Inflammatory Role of Transforming Growth Factor Beta-1 (TGF-β1) in Multistep Progression of Gastric Oncogenesis: An Immunohistochemical Study

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***Abstract*—The purpose of this study was to investigate differential expression of TGF-β1 in gastric tissues resected from patients diagnosed with chronic gastritis and gastric cancer (adenocarcinoma) and elucidate the potential role of TGF-β1 in steering towards cancer promoting or cancer suppressing microenvironment. Each group of disease serves as representative model involved in multistep progression of gastric oncogenesis. Immunohistochemistry of TGF-β1 on 162 gastric tissues were performed and analyzed microscopically using semi-quantitative system. Tabulated values which represent each pathological group were then assessed statistically to detect significance of expression. Significance association of expression among groups of age and gender were also included in this study. Chi-square and Mann-Whitney U tests with ($p > 0.05$) confirmed that TGF-β1 expression was dependent on tissue types and decrement of expression was detected between normal tissues to gastritis and cancer. Higher expression was also observed in diffuse type in comparison to intestinal cancer type. For normal samples, significant difference of expression was found between the two genders; male and female. TGF-β1 have the potential as molecular marker for detecting and monitoring inflamed microenvironment. Expression level in gastric cancer may be target for future therapeutic strategy in preventing cancer progression.**

***Index Terms*—transforming growth factor beta 1 (TGF-β1), chronic gastritis, gastric cancer, immunohistochemistry, oncogenesis**

I. Introduction

Worldwide epidemiological studies on gastric cancer have revealed that it is ranked second highest for cancer causing mortality rate (Brenner *et al*. 2009). This owes to the fact that most patients' exhibit no apparent symptoms until the cancer has attain advanced stages with poor prognosis. Current detection of such pathological condition involves costly and impractical invasive procedure. The needs for early detection and impediment measures are necessary for better management of the disease.

Tumorigenesis for most types of cancer has been proven to develop in a multistep manner (Vogelstein and Kinzler, 1993). Similarly, model of gastric tumorigenesis has been established in which gastric cancer progresses from normal to inflamed microenvironment; chronic gastritis, intestinal metaplasia, dysplasia and subsequent tumor formation. In line with morphological changes, aberrant alterations at genetics and protein level have prompted researchers to characterize tumor behaviours through molecular aspect (Khan and Shukla, 2006).

TGF-β protein is secreted by most cells in its inactive latent large complex (LLC). Activation requires proteolytic cleavage of LLC from small latent associated protein (LAP). TGF-β1 co-exists together as inactive pre-cursor LLC. For it to exert its effect and bind to its respective receptor, LAP has to dissociate from LLC via activation (Annes *et al*. 2003). Though mechanism of activation is complex, TGF-β1 isoform is the most abundantly expressed and studied in many pathological conditions in which it was previously observed that reduced TGF-β expression is associated with various inflammatory conditions such as inflammatory bowel disease (Hahm *et al*. 2001; Fiocchi, 2001). TGF-β has been proven to display both context dependent tumor promoting and tumor suppressing properties depending on cell types and stage of tumor development (Elliott and Blobe, 2005). This study focuses to find differences of expression of the protein in normal, chronic gastritis and gastric cancer tissues in an attempt to elucidate its role in gastric carcinogenesis.

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II. Materials and Methods

A total of 162 formalin-fixed paraffin embedded (FFPE) samples of gastric tissues were collected from Kuala Lumpur Hospital (HKL). Samples comprised of 57 gastric tissues which showed no significant pathology (normal), 65 chronic gastritis consisting 23 *H. pylori* associated gastritis, 42 non *H*. pylori gastritis and 40 gastric cancer (adenocarcinoma). Gastric cancer samples were grouped based on Lauren's classification where 25 samples were identified as diffuse-type adenocarcinoma, 13 intestinal and 2 indeterminate. Approval of research ethics from National Medical Research Register (NMRR) was obtained before stated procedures were commenced.

