nucleus and the cytoplasm. In dysplasia, STAT3 expression was also detected in stromal cells **(Fig. 2C, 2D 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 ($p<0.001$) higher STAT3 and pSTAT3 immuno-positivity were invariably detected in both nucleus and cytoplasm of adenocarcinoma cells as compared to precancerous and normal lesions (**Fig. 2C, 2D and Table S7**).

The habit of tobacco chewing/ smoking/ alcoholism was significantly associated with increased nuclear and cytoplasmic STAT3/pSTAT3 positivity **(Table S6 and S7)**. However, both STAT3, and pSTAT3 positivity (cytoplasmic and nuclear) invariably showed a stronger correlation ($p<0.001$) with *H. pylori* positivity not only in adenocarcinoma lesions **(Table S7)** but in all other grades of tissues **(Table S5 and S6)**.

Evaluation of survivin, STAT3 and pSTAT3 as diagnostic markers for pre-cancerous states and adenocarcinoma in gastric lesions

Receiver Operating Characteristic (ROC) analysis was used to determine the potential of survivin, STAT3, and pSTAT3 expression to distinguish pre-cancerous 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, pre-cancer and gastric adenocarcinoma samples with increased area under the curve (AUC) values **(Fig. 3A, 3B)**.

Interestingly, the nuclear survivin, STAT3, and pSTAT3 showed AUC values of 0.822, 0.800 and 0.809 ($p < 0.001$) with a positive predictive value (PPV) of 89.06, 89.66 and 91.07 respectively to discriminate between gastric pre-cancer and adenocarcinoma lesions **(Fig. 3B)**.

H. pylori infection is correlated with survivin, STAT3 and pSTAT3 expression in pre-cancerous 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 non-malignant gastric tissues **(Table 2).** Pearson's correlation was used to analyze the variability of expressions of survivin, STAT3, pSTAT3 by IHC and PCR-based detection of *H. pylori* infection in gastric tissues. The expressions of *ureC* and *cagA* are congruent ($r=0.490$, $p<0.001$) in all tissue samples.; ($r=0.516$, $p<0.001$). High concordance was observed between the two PCR experiments in adenocarcinoma tissues (**Table S8).**

The *ureC* expression was correlated with cytoplasmic survivin particularly in pre-cancer and adenocarcinoma cases ($r=0.381$, $p=0.038$ & $r=0.324$, $p<0.01$) (**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 pre-cancerous lesions. Interestingly, *cagA* positivity was only strongly associated with STAT3 and pSTAT3 positivity in adenocarcinomas. Interestingly, survivin expression was found strongly associated with STAT3 and pSTAT3 levels irrespective of their subcellular localization (**Table S8**).

Discussion

We observed overexpression of survivin and STAT3 proteins during gastric tumor progression, specifically cytoplasmic survivin along with nuclear pSTAT3 in gastric pre-cancerous lesions. A strong correlation was observed between *H. pylori* positivity as measured

by PCR based detection of its two 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 pre-malignant 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 pre-cancerous lesions expressed survivin predominantly in the cytoplasmic compartment, compared to 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 pro-survival phase, possibly reflecting survivin's distinct functions in different cellular compartments (16). Based on its anti-apoptotic role in the cytoplasm, it is likely that cytoplasmic survivin dominantly seen in pre-cancer lesions assists in preventing cell death and imparting chemo-resistance 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 anti-tumor 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 pre-cancerous tissues. *CagA* activates survivin expression via activation of the STAT3 pathway (21) which in concert with survivin promote gastric cell survival (20). Interestingly, cells incapable of overexpressing survivin underwent apoptotic death at a very early stage (33) through γ-glutamyl transpeptidase (GGT) activity that leads to proteasome-mediated degradation of survivin (34). These observations suggest a strong pro-survival axis mediated by *cagA* that is manifested through active STAT3 and survivin, which, if located in the cytoplasm, may impart cytoprotection to pre-cancerous 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 were 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 non-canonical presence of unphosphorylated STAT3 in 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 intestinal metaplasia 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 TNFalpha 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 pre-cancerous 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 where the direct action of *cagA* on ligand independent gp130 activation (44), loss of its feedback inhibitors SOCS-2/3 or through direct activation by EFGR (45). Incidentally, we observed a strong correlation between *H. pylori* positivity and STAT3 or pSTAT3 irrespective of their sub-cellular location. Although *H. pylori* infection may not be essential in advanced gastric lesions as seen in the present study, our observations suggest that 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 nuclei of pre-cancer 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 co-evaluated 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 pre-cancer 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. ROC 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.

