GENETIC ASSOCIATION STUDY OF TUMOUR NECROSIS FACTOR POLYMORPHISMS IN CHRONIC RHINOSINUSITIS WITH AND WITHOUT NASAL POLYPS

By

DR KHAIRUNNISAK BINTI MISRON

Dissertation Submitted In
Partial Fulfillment Of The
Requirements For The Degree Of
Master Of Medicine
(Otorhinolaryngology – Head And Neck Surgery)

UNIVERSITI SAINS MALAYSIA
2016

ACKNOWLEDGEMENT

In the Name of Allah, the Most Compassionate, the Most Merciful. Alhamdulillah I managed to complete my dissertation. All these efforts would never been possible without the support and guidance of various people in my life.

First and foremost, I would like to gratefully and sincerely thank my supervisor, Prof Dr Suzina binti Sheikh Ab Hamid, a lecturer in Department of Otorhinolaryngology - Head and Neck Department, School of Medical Sciences, Universiti Sains Malaysia, for her guidance, understanding, patience and most importantly, her friendship during my graduate study in Universiti Sains Malaysia. Her mentorship was paramount in providing a well-rounded experience consistent my long-term career goals.

I would also like to thank my co-supervisors, Dr Azlina binti Ahmad, a senior lecturer in Basic Science and Oral Biology Unit, School of Dental Sciences, Universiti Sains Malaysia and Dr Ramiza Ramza bin Ramli, a senior lecturer in Department of Otorhinolaryngology - Head and Neck Department, School of Medical Sciences, Universiti Sains Malaysia for their assistance and guidance in the completion of my dissertation paper. My appreciation to Dr Kueh Yee Cheng from Department of Biostatistic for guiding me in methodology and statistical analysis.

Not to forget, the lecturers and colleagues at Otorhinolaryngology - Head and Neck Department, School of Medical Sciences, Universiti Sains Malaysia for the friendship, hardwork, expertise and patience. Special thanks to the staffs at Craniofacial Laboratory, School of Dental Sciences, Universiti Sains Malaysia in helping me for DNA analysis throughout this study.

Finally and most importantly, I would like to thank my parents and my siblings. Their support, encouragement and unwavering love were undeniably the bedrock upon which the past four years of my life have been built.

TABLE OF CONTENTS **PAGE**

LIST OF ABBREVIATIONS

A	Adenosine
ASA	Acetylsalicylic acid
bp	Base pair
C	Cytosine
CRS	Chronic rhinosinusitis
CRSsNP	Chronic rhinosinusitis without nasal polyp
CRSwNP	Chronic rhinosinusitis with nasal polyp
CT	Computed tomography
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
ECP	Eosinophilic cationic protein
EPOS	European Position Paper on Rhinosinusitis and Nasal Polyps
et al	And other people
FoxP3	Forkhead box P3
G	Guanine
GA2LEN	Global Allergy and Asthma Network of Excellence
HLA	Human leukocyte antigen
ICAM	Intracellular cell adhesion molecule
IFN	Interferon
IL	Interleukin

LB	Lithium borate
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RANTES	Regulate on activation normal T cell expressed and secreted
RFLP	Restriction fragment length polymorphism
SNPs	Single nucleotide polymorphisms
T	Thymine
TGF	Transforming growth factor
Th	T helper cell
TIMP	Tissue inhibitor of matrix metalloproteinase
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
Treg	T regulatory cell
USM	University Sains Malaysia
VCAM	Vascular cell adhesion molecule

LIST OF TABLES **PAGE**

LIST OF FIGURES **PAGE**

ABSTRAK

Rinosinusitis kronik adalah penyakit radang kronik yang mengawal proses keradangan menyebabkan penghasilan pelbagai sitokin prokeradangan. Faktor nekrosis tumor adalah salah satu sitokin prokeradangan yang memainkan peranan penting dalam pathogenesis rinosinusitis kronik.

Kajian kes control ini bertujuan untuk mengenalpasti kewujudan dan perkaitan polimorfisma gen TNFα -1031 dan TNFβ +252 di antara rinosinusitis kronik dan kontrol yang sihat, seterusnya di antara rinosinusitis kronik yang mempunyai dan tidak mempunyai polip hidung. Selain itu, kajian ini ingin menyelidik perkaitan di antara gen-gen ini dan factor-faktor berkaitan dengan rinosinusitis kronik.

