

EXPRESSION OF PROTO ONCO GENE C-KIT AND CANCER ASSOCIATED FIBROBLASTS ARE ASSOCIATED WITH METASTATIC POTENTIAL OF HUMAN GASTRIC CANCER

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ABSTRACT

Background: The proto onco gene c-kit is a tyrosine kinase receptor plays significant role in embryo and postnatal development and also found to be expressed in solid tumours and hematological malignancies. Cancer associated fibroblasts (CAFs) have been found to be involved in promoting tumour growth, invasion and metastasis. The aim of study was to establish the expression pattern of c-kit and specific markers for CAFs in gastric epithelium tissues and their correlation with clinicopathological factors to establish their possible role in prognosis of gastric cancer.

Methods: Expression of c-kit and specific markers for CAF or proteins secreted by CAF were assessed by immunohistochemical and immunofluorescence staining of paraffin-embedded tissues, and RT-PCR in 64 tumours (observed group) and 33 normal gastric samples. The clinical pathological data was statistically analyzed by chi-square methods.

Result: The positive rate of c-kit and CAF protein expression in the observed group as 84.38% (54/64) and 96.88% (62/64) respectively. RT-PCR results also showed elevated expression of c-kit, α -SMA, Vimentin, and SDF-1 in gastric cancer than that of normal gastric tissues. The expression of c-kit and α -SMA was correlated with age, & gender of patients, position, size, & depth of the tumour, lymphatic invasion and node metastasis. c-kit and α -SMA prevalence is significantly correlated with size, & depth of the tumour, lymphatic invasion and node metastasis ($p < 0.05$).

Conclusion: Overall this data suggest that increased expression of c-kit and CAFs might be attributed with disease progression; malignancy transformation of gastric epithelium cells and prognosis of gastric cancer.

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INTRODUCTION

Stomach cancer, also called gastric cancer, is a malignant tumour arising from the lining of the stomach. Gastric Cancer is considered as one of the significant global health problems with a dismal prognosis. Though the incident rate of gastric cancer has steadily declined in the past decades, this still remains a major point of concern for mankind and draws attention for thorough research. It occurs with a high incidence in Asia and it is the second most common cause of cancer related death [1]. Even though modern surgical procedure like total gastrectomy and extended radical gastrectomy has shown to be useful for early gastric cancers, but the prognosis of these patients is poor due to the recurrence of the disease subsequent to surgery. The recurrence might happen in the form of lymphatic spread, blood-borne metastasis, or peritoneal dissemination [2].

The proto onco gene c-kit (CD117) encodes receptor tyrosine kinase which is a transmembrane protein

and related to platelet-derived growth factor receptor and macrophage growth factor receptor family of proteins. Stem cell Factor (SCF) also known as mast cell growth factor, steel factor and kit ligand is the cognate ligand for c-kit [3]. Upon receptor ligand interaction c-kit receptor kinase undergo autophosphorylation and transduce signal for multiple signal transduction pathway which include signal transducers and activators of transcription (STAT), phosphatidylinositol 3-kinase (PI3K)/Akt, and p44/42 mitogen-activated protein kinase (MAPK) [4]. c-kit signalling system sustain proliferation, differentiation and survival of various cell types including hematopoietic progenitors, mast cells, melanocytes germ cells and intestinal cells of Cajal [5]. C-kit expression is associated with various solid and haematological malignancies like gastrointestinal stromal cells (GIST), Ewing's tumour, chronic myelogenous leukemia (CML), small-cell lung cancer, and neuroblastoma [6-10]. On the other hand loss

of c-kit expression is associated with prognosis of breast cancer and thyroid cancer [11-12].