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 pre-cancerous 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 chemo-resistance (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.

Acknowledgements

The study was supported by research grants from (extramural from DST – PURSE Phase II/RC/2016/944; from DBT – 6242-P34/RGCB/PMD/DBT/ALCB/2015; ICMR – 5/13/38/2014 NCDIII-Eoffice73143; and intramural from University of Delhi) to Prof. Alok C. Bharti and ICMR- Senior Research Fellowship (3/2/2/11/2010/NCD-III) to AP. The authors are grateful to Dr. Alok Mishra and the staff of Department of Gastroenterology and Pathology, MLN Medical College, Allahabad, for their valuable support.

Disclosure of potential conflicts of interest- The authors declare that there is no competing interest.

Author's contribution

AP- Participated in study design, collected samples, performed major experimental work, assisted in data analysis and manuscript preparation, SCT- participated in IHC data analysis, assisted in manuscript drafting and statistical analysis, SS, SM and KV - assisted in experimental work related to western blotting, data analysis and manuscript preparation, SPM- participated in clinical evaluation, endoscopy and sample collection, VM- participated in histopathology and data analysis, SM- critical evaluation of the manuscript, MD- performed clinical evaluation, endoscopy, subject selection, sample collection, clinical data analysis, participated in design of the study, ACB- conceived and designed the study, critically reviewed and communicated the final manuscript. All authors have read and approved the final manuscript.

Ethical Standards- Written informed consent was obtained from all of the subjects. The study was carried out in accordance with the principles of Helsinki Declaration. The Institutional Ethics Committees of Motilal 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 proforma document.

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Tables:

Table 1 – Clinico-epidemiological details of the subjects enrolled in the study.

Table 2 – Distribution of survivin, STAT3 and pSTAT3 in different gastric tissue types with respect to the status of *H. pylori* infection.

Figure legends:

Fig. 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 adenocarcinoma (DC) and intestinal adenocarcinoma (IC) tissues. Blots were re-probed for β-actin, which was used as a loading control (50µg 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 to. Error bars indicate standard deviation. **p* value ≤0.001 vs. normal, $^{\#}p$ value = 0.004 vs. intestinal metaplasia, and †*p* value <0.05 vs. dysplasia. **(c)** Box plots showing distribution of nuclear and cytoplasmic survivin total scores based on immunohistochemical analysis. Normal, Pre-cancer, and Cancer. Scoring was performed on the scale of 0-7 on percent positivity and intensity of staining as described in the Methods section. 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 x200).

Fig. 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 adenocarcinoma (DC) and intestinal adenocarcinoma (IC). Blots were re-probed 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 ≤ 0.001 vs. normal for STAT3, $^{\#}p$ value ≤ 0.01 vs. normal for pSTAT3. **(c)** Box plots showing distribution of nuclear and cytoplasmic survivin total scores based on immunohistochemical analysis. Normal, Pre-cancer, and Cancer. Scoring was performed on the scale of 0-7 on percent positivity and intensity of staining as described in the Methods section. 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 x200).

Fig. 3: The biomarker analysis of survivin, STAT3 and pSTAT3 in gastric pre-cancer and cancer (adenocarcinoma) cases. (a) The Receiver Operating Characteristic (ROC) 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 cut off.