Kajian ini melibatkan 48 peserta yang menghidap rinosinusitis kronik yang terdiri dari 24 peserta rinosinusitis kronik dengan polip hidung dan 24 peserta rinosinusitis kronik tanpa polip hidung, beserta 48 kontrol yang sihat. Sampel ujian asid deoksiribonuklik diambil dari mukosa dinding pipi. Seterusnya, kaedah mengekstrak gen telah dijalankan pada sampel ini melalui kaedah 'Polymerase Chain Reaction' dan 'Restriction Fragment Length Polymorphisms'. Analisis statistik telah dilakukan menggunakan ujian 'Chi-square' atau 'Fisher's exact' dan 'multiple logistic regression' untuk menentukan perkaitan polimorfisma gen TNFα -1031 dan TNFβ +252 dengan rinosinusitis kronik.

Kajian ini mengesahkan kewujudan polimorfisma gen TNFα -1031 di mana homozigot normal (TT) dan heterozigot mutan (TC) mempunyai pengagihan sama rata di antara peserta rinosinusitis kronik dan kontrol serta di antara rinosinusitis kronik yang mempunyai dan tidak mempunyai polip hidung. Homozigot mutan (CC) tidak wujud dalam populasi kami. Di samping itu, distribusi alel normal (T) dan alel

mutan (C) juga seimbang. Berkenaan polimorfisma gen TNFβ +252, heterozigot mutan (AG) merupakan genotip yang paling banyak dijumpai berbanding homozigot normal (AA) dan homozigot mutan (GG). Alel normal (A) dan alel mutan (G) mempunyai distribusi yang lebih kurang sama. Analisis mendapati genotip dan alel polimorfisma gen TNFα -1031 dan TNFβ +252 tidak menunjukkan perkaitan di antara rinosinusitis kronik dan kontrol yang sihat, seterusnya di antara rinosinusitis kronik yang mempunyai dan tidak mempunyai polip hidung. Walau bagaimanapun, polimorfisma gen TNFα -1031 menunjukkan manfaat signifikan pada peserta rinosinusitis kronik yang mempunyai atopi (p-value = 0.037) tetapi tidak pada asma dan ketidaktoleransi aspirin. Tiada manfaat signifikan yang diperhatikan di antara polimorfisma gen TNFβ +252 dengan faktor-faktor berkaitan rinosinusitis kronik.

Kesimpulannya, kajian ini menunjukkan kewujudan polimorfisma gen TNFα -1031 dan TNFβ +252 tidak menunjukkan sebarang manfaat signifikan di antara rinosinusitis kronik dan kontrol, seterusnya di antara rinosinusitis kronik yang mempunyai dan tidak mempunyai polip hidung. Walaubagaimanapun, kajian ini mengusulkan bahawa kewujudan perkaitan di antara polimorfisma gen TNFα -1031 mempunyai risiko yang lebih tinggi kepada pesakit rinosinusitis kronik yang mempunyai atopi.

ABSTRACT

TITLE: GENETIC ASSOCIATION STUDY OF TUMOUR NECROSIS FACTOR POLYMORPHISMS IN CHRONIC RHINOSINUSITIS WITH AND WITHOUT NASAL POLYPS

Chronic rhinosinusitis (CRS) is a chronic inflammatory condition which initiates the cascade of inflammatory responses resulting in production of various proinflammatory cytokines. TNF is one of the proinflammatory cytokines that has crucial role in the pathogenesis of CRS.

This case-controlled study aimed to identify the presence and associations of TNFα -1031 and TNFβ +252 gene polymorphisms between CRS and healthy controls as well as between CRSwNP and CRSsNP. Another purpose of this study was to investigate the associations of these genes polymorphisms with factors related to CRS.

Forty eight CRS participants which comprised of 24 CRSsNP and 24 CRSwNP participants together with 48 healthy controls were enrolled in this study. All DNA samples were collected from buccal mucosa and subsequently, genotyped for TNFα -1031 and TNFβ +252 genes by mean of polymerase chain reaction (PCR) and restriction fragment length polymorphisms (RFLP). The statistical analysis were carried out using Chi-square Test or Fisher's exact test and multiple logistic regression to determine the associations of TNFα -1031 and TNFβ +252 gene polymorphisms in CRS with and without nasal polyps and risks of CRS.