Cancer associated fibroblasts (CAFs) are a subpopulation of cells which reside within the tumour microenvironment and act as major component of tumour stroma. It plays prominent role to endorse the transformation process by promoting tumour growth, angiogenesis, inflammation, and metastasis in various cancers [13]. Activated CAFs can be identified by typical spindle shaped phenotype and presence of molecular biomarkers like α -smooth muscle actin (α -SMA), fibroblast-specific protein 1 (FSP-1), desmin, vimentin, type I collagen, platelet derived growth factor receptor-B (PDGFR β) and fibroblast-activated protein (FAP) [14,15]. CAFs maintains communication among themselves or with cancer cells or inflammatory and immune cells through a complex network of either cell to cell contact or through paracrine/ exocrine signalling, proteases, and modulation of the extracellular matrix (ECM). This network system plays important role in maintenance of microenvironment and support tumorigenesis, angiogenesis, and metastasis [16,17]. Due to genetically homogenous nature of CAF, proteins which are specific or secreted by CAF might serve as both prognostic markers and targets for anticancer drugs. Different studies have suggested that CAF play pivotal role in metastasis of various human solid tumours like stomach, colon, breast, prostate, and pancreas and so on [18-22]. A detail study on underlying mechanism of CAF induced metastasis in human solid tumours will help to design effective therapeutic target for patients against reactive stroma which is specific predictor of disease recurrence.

Therefore, in the present study, immunohistochemistry and immunofluorescence method was used to study the expression of specific marker expressed by CAFs and c-kit in normal and malignant gastric epithelium tissues. RT-PCR analysis was also done to supports the expression pattern of both c-kit and proteins which are specific markers or secreted by CAFs. Discussion on the correlation of c-kit and CAF expression with tumour characteristics in gastric cancer tissues may be helpful to establish the biological behaviour and prognosis of gastric cancer.

Patient and Method

Study Population and Tissue Specimens

The surgically resected specimens following gastrectomy for stomach malignancy were obtained from 64 patients of Department of Gastroenterology, Stanley Medical College and Hospital, Chennai, Tamil Nadu, India. The study was approved by the ethics committee of the Stanley Medical College and Hospital. Written informed consents were obtained from all the patients for participation in the study. Another 33 cases of adjacent normal gastric mucosa were used as control.

The study group was consisted of 64 patients, male 48 cases and female 16 cases (25–75 years of age, the median age of 50 years). Stage, grade and histological types were defined after histopathological examination. There were 19 cases of upper gastric malignancy, 17 cases of middle malignancy and 28 cases of antral malignancy. In the all, 27 cases had tumour size (<4 cm) and the other 17 cases had large tumour size >8 cm, and 20 patients had tumour size in between 4-8 cm. There were mucosal tumour 12 cases, 23 cases of sub mucosal tumour, 18 cases of Muscularis Propria tumour and 11 cases of Sub Serosa

tumour. There were 39 cases with lymphatic invasion and 40 cases with node metastasis of tumour. Patients in the study group had no history of undergoing chemotherapy or radiation therapy.

Immunohistochemistry and Immunofluorescence Study

Experimental paraffin block samples were used for serial tissue sectioning, 4 μ m thicknesses underwent HE, as well as IHC staining respectively. The endogenous peroxidase activity was blocked by 3% H₂O₂ and non-specific binding was blocked with 3% BSA in 1x PBS at room temperature. The sections were then incubated overnight at 4°C with primary antibodies for anti α -SMA (1:300), (Santa Cruz Biotechnology-USA). The slides were washed in 1x PBS and incubated with the corresponding secondary antibody conjugated with HRP. The peroxidase reaction was developed by hydrogen peroxide in 1x PBS as substrate and 3,3'-diaminobenzidine tetrachloride as chromogen. The sections were counter stained with Mayer's haematoxylin and mounted with DPX after proper dehydration. Positive control sections were prepared to confirm the reactivity of the antibody whereas negative control sections were prepared by substituting PBS instead of primary antibody.

For immunofluorescence studies, after incubation with primary antibody [anti- c-kit (1:300), (Santa Cruz Biotechnology-USA)] fluorescent conjugated secondary antibodies (FITC- conjugated anti-mouse for c-kit) and the slides were counterstained using DAPI. The sections were mounted and examined under confocal microscopy (Leica TCS2- XL Spectra confocal-Multiphoton microscopy).