A. Immunohistochemistry

Immunohistochemistry was performed on 4µm sectioned tissues using monoclonal antibody against TGF-β1 (GeneTex, Inc.) in dilution of 1:1000. Heat induced antigen retrieval (HIER) process was carried out in microwave oven for 15 minutes using Citrate buffer, pH 6. Primary antibody was then stained using Dako REAL™ EnVision™ Detection System (Dako, Denmark), adhering to the manufacturer's protocol. Positive (breast carcinoma) and negative control tissues were included in every run of experiments.

B. Evaluation of Immunohistochemistry

Positive cytoplasmic glandular staining was evaluated semi-quantitatively by adapting established TGF-β1 scoring system (Culhaci *et al*. 2005). Observed stained tissues were categorized into 4 levels of intensity: 0: no staining, 1: weak staining, 2: moderate staining, 3: intense staining. Percentage of positive stained tissue was classified based on: 0: negative, 1: less than 33% positive staining, 2: 33%-66% positive staining, 3: more than 66% positive staining. Summed values of both intensity and positivity were classified as 0 to 1: negative, 2: weakly positive, 3 to 4: moderately positive and 5 to 6 as strongly positive.

C. Statistical Analysis

Nominal positive or negative expression of scores correlation between tissue groups was tested using Chi-square test. For quantitative value differences, Mann-Whitney U test was applied on comparing two pair of groups and Kruskal-Wallis test as whole comparison of groups. Spearman rank correlation test was applied on the three groups of samples to test correlation on two selected demographical parameters: age and gender. For all tests, $P < 0.05$ was considered as statistically significant. Analyses were performed using SPSS version 19.0.

TABLE I. Correlation of TGF-b1 Expression to Demographic Data

Groups	Description		Total (*n*)	Total Score Class: Negative	Weak	Moderate	Strong	*P**
Normal	Total		57	29	6	15	7	
	Age							
		≤50	19	8	4	4	3	0.549
		>50	38	21	2	11	4	
	Gender							
		Male	28	10	3	12	3	**0.048**
		Female	29	19	3	3	4	
Chronic Gastritis	Total		65	49	4	9	3	
	Age							
		≤50	17	14	0	2	1	0.646
		>50	48	35	4	7	2	
	Gender							
		Male	39	29	1	7	2	0.680
		Female	26	20	3	2	1	
Gastric Cancer	Total		40	32	4	3	1	
	Age							
		≤50	16	11	2	3	0	0.151
		>50	24	21	2	0	1	
	Gender							
		Male	28	22	3	2	1	0.724
		Female	12	10	1	1	0	

**P* value using Spearman's rank order correlation. Significant value is shown in bold.

III. Results

Combined tabulated scores yielded 50.8% (n=29) of normal, 75.3% (n= 49) of gastritis and 82.5% (n=33) gastric cancer tissues with negative expression (Table I). The table also depicts results of Spearman rank order correlation test assessed on two demographical parameters, where significant correlation was only found in group of normal tissue expression when paired to gender parameter. Mean rank scale values of expression are shown in Fig. 1. Significant reduction of expression among the three groups of samples were further verified with significant values of Chi-Square (p= 0.003), 2-paired Mann-Whitney and Kruskal Wallis tests. On Mann-Whitney test, significant decrease of expression was detected from normal towards tumor development stages. P values of normal tissues when paired to gastritis and gastric cancer tissues are 0.002 and 0.018 respectively (Table II). Though there is no significant decrement of expression between groups of gastritis and gastric cancer, Kruskal-Wallis test with p value of 0.003 implies significance among the whole groups. Mann-Whitney intra-group comparison values of expression on gastritis and cancer tissues are presented in Table III. Higher significance of expression was detected only between subset groups of cancerous tissues in which diffuse type cancer expressed higher level of TGF-β1.