Our findings confirmed the presence of TNFα -1031 gene polymorphisms in which the homozygous wild-type (TT) and heterozygous mutant-type (TC) were

evenly distributed between CRS and healthy controls as well as in CRSsNP and CRSwNP. Homozygous mutant-type (CC) was absent in our population. Similarly, the wild-type allele (T) and mutant allele (C) revealed balance distributions. As for TNFβ +252 gene polymorphisms, the heterozygous mutant-type (AG) was identified to be more prevalent in comparison to homozygous wild-type (AA) and homozygous mutant-type (GG). The wild-type allele (A) and mutant-type allele (G) distributed uniformly. Statistical analysis of genotype and allele frequencies of TNFα -1031 and TNFβ +252 gene did not show any significant associations between CRS and healthy controls as well as between CRSwNP and CRSsNP. However, a significantly statistical difference of TNFα -1031 was observed in CRS participants with atopy (p-value = 0.037) but not asthma and ASA intolerance. There were no significant associations of TNFβ +252 gene polymorphisms with factors related to CRS.

In conclusion, the presence of TNFα -1031 and TNFβ +252 gene polymorphisms in current study did not render any significant associations between CRS and control as well as CRSsNP and CRSwNP. However, this study suggests that the presence of TNFα -1031 gene polymorphisms in CRS patients with atopy may be associated with increase susceptibility towards CRS.

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Chronic rhinosinusitis

1.1.1 Epidemiology

Chronic rhinosinusitis (CRS) is one of the commonest upper respiratory tract diseases encountered by Otorhinolaryngologists as well as primary care physicians. The accurate prevalence of CRS worldwide is unknown. The Global Allergy and Asthma Network of Excellence (GA2LEN) conducted a survey based on European Position Paper on Sinusistis (EPOS) criteria in 19 centres in Europe involving 57128 adults aged 15 to 75 years old. They found that the prevalence of CRS by EPOS criteria was 10.9%. This survey also mentioned that prevalence of self-reported physician-diagnosed CRS within these centres was substantially related to the EPOS diagnosed CRS (Hastan *et al.*, 2011). Another survey done by the Korea National Health and Nutrition Examination Survey reported the overall prevalence of CRS was 6.95% (Kim *et al.*, 2011b). A comparative study in north of Scotland and Southern Caribbean found similar prevalence of CRS among patient who attended ORL clinics, which were 9.6% and 9.3%, respectively (Ahsan *et al.*, 2004).

The prevalence of CRS worldwide is more likely to be overestimated. It is because most of the researchers are focusing mainly on the clinical presentations of CRS without considering nasoendoscopic and Computed Tomography (CT) scan findings. However, studies showed there were poor associations between symptoms and nasoendoscopic or CT scan features among CRS patients (Stankiewicz and Chow, 2002).

In contrary, the prevalence of nasal polyps seems to be underestimated. It is owing to the need of endoscopic examination to assess the existence of nasal polyps inside the nasal cavity. The prevalence of nasal polyps was estimated approximately 2.7% in Sweden (Johansson *et al.*, 2003). However, in an examination of autopsies, the occurrence of nasal polyps was found to much higher where nasal polyps was detected in 22 out of 69 autopsies (Larsen and Tos, 2004).

CRS and nasal polyps were found to be more common among male than female in Sweden and Korea (Johansson *et al.*, 2003; Kim *et al.*, 2011b). However, a study among Canadians with CRS reported female is more susceptible towards CRS than male (Chen *et al.*, 2003). The prevalence of CRS and nasal polyps were also higher with increasing age (Chen *et al.*, 2003; Johansson *et al.*, 2003).

Studies have shown that CRS has significant socioeconomic impact as well as impairment in patients' quality of life. It has been estimated that the cost for ongoing treatment for CRS in the United States to be approximately USD2449 per patient but it has dropped markedly following endoscopic sinus surgery. However, the cost related to surgical procedure is still significantly expensive (Bhattacharyya *et al.*, 2011). Similarly, another study revealed the impact of CRS on insurance status, work absenteeism and resource use among race or ethnic group in United States (Soler *et al.*, 2012).

