UNIVERSITI PUTRA MALAYSIA

PROTEIN EXPRESSION OF TGF-β, SMAD2 AND RUNX3 IN NORMAL STOMACH, CHRONIC GASTRITIS AND GASTRIC ADENOCARCINOMA

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By

AHMAD ZHARIF ISMAIL

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

January 2016

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Specially dedicated to
My parents, sisters, brothers and
the scientific community who have endured a never ending journey in pursuit of scientific and medical knowledge and progress.

,Abstract of the thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

PROTEIN EXPRESSION OF TGF-β, SMAD2, AND RUNX3 IN NORMAL STOMACH, CHRONIC GASTRITIS AND GASTRIC ADENOCARCINOMA

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January 2016

Chair : Hairuszah Ithnin, MD, MPATH, FAMM
Faculty : Medicine and Health Sciences

Though recorded as 7th most common type of cancer worldwide, gastric cancer ranked 2nd for the highest mortality rate for cancer related cases. With medical evidences that supported higher curability chances should tumor were detected in earlier initial stages, current medical technology development to facilitate early detection of gastric cancer remain infeasible and costly. Thus discovery of biomarker that is concurrently altered from normal physiological condition in tumor progression would be an ideal solution to address the problem. This study focuses on controversial pleiotropic protein expression, TGF-β and its downstream associated products Smad2 and RUNX3 in samples that represent stages of multistep tumorigenesis. A total of 162 tissue samples in the form of formalin fixed paraffin embedded (FFPE) were employed. Out of these, 57 were screened negative for any pathological symptoms (normal), 23 *Helicobacter pylori* associated chronic gastritis, 42 non *Helicobacter pylori* chronic gastritis and 40 gastric adenocarcinoma tissues. Through semiquantitative score immunostaining, decreasing percentage of TGF-β1 immunostaining positive were detected from normal (49.1%) to chronic gastritis (24.6%) and adenocarcinoma (17.5%) samples. These alterations were proven to be statistically significant with p values less than 0.05 at every successive stages.SMAD2 however expressed inverse relation in which samples with strongly positive immunostain increases from 5.3% in normal to 7.7% in chronic gastritis and 16.2% in gastric adenocarcinoma. In spite of these increment, unlike samples stain for TGF-β, only one pair of group (normal, chronic gastritis) showed significant increment for 2 paired Mann Whitney test. In addition, no correlation of TGF-β- SMAD2 expression tested in the same samples were found using Spearmann correlation test. For RUNX3 we report negative expression on all types of gastric tissues amidst some positive lymphocytes stain present in certain number of samples. For these significant changes of TGF-β and SMAD2 expression from normal gastric to inflamed conditions, we report that these proteins could become potential inflammatory biomarkers in conclusion to this study.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains

EKSPRESI PROTEIN TGF-β, SMAD2, DAN RUNX3 DALAM GASTRIK NORMAL, GASTRITIS KRONIK DAN GASTRIK ADENOKARSINOMA

Oleh

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Pengerusi : Hairuszah Ithnin, MD, MPATH, FAMM
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Walaupun direkodkan menduduki tempat ke-7 sebagai insiden kanser paling kerap berlaku, kanser gastrik mencatatkan rekod ke-2 tertinggi kematian bagi kes yang melibatkan penyakit kanser. Dengan bukti-bukti kajian perubatan yang menyokong kadar keberhasilan rawatan adalah lebih tinggi sekiranya kanser dikesan pada peringkat awal, teknologi perubatan terkini bagi pengesanan kanser pada tahap awal masih berada pada tahap kurang praktikal dan berkos tinggi. Oleh itu penemuan biomarker yang mengalami perubahan sifat selari dengan penyimpangan fisiologi normal dalam proses perkembangan tumor adalah penyelesaian yang ideal bagi permasalahan ini. Kajian ini menumpukan ekspresi protein yang bersifat pleiotropik, TGF-β serta protein-protein isyarat bawahannya, SMAD2 dan RUNX3 di dalam sampel yang mewakili peringkat-peringkat dalam proses perkembangan tumor (tumorigenesis). Sejumlah 162 sampel tisu yang diproses dalam bentuk *formalin fixed paraffin embedded* (FFPE) digunakan. Daripada jumlah ini, 57 telah disahkan negative daripada sebarang symptom patologi (normal), 23 disahkan gastritis kronik berkait dengan *Helicobacter pylori*, 42 gastritis kronik bebas *Helicobacter pylori* dan 40 tisu adenokarsinoma gastrik. Melalui kaedah skor separa kuantitatif *immunostain*, penurunan peratus immunostain positif bagi TGF-β1 dikesan dari sampel normal (49.1%), gastritis kronik (24.6%) dan adenokarsinoma (17.5%). Perubahan ini dibuktikan signifikan melalui analisa statistik dengan nilai p kurang daripada 0.05 pada setiap peringkat perkembangan. Walaubagaimanapun ekspresi SMAD2 adalah berlawanan kerberkaitan di mana sampel yang menunjukkan tahap positif tinggi meningkat dari sampel normal (5.3%) kepada gastritis kronik (7.7%) dan gastrik adenokarsinoma (16.2%). Sungguhpun mencatatkan peningkatan peratusan, hanya satu pasang kumpulan (normal, gastritis kronik) menunjukkan peningkatan signifikan berdasarkan ujian berpasang Mann Whitney. Tambahan pula, tiada sebarang korelasi ekspresi TGF-β- Smad2 dikesan apabila diuji dalam sampel sama menggunakan ujian korelasi Spearmann. Bagi RUNX3, kajian mendapati kesemua jenis tisu gastrik menunjukkan ekspresi negatif walaupun terdapat segelintir sel limfosit yang menunjukkan kesan positif dalam sesetengah sampel. Sebagai