Evaluation of Immunostaining

The intensity and cellular localization of immunostained c-kit and α -SMA was evaluated in a semi-quantitative fashion. Staining intensity was graded in 0-3 scale (0- no staining, 1- shallow brown, 2- Brown, 3- dark brown). Positive cells were also graded in 0-3 scale (0- no staining, 1< 25% staining cells, 2< 25-50% staining cells and 3> 50% staining cells). The final scores were given as -, +, ++ and +++ which were summation of staining percentages and intensity; and considered as negative, mild, moderate and intense respectively. Distinct membranous to cytoplasmic expression of c-kit was considered as positive expression whereas presence of α -SMA positive stromal cells were considered as positive for CAFs.

Reverse transcriptase PCR

RNA was extracted using Isol-RNA Lysis Reagent (5'PRIME Inc, Gaithersburg, USA) according to the manufacturer's protocol. The RNA concentration was determined by Biophotometer (Eppendorf, Germany) and total RNA (1 mg) was used to generate c-DNA using standard method. Specific primer sequences for c-kit, α -SMA, Vimentin and SDF-1 were used to amplify gene transcript. The reactions were cycled after an initial denaturation at 94°C for 5 min followed by 39 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, and polymerization at 72°C for 60 sec. Final extension was done at 72°C for 5 min. β -actin was used as housekeeping gene. The PCR products were electrophoresed on 1.2% agarose gels with ethidium bromide and visualized in UV light (VilberLourmat, France).

Statistical Analysis

The χ^2 test was used for the percentage of samples with positive staining among lesions of different

histological grades using StatCalc 5.0.4 (AcaStat software). $p < 0.05^*$ was considered statistically significant and $p < 0.01^{**}$ was considered as highly significant.

Results

Presence of c-kit was prevalent in gastric carcinoma tissues

To determine the extent of c-kit in gastric cancer, paraffin embedded sections were used for immunofluorescence study. In addition to this Reverse transcriptase PCR was carried out to establish expression of c-kit in gastric epithelium tissue.

c-kit was expressed in cytoplasm and membrane of cancer stomal cell and play significant role in cancer cell invasion. Immunofluorescence result had showed that presence of c-kit was more predominant in carcinoma tissues rather than in normal gastric epithelium. Eighteen out of thirty three normal gastric specimens were negative (-) for c-kit whereas fifteen specimens were very mild (+). None of the normal gastric specimens were expressing moderate or strong expression. While cancer specimens were concern, there were 10, 13, 18, 23 cancer specimens were negative (-), mild (+), moderate (++), strong (+++) positive for c-kit respectively [Figure 1, Figure 3].

To determine mRNA expression of c-kit, RT-PCR results showed that the expression levels were elevated at carcinoma specimens rather than in normal gastric tissues [Figure 5].

c-kit prevalence is associated with invasive property of gastric cancer

In gastric cancer specimens, Multivariate correlation analysis was carried out to establish c-kit prevalence as predictor for gastric cancer prognosis. During this study, expression of c-kit were correlated with various clinicopathological parameters like age, & gender of patients, position of tumour, size and depth of tumour, lymphatic invasion and node metastasis. In Table I, it was shown that, age, gender of the patients and position of the tumour were not correlated with c-kit expression. While concerning the size and depth of the tumour, lymphatic invasion and node metastasis, there were statistically significant correlation found between c-kit expression and tumour characteristics ($p < 0.05$). High c-kit prevalence was found in 81.5% small tumour and 58.8% of big tumour size; 83.33% of mucosal tumour and 94.44% of muscularis propia tumour. The high expression of c-kit was positively correlated with 74.36% lymphatic and 75% node metastasis specimens. These results suggest that c-kit could be used as marker for gastric cancer prognosis [Table I].

Reactive Cancer associated Fibroblasts were prevalent in gastric carcinoma tissue

To determine the extent of CAFs in gastric cancer paraffin embedded sections were used for immunohistochemistry study of α -SMA. In addition, Reverse transcriptase PCR was carried out to establish the expression of several proteins which is specific or secreted by CAFs like α -SMA, vimentin, and SDF-1.