1.1.2 Definition and classification

CRS is recognized as a chronic inflammatory disease affecting nasal and paranasal sinuses mucosa. The terminology of rhinosinusitis is being used instead of

sinusitis alone because rhinitis and sinusitis usually occur concurrently (Bachert *et al.*, 2003; Chan and Kuhn, 2009; Fokkens *et al.*, 2012).

The European Position Paper on Rhinosinusitis and Nasal Polyps 2012 defined CRS as an inflammation involving nose and paranasal sinuses that occur 12 weeks or more which characterised by two or more symptoms, one of which should be either nasal blockage, nasal congestion, or nasal discharge. Other symptoms include presence or absence of facial pain or reduction of smell. The diagnosis must be supported by either positive nasoendoscopic findings or CT scan changes. The endoscopic signs include presence of either nasal polyps or mucopurulent discharge or mucosal obstruction primarily in the middle meatus. Similarly, the positive CT scan findings include mucosal changes within the osteomeatal complex or sinuses (Fokkens *et al.*, 2012).

In European Position Paper on Rhinosinusitis and Nasal Polyps 2012, CRS is subclassified into two groups, which are chronic rhinosinusitis without nasal polyp (CRSsNP) and chronic rhinosinusitis with nasal polyp (CRSwNP) (Fokkens *et al.*, 2012). This classification indicates nasal polyps as one of the subset of CRS. It reflects that nasal polyps cannot occur without concurrent inflammation of nasal and paranasal sinuses mucosa.

Both CRSsNP and CRSwNP present with similar symptoms but the diagnosis of nasal polyp are made based on nasoendoscopic findings in majority of cases unless the nasal polyp is very extensive and able to be visualised during anterior rhinoscopy. As for nasal polyp, it is further graded based on the endoscopic appearance (Lund, 1995). According to this classification, Grade I is when the polyp is restricted to middle meatus, Grade II is when polyp is beyond middle meatus, while Grade III is massive polyp seen in the nasal cavity.

1.1.3 Factors associated with CRS

Despite being one of the most prevalent diseases worldwide, the pathogenesis of CRSsNP and CRSwNP is still not well understood and appears to be multifactorial. Blockage of paranasal sinus ostia is thought to be the key to the inflammatory response in CRS. Mucosal oedema following inflammation results in obstruction of the paranasal sinuses ostia which eventually leads to retention of secretion and impaired sinus ventilation. This condition creates acidic and anaerobic environment within the sinuses. As a result, it enhances the immune reaction and infection of sinuses and thus promotes development of CRS (Naclerio and Gungor, 2001).

The role of atopy in the development of CRS has been discussed extensively in the literature (Chen *et al.*, 2003; Houser and Keen, 2008; Pearlman *et al.*, 2009). In addition, the association between allergic rhinitis and CRS patients had also been described in previous studies (Houser and Keen, 2008; Kim *et al.*, 2011b). Approximately half of the patients with CRS have positive skin prick test (Pearlman *et al.*, 2009). The prevalence of positive skin prick test was reported even higher in CRS patient who failed medical treatment as compared to the general population (Shkoukani and Krouse, 2013). These findings suggest that allergic inflammation of nasal mucosa plays a vital role in the development of CRS. It owes to the swelling of nasal mucosa which leads to narrowing of the sinus ostia and retention of mucous within sinuses.

Asthma has been implicated as one of the factors that contribute to the susceptibility towards development of CRS. Due to awareness of 'one airway, one disease' concept, many studies have been conducted to observe the relationship between CRS and asthma (Chen *et al.*, 2003; Johansson *et al.*, 2003). GA2LEN

survey in 12 European countries showed that there was a high preponderance of asthma in CRS patients with the odd ratio of 3.47 and the association was even stronger in patients who had concurrent allergic rhinitis in which the odd ratio was estimated to be 11.85 (Jarvis *et al.*, 2012). A study on 553 Japanese had shown that the prevalence of CRS in asthmatic patients was 23.1% (Yoshimura *et al.*, 2001). Moreover, the incidence of nasal polyps is also likely to occur in asthmatic than non asthmatic patients (Pearlman *et al.*, 2009). These findings suggest that the upper and lower airway diseases form an integrated airway syndrome model because the nasal, paranasal sinuses and bronchus are a continuous structures that are lined by ciliated columnar epithelium (Vinuya, 2002; Togias, 2003).