kesimpulan, disebabkan perubahan signifikan dalam ekspresi TGF-β dan SMAD2 dari keadaan normal kepada keradangan, kami laporkan bahawa protein-protein ini berpotensi untuk dijadikan *biomarkers* bagi mengesan keradangan.

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I certify that a Thesis Examination Committee has met on 28 January 2016 to conduct the final examination of Ahmad Zharif bin Ismail on his thesis entitled "Protein Expression of TGF-β, SMAD2 and RUNX3 in Normal Stomach, Chronic Gastritis and Gastric Adenocarcinoma" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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LIST OF ABBREVIATIONS

µm	Micrometer
CagA	*Cytotoxin-associated gene A*
CIC	Cancer-initiating cells
C_{power}	Constant defined by chosen P value and power
CRC	Clinical Research Centre
CTGF	Connective tissue growth factors
DAB	3,3'-Diaminobenzidine
DNA	Deoxyribonucleotide acid
EGJ	Esophagogastric junction
FDA	Food and Drug Administration
FFPE	Formalin-fixed paraffin embedded
GIST	Gastrointestinal stromal tumor
HIER	Heat induced epitope retrieval
hTERT	Human telomerase reverse transcriptase
IARC	International Agency for Research on Cancer
IL	*Interleukin*
iNOS	Inducible nitric oxide synthases
LAP	Latent associated protein
LLC	Large latent complex
LOH	Loss of heterozygosity
LTBP	Latent TGF-β binding protein
MH	Mad homology
MHC	Major Histocompatibility Complex
NIH	National Institute of Health
RUNX	Runt-related transcription factor
SARA	SMAD Anchor for Receptor Activation
SLC	Small latent complex

TBS	Tris-Buffered Saline
TGF-β	Transforming Growth Factor Beta
TNF	*Tumor necrosis factor*
VacA	Vacuolating cytotoxin
VEGF	Vascular endothelial growth factor
WHO	World Health Organization
HCC	Hepatocellular carcinoma

CHAPTER 1

INTRODUCTION

1.1 Background

Tumor or neoplasm refers to progeny of cells derived from a single faulty cell with the potential to proliferate indefinitely. Successive proliferation of these cells would later give the ability to invade surrounding tissues, a phenomenon commonly termed as metastasis (Kinzler and Vogelstein, 2002). Tumor classifications are term in accordance to the origin site of development: epithelial origin (carcinoma), mesenchymal origin (sarcoma) and hematopoietic (lymphoma). Process of tumor formation (tumorigenesis) has been outlined classically to develop in a multistep manner on both histological and molecular level. At histological level, tumor progresses from normal, to neoplastic precursors which includes metaplasia and dysplasia before setting into neoplastic condition (Cohen, 2002) while at molecular level, tumorigenesis are arranged in particular sequence: initiation, promotion and progression (Becker *et al*., 2006).