α -smooth muscle actin is the filamentous protein which expressed in stomal cell and play significant role in cancer cell invasion. Immunohistochemistry result had showed that presence of α -SMA expressing fibroblast are more predominant in carcinoma tissues rather than in normal gastric epithelium. Twenty one out of thirty three normal gastric specimens were negative (-) for α -SMA whereas twelve specimens were very mild (+). None of the normal gastric specimens were expressing moderate or strong expression. While cancer specimens were concern, there were 2, 4, 20, 38 cancer specimens were negative (-), mild (+), moderate (++), strong (+++) positive for fibroblast expressing α -SMA respectively [Figure 2, Figure 4].

To determine mRNA expression of CAFs proteins, results showed that the expression level of all these proteins were elevated at carcinoma specimens rather than in normal gastric tissues. There were elevated expression of α -SMA, vimentin and SDF-1 was found in gastric cancer specimens than in normal specimens [Figure 5].

From this result, it can be concluded that reactive CAFs were predominant in gastric cancer tissues, and it secret proteins which are essential for growth, invasion and metastasis of gastric tumours.

CAF prevalence is significantly associated with invasive property of gastric cancer

Multivariate correlation analysis was carried out to determine whether the CAFs prevalence in gastric cancer specimens could serve as predictor for gastric cancer prognosis. During this study, expression of α -SMA were correlated with various clinicopathological parameters like age & gender of patients, position of tumour, size and depth of tumour, lymphatic invasion and node metastasis. In Table I it was shown that, age, gender of the patients and position of the tumour were not correlated with α -SMA expression. While concerning the size and depth of the tumour, lymphatic invasion and node metastasis, there were statistically significant correlation found between CAFs expression and tumour characteristics ($p < 0.05$). The high incidence of α -SMA expression was correlated with 66.665% lymphatic invasion and 77.5% of node metastasis specimens. These results suggest that above discussed proteins could be used as marker for gastric cancer prognosis [Table I].

Table I: Relation of c-kit & α -SMA expression and clinicopathological factors in gastric cancer

Parameters	n = 64	c-kit (+ve)	c-kit (-ve)	χ^2	p value	α SMA (+ve)	α SMA (-ve)	χ^2	p value
Age									
<=50	40	32	8	3.48	0.062	30	10	2.95	0.086
>=50	24	14	10						
Gender									
Male	48	37	11	2.58	0.108	35	13	0.103	0.748
Female	16	9	7						
Tumour Position									
Upper	19	15	4	2.56	0.278	15	4	1.79	0.409
Middle	17	12	5						
Lower	28	16	12						
Tumour Size									
<4	27	22	5	6.92	0.031*	22	5	8.59	0.014*
4-8	20	9	11						

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>8	17	10	7			11	6		
Tumour Depth									
Mucosa	12	10	2	9.05	0.029*	8	4	10.8	0.013*
Sub Mucosa	23	17	6			23	0		
Muscularis Propia	18	17	1			17	1		
Subserosa	11	7	4			10	1		
Lymphatic Invasion									
(+ve)	39	29	10	5.99	0.014*	26	13	4.40	0.036*
(-ve)	25	11	14			10	15		
Node Metastasis									
(+ve)	40	30	10	7.11	0.008**	31	9	5.15	0.023*
(-ve)	24	10	14			12	12		

a. *Denotes significance at 5% level, b. ** Denotes significance at 1% level.

DISCUSSION

c-kit expression has been well studied in various types of tumours which include small-cell lung cancer, neuroblastoma, and GIST and CML [6,7,9,10]. In this study we examined the relation between c-kit expression and malignancy potential of gastric epithelium specimens and also we had discussed the correlation between c-kit influence and clinicopathological characteristics of gastric cancer prognosis.

A study by Akira Y (2006) had shown that c-kit expression is high in pancreatic cancer and is correlated with the disease prognosis [23]. In concomitant to this study our immunofluorescence findings also suggests that the gastric adenocarcinoma tissues were expressing high level of c-kit than that of normal gastric epithelium. Our RT-PCR result supports this finding and showed prevalence of c-kit m-RNA expression in carcinoma than that of normal gastric epithelium. The prevalence of c-kit expression in carcinoma might be correlated with malignancy transformation of the disease and acquire of mesenchymal phenotype of gastric tumours. Conflict to our report, other studies in breast, and colon cancer had showed that loss of c-kit expression was associated with the disease progression [24].