Aspirin sensitivity has been recognised as one of the associated factors in nasal polyps. Previous study demonstrated 36.0% of patients with aspirin intolerance had concurrent nasal polyps (Settipane, 1996). However, more recent studies reported contradicting data regarding this association among Skovde population in Sweden (Johansson *et al.*, 2003). Another study among Chinese population also documented low prevalence of non steroidal anti inflammatory drugs intolerance in CRS patients (Lu *et al.*, 2014). The possible explanation for this poor association is the rarity of aspirin usage in clinical practice nowadays. Hence, the association between aspirin intolerance and CRS may be underestimated in many of the studies.

Many studies have pointed smoking as another important predisposing factor for the development of CRS. A number of studies corroborated strong association of cigarette smoking among CRS and nasal polyps patients (Chen *et al.*, 2003; Houser and Keen, 2008; Hastan *et al.*, 2011). The prevalence of CRS was reported to be higher in not only among active smokers but also in passive smokers (Reh *et al.*, 2012). Cigarette smoke, either actively or passively inhaled is thought to cause

alterations in respiratory epithelium which subsequently impair the mucociliary activity along the respiratory tract. However, a systematic review on the effects of smoking and CRS found out that there might be individual susceptibility in the development of CRS among smokers (Tamashiro *et al.*, 2009).

Genetically determined susceptibility undoubtly plays an important role in the development of CRS. Supportive evidence for genetic basis of nasal polyp was proposed by Qu *et al.* (2007) where they found that first degree and second degree relatives have higher chances to develop nasal polyp as compared to control group. Similarly, it has been reported identical twins have similar chances to develop nasal polyps despite different social lifestyles (Drake-Lee, 1992). At present, some efforts have been focusing on genetic studies of both CRSsNP and CRSwNP which are believed to have promising correlation that might contribute to the pathogenesis of this disease (Hsu *et al.*, 2013).