An epidemiology report of the World's Health Organization (WHO) in 2012, documented gastric cancer as third most common cancer types (Stewart and Wild, 2014). Distinct pattern of gastric cancer incidents show differences in accordance to geographic distribution where Asian countries were recorded to have the highest rate of incidence in comparison to their western counterparts. In terms of gender and age, male individuals make up twice to the number of female patients and elderly are at higher risk (Hohenberger and Gretschel, 2003). Due to its unapparent nature of symptoms manifestation in early development stages, mortality rate continues to rise hence further complicates management of the disease. Therefore effective detection method is crucial as invasive surgery such as endoscopy for early screening is costly and labor intensive. For local cases in Malaysia, Kandasami *et al*., 2003 reported that 82% of gastric cancer cases in Malaysia were diagnosed at the final stage which translates into poor prognosis for curability.

One potential alternative approach to such issue is to search for suitable biomarkers where molecular causes for a particular tumorigenic pathology are discerned and applied for clinical detection, therapy and monitoring. Unlike malignancy of hematopoietic origin (lymphoma) where tumor samples are readily extracted from peripheral blood, selection and clinical application of ideal tumor biomarkers for solid tumors impose several challenges. One such challenge is obtaining additional tissue samples post treatment for tumor progression assessment (Sanders, 2008). Therefore conventional method of withdrawing tumor would heavily rely on random sampling of rare

circulating tumor cells in peripheral blood where serum has to be withdraw in high volume for tumors to be detected efficiently (Shaffer *et al*., 2007; Cristofanilli *et al*., 2004). Furthermore the high risk of cancer gaining upper hand of metastasis and progressed to advanced stages defeat the purposes for early detection of biomarkers. Alternative to tumour cells sampling, profiling molecular composition (cytokines, hormones) alteration concomitant to carcinogenesis offers promising hope in search for potential biomarker candidate.

Transforming growth factor beta (TGF-β) have been extensively studied to have pleiotropic functions exhibiting both tumor suppressive and pro-oncogenic features at both extreme microenvironments; normal and tumor (Elliott and Blobe, 2005; Wakefield and Roberts, 2002). However not many study are done on its role in intermediate stages of multistep tumor progression model. In this study, gene products associated to TGF-β and two other downstream signaling molecules expressions will be studied. Expression and prognosis on each stage (normal tissues, inflamed tissues and tumor tissues) of tumor progression model are postulated based on established literatures. For example, by enumerating normal tissue samples expression of TGF-β as basal expression, tumors are expected to gain upper hand in malignancy if higher TGF-β expression is detected in tumor group samples. In intermediate grey stages such as chronic gastritis, conclusion can be drawn based on statistical analysis correlation, as to whether group of chronic gastritis samples have higher statistical expression correlation to tumor or normal sample group. Concurrently, distinct expression or deviations from normal group expression of any TGF-β-associated downstream proteins such as SMAD2 and RUNX3 render potentiality as biomarker candidate for tumor detection.

1.2 Problem Statement

Due to its nature of unapparent symptoms, current technologies for detecting early formation of gastric cancer are limited to costly and invasive endoscopic procedure. With identification of potential biomarkers, hindrance of such problem is expected to be alleviated for improved disease management in early detection. Exhibiting opposing roles in both extreme phases in multistep tumorigenesis model, TGF-β and its downstream associated products including SMAD2 and RUNX3, documented to have underpinning roles in carcinogenesis are potential candidates for predicting onset of tumor formation.

1.3 Research Objectives

General Objective

To determine difference in expression of TGF-β signaling pathway and postulate its effects in gastric oncogenesis as potential future biomarker study.

Specific Objectives

1. To determine the expression of TGF-β1, SMAD2 and RUNX3 protein in normal stomach, chronic gastritis and gastric adenocarcinoma by immunohistochemistry.

2. To correlate the expression of TGF-β1, SMAD2 and RUNX3 protein within samples of normal stomach, chronic gastritis and gastric adenocarcinoma.

3. To correlate the expression of TGF-β1, SMAD2 and RUNX3 protein in chronic gastritis which includes (*H. pylori* and non *H. pylori*) and intestinal and diffuse for adenocarcinoma.

4. To correlate the expression of TGF-β1, SMAD2 and RUNX3 with demographic factors in normal stomach, chronic gastritis and gastric adenocarcinoma.

1.4 Research Hypothesis

1. TGF-β1 and its downstream proteins; SMAD2 and RUNX3 expression is detected in normal, chronic gastritis and gastric adenocarcinoma.