High incidence of c-kit expression was associated with VEGF expression in progressive stages of GIST and gastric carcinosarcoma [25,26]. In the light of these findings our report suggest that high expression of c-kit in cytoplasm and membrane of cancer stromal cells are believed to be necessary for tumour growth, invasion and metastasis and also might play significant role in tumour angiogenesis. In this study, prevalence of c-kit expression was correlated with clinicopathological characteristics of tumour and the finding suggest that lymphatic invasion and node metastasis were more severe in c-kit positive group than that of c-kit negative group. The high c-kit expression was also statistically significant with size & depth of the tumour ($p < 0.05$). These findings suggest that during malignancy transformation cancer stromal cell express high amount of c-kit which in turn play prominent role in invasion and metastasis of gastric cancer and might serve as better therapeutic target for prognosis of gastric cancer.

Studies in molecular and cellular biology have been elicited that tumour microenvironment is consisted of cancer cells as well as stromal cells containing vascular cells, fibroblast and smooth muscle cells which in turn play prominent role in tumour growth and metastasis [27]. Studies have been shown that Fibroblast are major component of stromal cells and play significant role in tumour growth, invasion and metastasis of breast cancer and colon cancer and also found to be involved in angiogenesis of gastric cancer model [28]. These findings are consistent with our result which showed that

accumulation of fibroblast was associated with progression of gastric cancer.

In tumours, Fibroblast mainly consists of biologically active reactive fibroblast, myofibroblast and tumour associated fibroblasts. α -smooth muscle actin is considered as marker for activated myofibroblast also known as cancer associated fibroblast. According to Bissel *et al*, wounded microenvironment or desmoplastic stroma acts as promoter for tumour growth and can be characterized by the presence of α -smooth muscle actin reactive myofibroblast [29]. In the present study immunohistochemical studies showed the presence of scattered or mild expression of α -smooth muscle actin in normal gastric mucosa and actin expression got increased significantly in gastric adenocarcinoma and distributed irregularly in the tumour nest. Recently it was proved that the stroma provides continuous support to carcinoma cells throughout the different pathophysiological processes and modulates tumour progression [30]. These findings suggest that α -smooth muscle actin reactive fibroblast cells irreversibly activated by tumour microenvironment and could contribute to the invasive potential of cancer cell. According to Kuang *et al*, α -smooth muscle actin expression and its relation with depth of lesion is involved in cancer metastasis [31]. The results of our study are similar to that reported by Nakayama *et al*, who found a correlation between presence of α -smooth muscle actin reactive myofibroblast cell in intestinal type gastric cancer [32] and the study by Anca MC *et al*, in breast cancer [33]. Several other studies had shown that this kind of activated myofibroblast present in the stromal region of the genetically mutant tumour cell may auxiliaries the expression of various chemokines, cytokines, growth factors, remodelling of the extracellular matrix and matrix metalloproteases which in turn induce tumour immune escape and control tumour development, angiogenesis [34] and metastasis [35]. α -smooth muscle actin expressive stromal cells also can promote the switch between epithelial cell to mesenchymal phenotype via activation of various transcription factors during the advanced stage of carcinoma progression and correlates with metastatic potential of the tumour cells [36]. The association between clinicopathological factor of tumour and α -SMA expression suggest its prominent role as prognostic marker of gastric cancer.

To determine the expression of other CAF associated proteins RT-PCR was performed. Vimentin is a intermediate filament protein and characteristic component of mesenchymal cells. Fibroblast like cells express high amount of vimentin and elevated expression of this protein is significantly associated with tumour invasion and metastasis [37]. According to our findings m-

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RNA expression of Vimentin was elevated in gastric cancer tissue than that of normal gastric epithelium. The next protein was SDF-1 which was expressed by stromal cells and play prominent role in proliferation of tumour cells, angiogenesis, invasion and metastasis through CXCR4 receptor expressed on the tumour cells [38]. Our findings

suggest that, expression of SDF-1 was elevated in gastric cancer tissue than that of normal gastric epithelium. These results suggest that up regulation of CAFs are associated with malignant transformation of gastric cancer and would serve as predictive marker for invasion.