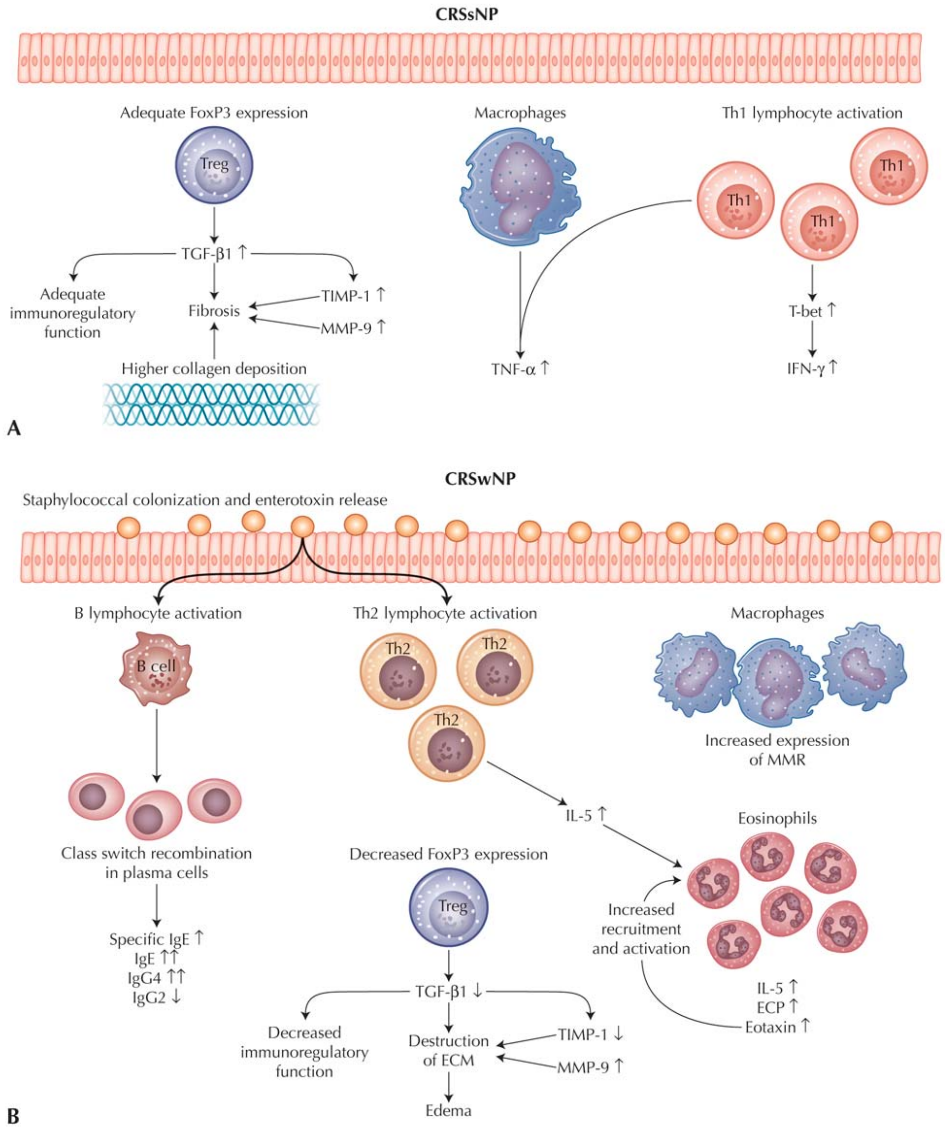

Figure 1.1: Inflammatory process in CRSsNP and CRSwNP. ECM, extracellular matrix; ECP, eosinophilic cationic protein; FoxP3, forkhead box P3; IFN, interferon; IL, interleukin; MMP, matrix metalloproteinase; MMR, macrophage mannose receptor; T-bet, T-box transcription factor; TGF, transforming growth factor; Th, T helper cell; TIMP, tissue inhibitor of matrix metalloproteinase; TNF, tumour necrosis factor; Treg, T regulatory cell.

(Adapted from Huvenne *et al* (2009). Chronic Rhinosinusitis With and Without Nasal Polyps: What Is the Difference? *Curr Allergy Asthma Rep*, **9(3)**, 213-220.)

1.2 Molecular biology of CRS

1.2.1 Inflammatory process in CRS

Inhalation of noxious substances such as bacteria, viruses, fungi and allergens result in alteration of respiratory epithelium. This mucosal irritation of airway epithelium triggers the initial mucosal event of inflammatory process in CRS. Subsequently, T lymphocytes are activated in response to inflammation both in CRSsNP and CRSwNP (Figure 1.1). However, the distinct molecular biologic event in CRSsNP and CRSwNP suggest that these two conditions have separate entities. The differences between CRSsNP and CRSwNP are summarized in Table 1.1.

T helper 1 (Th1) and T helper 2 (Th2) cells recognise major histocompatibility complex Class II (MHC II) that bind on cell surface of pathogen following inflammation. CRSsNP is characterised by Th1 as compared to CRSwNP which mainly involves Th2 polarisation (Huvenne *et al.*, 2009; Crombruggen *et al.*, 2011; Eloy *et al.*, 2011). Th1 and Th2 cells commitments are specified by T box transcription factor (Tbet) and GATA binding protein (GATA-3), respectively. Another subpopulation of T cell, regulatory T cell (Treg) which is characterised by forkhead box protein 3 (FoxP3) responsible in modulating inflammatory process in chronic illnesses. It has been postulated that Treg can migrate to the site of inflammation and regulate the Th1 and Th2 cells (Umetsu and DeKruyff, 2006).

Inflammation mediated by Th2 is the main feature in CRSwNP (Ramanathan *et al.*, 2008). Th2 cells stimulate the release of cytokines mainly IL5. These cytokines promote the recruitment of eosinophils and release of eosinophilic cationic protein (ECP). ECP results in epithelial damage, oedema formation and albumin deposition.