Supporting Information:

Supplementary Tables – as S1-S8 Table

Supplementary Figure – as Supplementary Figure F1

Supplementary Methods – as Supplementary Methods

Table 1 – Clinico-epidemiological details of the subjects enrolled in the study

Clinico-epidemiological Characteristics	Normal	Pre-cancer	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%)
Histo-pathological classification of lesions			
Intestinal metaplasia-IV	-	15 (50%)	-
Dysplasia	-	15 (50%)	-
Diffuse adenocarcinoma	-	-	44 (67.7%)
Intestinal adenocarcinoma	-	-	21 (32.3%)
Age in years (median)	07-85 (40)	13-78 (41.5)	22-90 (55)
Habits*			
Tobacco chewers	28 (45.9%)	15 (50%)	24 (37%)
Tobacco non-chewers	33 (54.1%)	15 (50%)	41 (63%)
Tobacco Smokers	22 (36.1%)	11 (36.7%)	20 (30.8%)
Tobacco Non-smokers	39 (64%)	19 (63.3%)	45 (69.2%)
Alcoholic	15 (24.6%)	8 (26.7%)	16 (24.6%)
Non-alcoholic	46 (75.4%)	22 (73.3%)	49 (75.4%)
Multiple habits	22 (36.1%)	9 (30%)	16 (24.6%)
***H. 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%)

*Tobacco chewing habits include betel quid, areca nut and/or pan masala use; multiple habits include two or more of any tobacco and/or alcohol habits

Table 2 – Distribution of survivin, STAT3 and pSTAT3 in different gastric tissue types with respect to the status of *H. pylori* infection

		Normal (n=61)			Pre-cancer (n=30)			Cancer (n=65)			Total Cases (n=156)		
		HP+ve (%)	HP–ve (%)	*p* value	HP+ve (%)	HP–ve (%)	*p* value	HP+ve (%)	HP–ve (%)	*p* value	HP +ve (%)	HP–ve (%)	*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*	**Subcellular Localization**												
Survivin	Cytoplasmic (A)	3 (8.1)	0 (0)	0.153	17 (60.7)	0 (0)	0.094	34 (100)	25 (80.6)	**0.007**	54 (54.5)	25 (43.9)	0.199
	Nuclear (B)	10 (27)	0 (0)	**0.005**	7 (25)	0 (0)	0.419	34 (100)	23 (74.2)	**0.002**	51 (51.5)	23 (40.4)	0.179
	Both (A∩B)	3 (8.1)	0 (0)	0.153	7 (25)	0 (0)	0.419	34 (100)	23 (74.2)	**0.002**	44 (44.4)	23 (40.4)	0.619
STAT3	Cytoplasmic (A)	0 (0)	0 (0)	--	9 (32.1)	0 (0)	0.338	33 (97.1)	12 (38.7)	**≤0.001**	42 (42.4)	12 (21.1)	**0.007**
	Nuclear (B)	5 (13.5)	0 (0)	0.060	6 (21.4)	0 (0)	0.464	34 (100)	18 (58.1)	**≤0.001**	45 (45.5)	18 (31.6)	0.089
	Both (A∩B)	0 (0)	0 (0)	--	5 (17.9)	0 (0)	0.513	33 (97.1)	12 (38.7)	**≤0.001**	38 (38.4)	24 (21.1)	**0.026**
pSTAT3	Cytoplasmic (A)	0 (0)	0 (0)	--	7 (25)	0 (0)	0.419	33 (97.1)	10 (32.3)	**≤0.001**	40 (40.4)	10 (17.5)	**0.003**
	Nuclear (B)	0 (0)	1 (2.7)	0.417	5 (17.9)	0 (0)	0.513	34 (100)	17 (54.8)	**≤0.001**	40 (40.4)	17 (29.8)	0.186
	Both (A∩B)	0 (0)	0 (0)	--	4 (14.3)	0 (0)	0.566	33 (97.1)	10 (32.3)	**≤0.001**	37 (37.4)	10 (17.5)	**0.009**

* The tissue sections were scored on the scale of 0-7 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 semi-quantitatively on the basis of staining intensity as no-stain = 0; mild = 1; moderate = 2; intense = 3. Sections were considered positive when total score was ≥2 for survivin and ≥3 for STAT3 and pSTAT3.