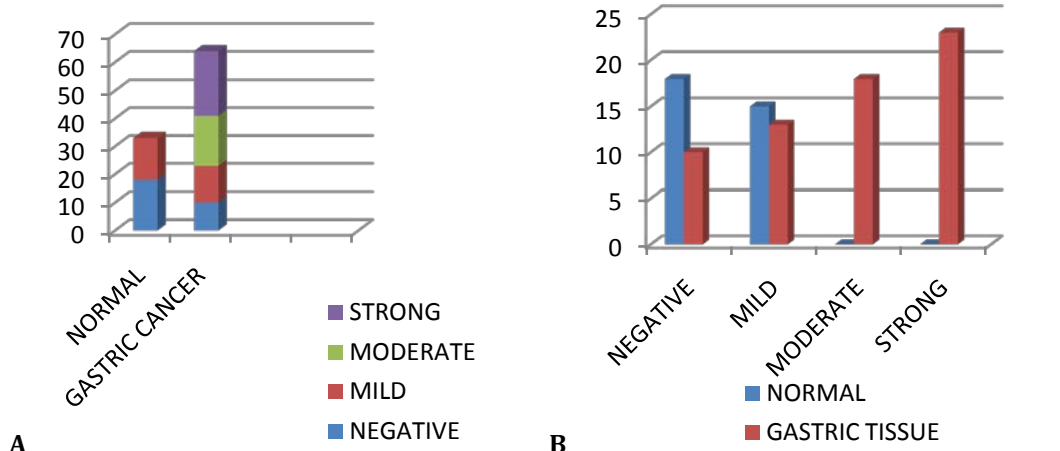


Figure 1. Immunofluorescence Analysis of c-kit in Normal and Gastric Tissues Paraffin embedded surgically resected Normal (33) and Gastric cancer tissues (64) were stained for c-kit and the frequency of staining pattern was graded as Negative, Mild, Moderate and Strong. A. Distribution of these four grades in Normal and Cancer tissues were analyzed. B. The number of normal or cancer tissues graded as Negative, Mild, Moderate and Cancer were compared.

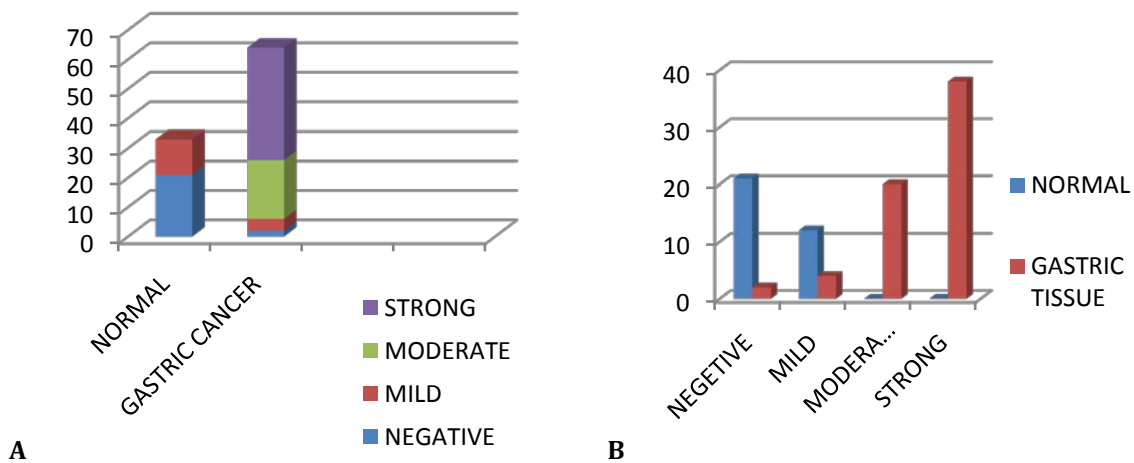
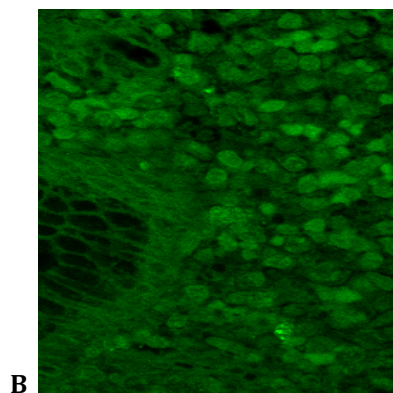
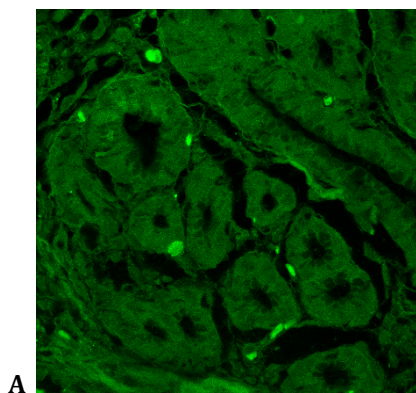