Table 1.1: Immunobiologic phenomena and histological features of CRSsNP and CRSwNP

	CRSsNP	CRSwNP
T cell profile	Th1	Th2
Inflammatory cell profile	Mainly neutrophils, ↓eosinophils and mast cells	Mainly eosinophils, ↓B and T cell
Proinflammatory cytokines	TNFα, TNFβ, IL1β, IFNγ	IL4, IL13, TNFα
Endothelial adhesion molecules	↑ICAM-1	↑VCAM-1
Transcription factors	Adequate FoxP3, ↑Tbet	↓FoxP3, ↑GATA-3
Extracellular matrix proteins	↑MMP-9, ↑TIMP-1	↑MMP-9, ↓ TIMP-1
Histological features	Limited subepithelial oedema, prominent collagen deposition and fibrosis	Epithelial damage, tissue oedema, pseudocyst formation containing albumin, thickened basement membrane, less fibrosis

↑, increase; ↓, decrease

Abundance of IgE antibodies to *Staphylococcus aureus* are found in CRSwNP as compared than in CRSsNP patients suggest that nasal polyps involve eosinophilic inflammation (Bachert *et al.*, 2001). Thus, it hypothesises the potential effects of superantigen in the pathogenesis of CRSwNP (Wang *et al.*, 2008). Staphylococcal

superantigens activate both T and B cells with multiclonal IgE formation, thus eliciting massive inflammatory reaction in CRSwNP. In comparison, neutrophilic inflammation is more prominent in CRSsNP that shows increased Th1 cells polarisation.

CRSwNP is also associated with significant down regulation of forkhead box P3 (FoxP3) (Bruaene *et al.*, 2008). Reduction in FoxP3 level signifies the deficiency in Treg cells function in patients with CRSwNP and thus, results in low transforming growth factor β1 (TGFβ1) (Sejima *et al.*, 2012; Chuan *et al.*, 2013). TGFβ1 is important in controlling initiation and resolution of inflammatory responses. Lack of the TGFβ1 contributes to massive oedema formation and deficient of collagen formation in CRSwNP. On the other hand, increase TGFβ1 levels and adequate FoxP3 expression in CRSsNP may explain the excessive collagen deposition and fibrosis formation in this condition with minimal tissue oedema.

The remodeling process in CRS is characterised by alteration in the extracellular matrix (ECM) which is also played by transforming growth factor β1 (TGFβ1). It stimulates the balance between matrix metalloproteinase-9 (MMP-9) and tissue inhibitor of matrix metalloproteinase-1 (TIMP-1). TIMP-1 plays a role in preventing enzymatic breakdown of ECM. It has been demonstrated that MMP-9 and TIMP-1 are upregulated in CRSsNP while in CRSwNP only MMP-9 is upregulated but not TIMP-1 (Watelet *et al.*, 2004). The imbalance between MMP-9 and TIMP-1 in CRSwNP leads to ECM destruction and albumin accumulation which subsequently results in tissue oedema and pseudocyst formation. These distinguished immunological features showed that CRSwNP exhibit more severe inflammatory process than CRSsNP.

Recent literature demonstrated different inflammatory pattern among Asian patients with CRSwNP, whereby Th17 cells predominate in this population as compared to whites, which amplifying neutrophilic inflammation rather than eosinophilic (Zhang *et al.*, 2008). Similarly, another study in Malaysia also reported increase prevalence of neutrophilic nasal polyps based on histopathological findings (Tikaram and Prepageran, 2013).

1.2.2 Proinflammatory cytokines in CRS

The presence of proinflammatory cytokines has been advocated in the development of CRS. Although the precise molecular mechanisms by which the proinflammatory cytokines can induce mucosal response in CRS is still not well established, there are some evidences that show these cytokines may play roles in the disease process (Min and Lee, 2000; Perić *et al.*, 2010; Sejima *et al.*, 2012).

The initial event that triggers the inflammatory process in CRSsNP is the bacterial colonisation at the nasal and paranasal sinuses mucosa. It has been shown that the predominant inflammatory cells being secreted are neutrophils, followed by little contributions from macrophages, mast cells, eosinophils and basophils which are capable of releasing cytokines mainly tumour necrosis factor particularly tumour necrosis factor alpha (TNFα) and less commonly tumour necrosis factor beta (TNFβ) as well as interleukin 1 beta (IL1β) and interferon gamma (IFNγ) (Stierna, 2001; Pawankar and Nonaka, 2007). These cytokines upregulate the endothelial adhesion molecule namely intracellular cell adhesion molecule-1 (ICAM-1) (Eloy *et al.*, 2011). Consequently, there is influx of inflammatory cells in chronically inflamed tissue, thus enhancing the inflammatory response.