Figure 1

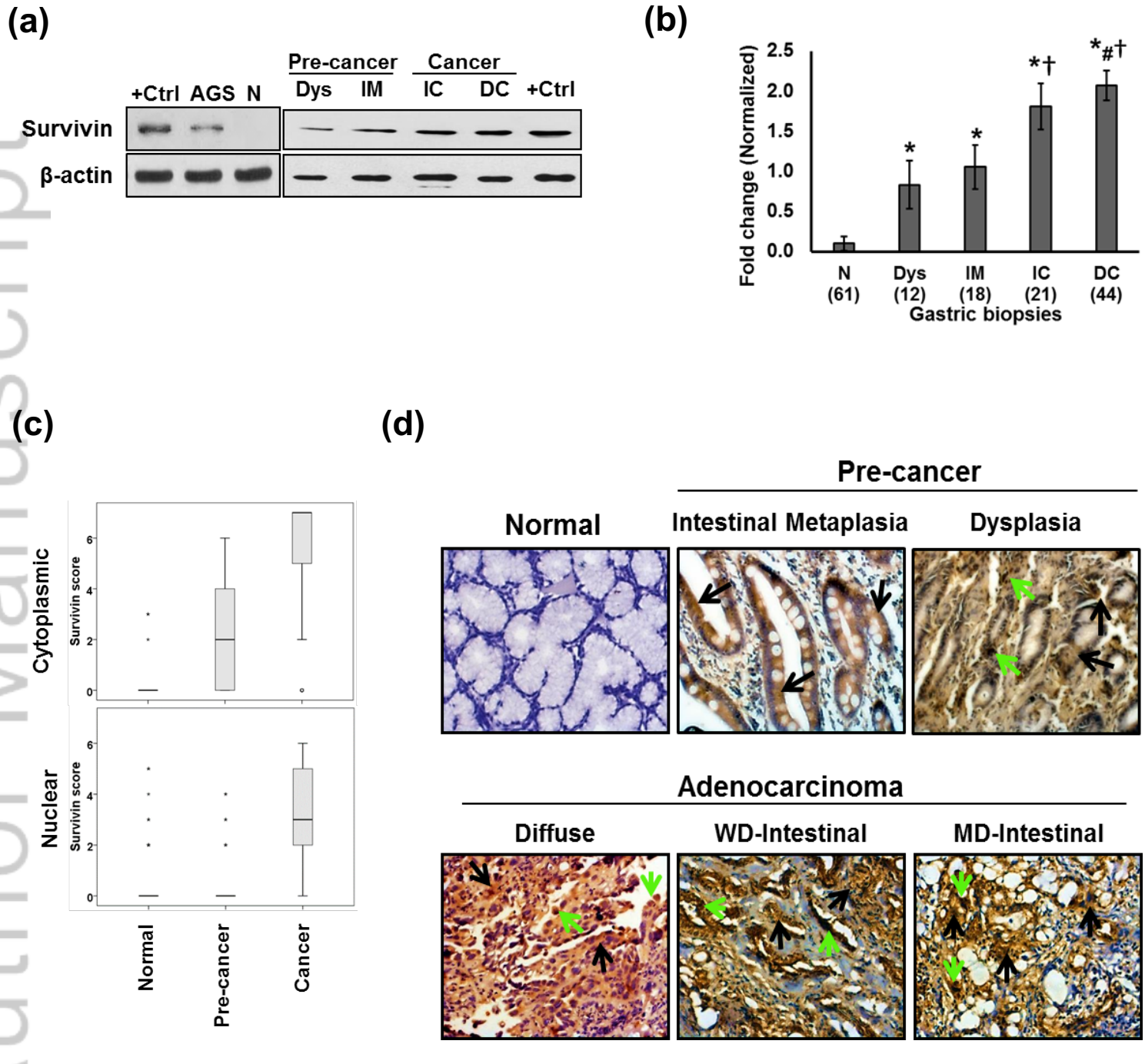


Figure 2