Figure 2. Immunohistochemistry Analysis of alpha-SMA in Normal and Gastric Tissues Paraffin embedded surgically resected Normal (33) and Gastric cancer tissues (64) were stained for alpha-SMA and the frequency of staining pattern was graded as Negative, Mild, Moderate and Strong. A. Distribution of these four grades in Normal and Cancer tissues were analyzed. B. The number of normal or cancer tissues graded as Negative, Mild, Moderate and Cancer were compared.



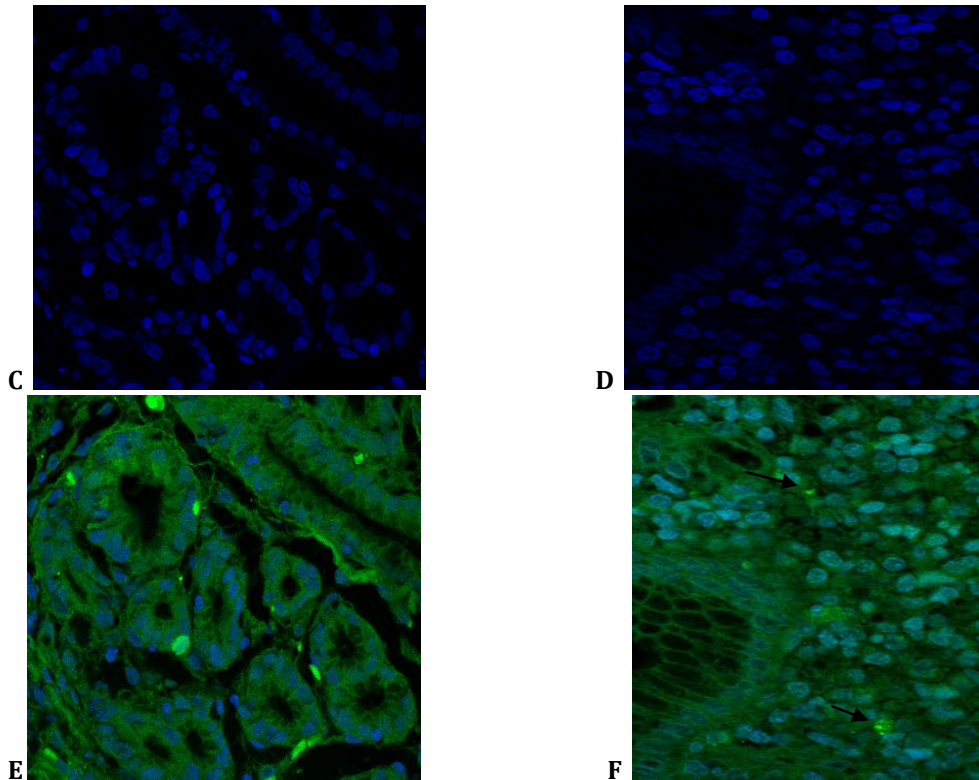


Figure 3. Immunofluorescence Analysis of c-kit in gastric epithelial tissues. The expression of C-kit in the same field of normal (A,C,E) and gastric cancer (B,D,F) tissue by immunofluorescence using FITC (green) (A-B). The tissues were counterstained with DAPI (C-D) Merged image of c-kit and DAPI (E-F). (Magnification in 60X)