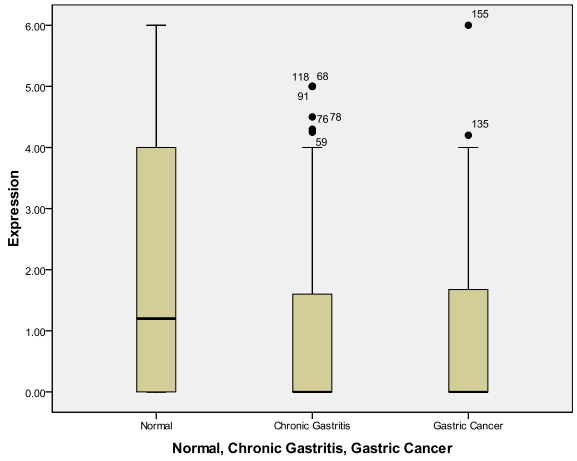

Figure 1. Box-plots of TGF-β1 expression among three classes of score distribution in gastric tissues depict reduction and no overlap and of median values between normal sample group and groups of chronic gastritis and gastric cancer. The significance is further validated using Mann-Whitney U test. Presence of samples with outlier values above the plot indicates extreme expression in comparison to other samples of similar class.

TABLE II. Paired Mann-Whitney Tests Among the 3 Groups of Sample

Sample Group	Mean Rank	P value
Normal	71.32	**0.002**
Gastritis	52.88	
Normal	54.34	**0.018**
Adenocarcinoma	41.39	
Gastritis	51.85	0.571
Adenocarcinoma	54.88	

TABLE III. Paired Intragroup Mann-Whitney Tests of Gastritis and Adenocarcinoma

Sample	Mean Rank	P value
H. pylori associated gastritis	31.22	0.505
Non *H. pylori* gastritis	33.98	
Diffuse Adenocarcinoma	22.26	**0.02**
Intestinal Adenocarcinoma	14.19	

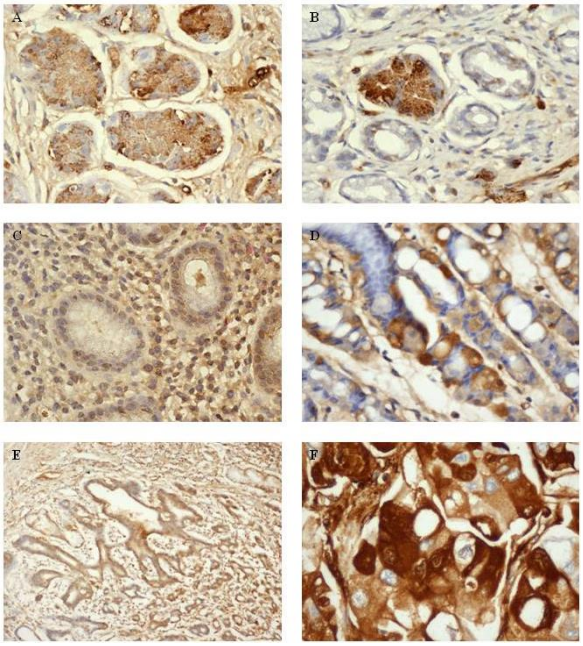

Figure 2. Representative Tissue Staining of Different Pathological Classification. (A) represents normal gastric tissue of moderate staining, with values of (2) for intensity and 3 for positivity scores. Tissue (B) shows staining of sample diagnosed with non H. pylori chronic gastritis with high intensity but low value for positivity score. In contrast, sample (C) represents a case of H. pylori associated gastritis which is more evenly stained with lower intensity but higher positivity score. Sample D and E each diagnosed with distinct types adenocarcinoma; diffuse (D) showing spectrum of strong staining intensity and intestinal (E) type with a strong staining intensity pattern. Breast cancer used as positive control in the experiment displays varying degree of cytoplasmic and membranous staining intensity. Magnification: 400×.

IV. Discussion

With significant values of both Chi-square and Spearman rank correlation test, our finding detected differences of TGF-β1 expression among gender which corroborate with Tyagi *et al*, 2009 finding where higher expression in males were observed in normal control group of rats. As evidenced in the increment values of non significance in Spearman correlation test, this associative expression pattern was lost as tissues progress into abnormal; inflamed and oncogenic conditions thus necessitating the needs to address gender differences for future pathological study of TGF-β1 expression.