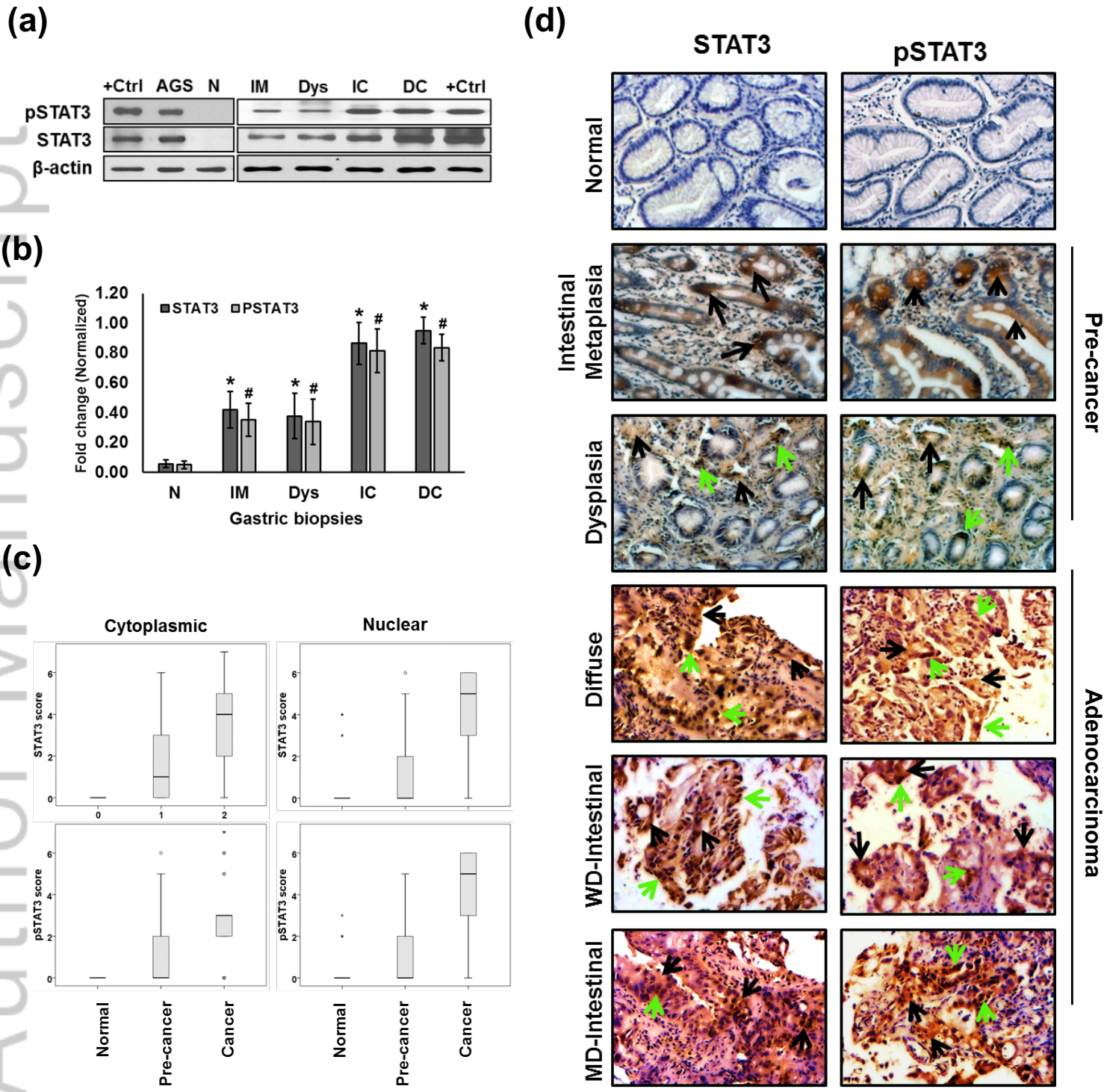
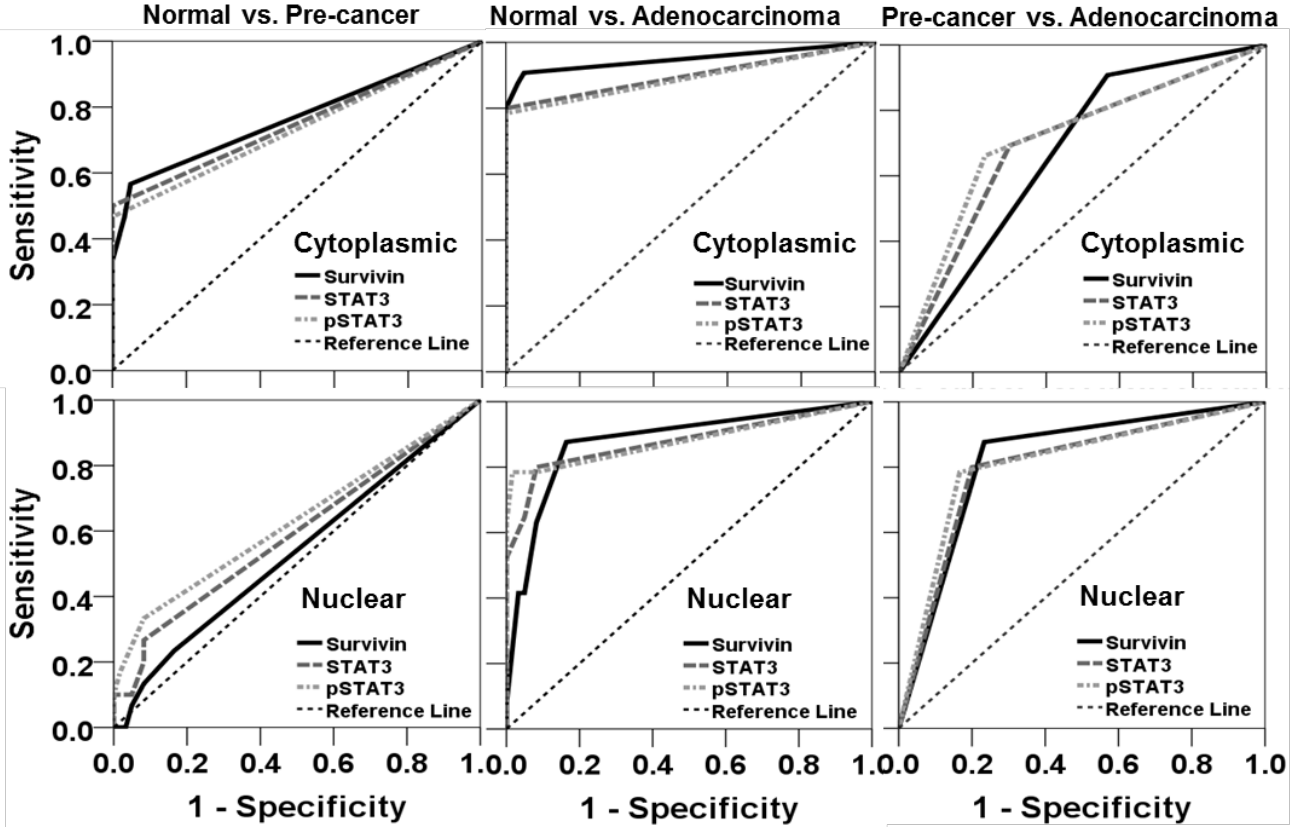


Figure 3

(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