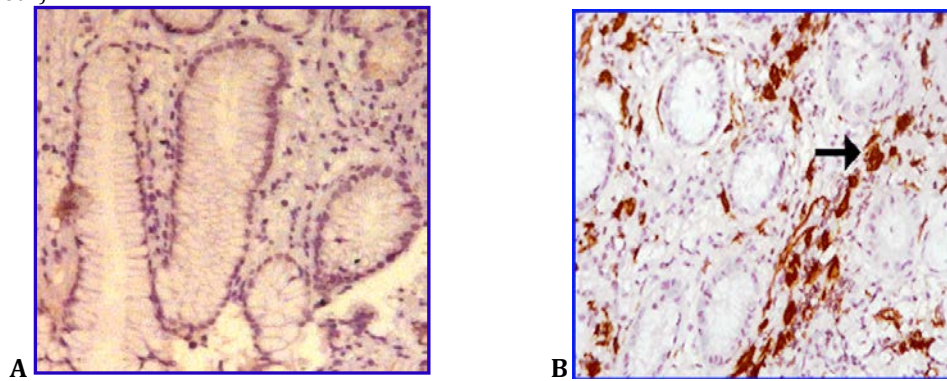


Figure 4. Immunohistochemical analysis shows expression of α -SMA in normal and Gastric cancer Tissues. A. Negative to mild expression of α -SMA was found in stromal cells. B. α -SMA prevalence was found in stromal cells of Cancer tissue. (Magnification in 20X)

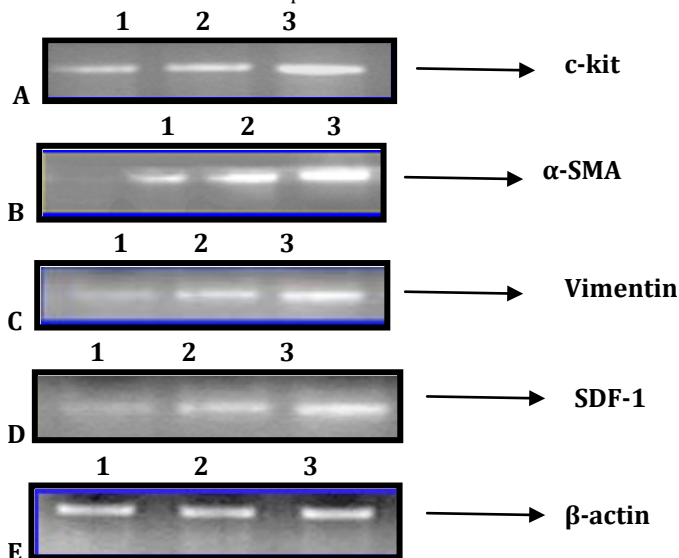


Figure 5. RT-PCR analysis shows c-kit, α -SMA, Vimentin, SDF-1 m-RNA expression in human gastric tissues. A. C-kit m-RNA transcript was amplified in normal and carcinoma tissue. B. α -SMA m-RNA transcript was amplified in normal and carcinoma tissue. C. Vimentin m-RNA transcript was amplified in normal and carcinoma tissue. D. SDF-1 m-RNA transcript was amplified in normal and carcinoma tissue. E. β -actin m-RNA transcript was amplified in normal and carcinoma tissue. (Lane 1- Normal, Lane 2,3 - Cancer tissue)

CONCLUSION

In conclusion, our reports are supportive to previous findings and demonstrate that elevated c-kit and CAF expression was associated with malignant transformation of gastric cancer and their correlation with tumour size, depth of the tumour, node metastasis and lymphatic invasion established them as cognate marker for gastric cancer invasion and metastasis. Furthermore studies on these markers will help to establish them as a potential therapeutic target for gastric cancer patients.

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