Our postulate of reduced TGF-β1 expression in inflamed tissues as the driving factor that promotes towards pro-oncogenic microenvironment is based on its ability to regulate anti inflammatory mechanism via arms

of immune cells; T cells (Nakamura *et al*. 2004; Li *et al*, 2007), macrophages (Gong *et al*. 2012; Reifenberg *et al*. 2012) and antigen presenting cells (Kosiewicz *et al*. 2004). Furthermore, TGF-β1 has a role in promoting re-epithelialization at such important wound healing phase (Reynolds *et al*. 2005). Future therapeutic strategy in managing this response would most probably be an intervention to restore TGF-β expression to normal level. It is also worth to note that our results of TGF-β1 expression in *H. pylori* associated gastritis tissues showed contrasting results to previous experiments done *in vitro* on *H. pylori* challenged cultured gastric cells (Beswick *et al*. 2011; Wu *et al*. 2007). We attribute these discrepancies to two possibilities which are differences of host-bacteria interaction and method of protein expression quantification. Based on the same literature we deduced that the downregulation of TGF-β1 have negative outcome on *H. pylori* colonization and pathogenesis but at the same time poses detrimental effects towards host tissue for the reduction of anti-inflammatory mechanism thus hinting for alternative early stage intervention of eradicating *H. pylori* colonization without twitching the optimum balance of the anti-inflammatory cytokine in future medical development strategy.

For chronic inflammation, restoring TGF-β1 expression in chronic gastritis would probably yield the most promising effect by targeting upstream signalling molecules since various studies (Fiocchi, 2001) have reported failure on containing inflammation by targeting downstream cytokines, alternative upstream targeted therapy may hold the potential key to revert inflammatory microenvironment to normal. Monteleone *et al*, (2004) attempt to contain inflammation in *H. pylori* associated gastritis proved to be successful by targeting negative regulator molecule, Smad7 in TGF-β signalling through upstream molecule, interferon-gamma induction.

Our observation on cancer tissues which showed no significant increment of expression in comparison to gastritis would probably have positive anti-oncogenic effects on its microenvironment. Late stage TGF-β expression in tumors have been correlated with poorer prognosis which include high metastatic ability of carcinoma, tumor immortality and promotion of angiogenesis for tumor growth (Wakefield, 2002; Derynck *et al*. 2001). As such, propose targeted therapy would be opposite to the strategy of curing chronic gastritis which is by lowering level of TGF-β in order to contain malignancy. Our data does not only suggest contradiction of expression from previous report (Hawinkels *et al*. 2007) in terms on observed elevated TGF-β1 expression of gastric cancers in comparison to normal tissues, but also inverse of expression between intestinal and diffuse adenocarcinoma in which we found higher expression in diffuse type compared to intestinal. This stratifies poorer prognosis patients with diffuse type adenocarcinoma thus suggesting some relevance of quantifying TGF-β1 expression as clinical biomarker as a prognostic tool and therapeutic target in future. A criteria for developing good biomarker is to allow easier access of protein expression quantification without resorting to invasive surgery and Ma *et al*. 2013 has recently proposed that gastric oncogenesis may have systemic effects on TGF-β1 serum level rather than limited to just sites oncogenesis. Nevertheless detail future conclusive study on correlations among gene expression to protein transcript level, activated against latent type of TGF-β and serum protein concentration level at every stage of the oncogenesis model are crucial for validating its clinical usefulness.

V. Conclusion

Decrement of TGF-β1 expression was detected when normal samples were compared to inflamed chronic gastritis samples thus presenting potential prospects for prognostic and therapeutic target of this protein on managing gastritis and gastric cancer. Despite conflicting results reported by other works, future conclusive study with larger sample size, constant methods and parameters of study are needed to verify this finding.

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