2. TGF-β1 and its downstream proteins; SMAD2 and RUNX3 should demonstrate significant difference of expression in normal, chronic gastritis and gastric adenocarcinoma.

3. TGF-β1, SMAD2 and RUNX3 expressions are observed in types of *H. pylori* and non *H. pylori* gastritis and diffuse and intestinal adenocarcinoma samples.

4. TGF-β1, SMAD2 and RUNX3 profile expression vary according to demographic factors.

1.5 Conceptual Framework

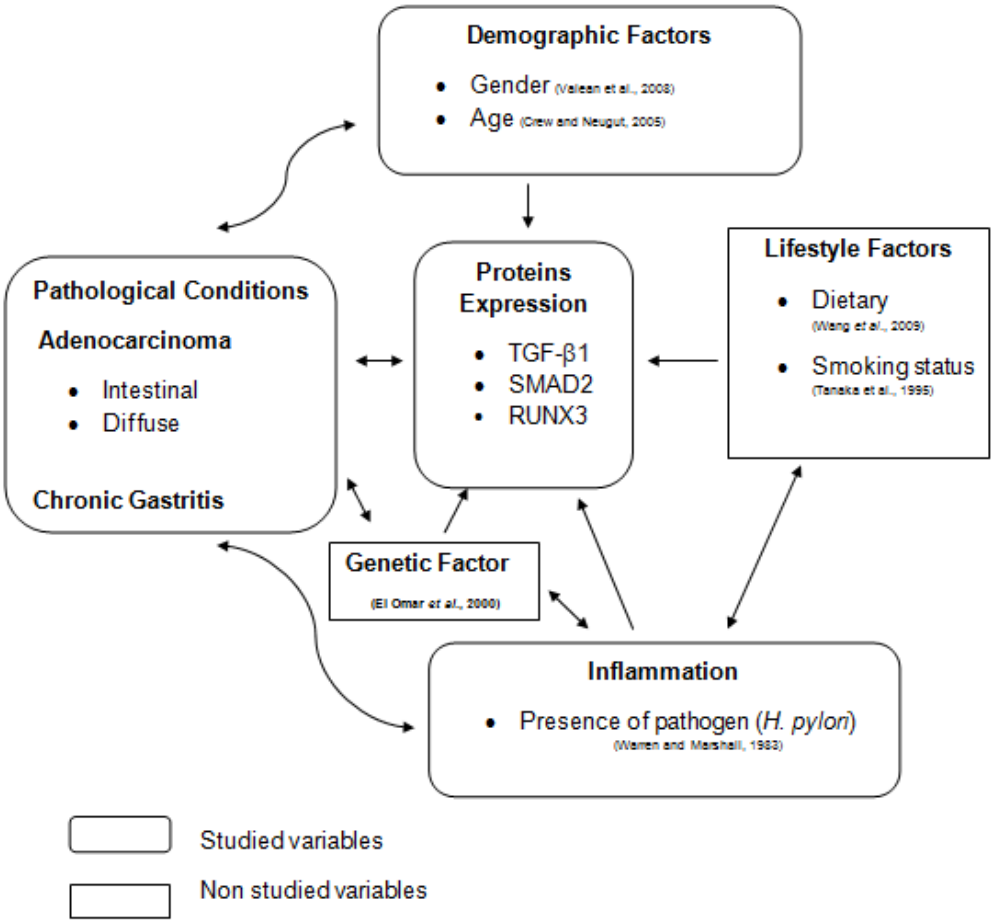

Figure 1.1 Conceptual framework of the study

1.6 Significance of study

Though there have been reports of sluggish growth of biomarker discovery for novel, reliable clinical application (Rifai *et al*., 2006), researchers have maintained optimistic of this field for the refinements made in techniques of study and technological advances achieved. One of the proven effective ways suggested for enhancing the reliability (specificity and sensitivity) of biomarkers for detecting onset of pernicious pathological conditions is to employ markers in set of panels rather than singling to just one for clinical setting (Mor *et al*., 2005; Xiao *et al*., 2005).

Despite all these hurdles on unraveling the potential mysteries, to date there are few established Food and Drug Administration, FDA-approved biomarkers used for early detection of plethora of cancers with varying specificity and sensitivity with none has yet to be developed for gastric cancer (Polanski and Anderson, 2007). Though this study does not delve into detail of biomarkers expression in blood plasma or other convenient sites for test sampling for clinical feasibility, it is hoped that the findings of *in situ* gastric expression would provide the preliminary step towards developing the goal.

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