On the other hand, the mechanisms promoting the accumulation of eosinophils in CRSwNP has been attributed by vascular cell adhesion molecule-1 (VCAM-1) (Hamilos, 2000; Bernstein, 2001; Pawankar and Nonaka, 2007). VCAM-1 participates in eosinophilic recruitment on nasal and paranasal endothelium. Activated mast cells facilitates the release of proinflammatory cytokines such as interleukin 4 (IL4), interleukin 13 (IL13) and TNFα (Pawankar and Nonaka, 2007; Otto and Wenzel, 2008). The activated mast cells also stimulate the upregulation of eotaxin and regulate on activation normal T cell expressed and secreted (RANTES), which subsequently promotes further infiltration of eosinophils to the tissue (Bernstein, 2005). The upregulation of eosinophils in CRSwNP suggests that allergy and asthma might be implicated in the pathogenesis of CRSwNP.

1.2.3 Role of TNF as proinflammatory cytokines in CRS

Although various cytokines have been proven to play pivotal roles in pathogenesis of CRS, this study concentrated on area relating to tumour necrosis factor (TNF). TNF was first described by Carswell EA *et al* in 1975 where they found that endotoxin-induced serum in mice release a substance that can cause necrosis in tumour tissues (Carswell *et al.*, 1975). However, only in 1985, Aggarwal *et al.* (1985) was the first who defined two distinct types of TNF, which are TNFα and lymphotoxin or also known as TNFβ. TNFα is derived from macrophages with molecular mass of 17 kDa while TNFβ is produced by lymphocytes with molecular mass of 20 kDa (Aggarwal *et al.*, 2012). Both have 50 percent amino acids sequence identity, thus they share many similar functions.

Various biological functions of TNF have been described in the literatures. TNF is thought to promote inflammation by releasing cyclooxygenase 2. Both TNFα and TNFβ share same receptors eventhough they show discrete binding affinity to the receptors. Once the TNF molecules bind to their receptors, they induce signaling interaction within the target cells. TNF display two different receptors, which are tumour necrosis factor receptor 1 (TNFR1) and tumour necrosis factor receptor 2 (TNFR2). TNFR1 are found in most of cells in human, in contrast, TNFR2 are confined to cells of immune system, endothelial cells and nervous cells (Aggarwal *et al.*, 2012). TNFα exhibit both soluble and transmembrane ligands while TNFβ express as soluble ligands only.

The cell signaling is important in controlling the inflammatory process. In physiological state, TNF production is important for normal response towards inflammation. However, excessive accumulation of TNF in human body can be harmful. In some cases, TNF signaling promotes lymphocytes apoptosis in response to infection, while in certain cases, it stimulates lymphocytes proliferations to combat the inflammation. Because of contradicting role of TNF, it is essential to control the balance of this cell signaling. Once the TNF signaling lost its control, the inflammation overwhelm and resulting in serious diseases (Goodsell, 2006).

1.3 Single nucleotide polymorphisms (SNPs)

1.3.1 Definition

SNPs are one of the most common polymorphisms seen in human genome (Marian, 2010). SNPs are defined as genetic variation in deoxyribonucleic acid

(DNA) where a single nucleotide is being replaced by another, either by substitution or deletion as depicted in Figure 1.2 (Brooks, 2003). They exist in normal population and this allele variant has a frequency of one percent or greater. SNPs differ from mutation in which mutation occurs in less than one percent of the population and they cause more serious manifestation of diseases (Crawford and Nickerson, 2005).

The polymorphism in the DNA sequence is known as allele. SNP can be assigned as major or minor allele depending on its occurrence in the observed population. There are variations of SNP allele frequencies among different populations. Haplotype is referred to allelic combination because human is diploid (each chromosome is carried from mother and father). A pair of haplotypes contribute to several genotypes in SNP which are homozygous for major allele (homozygous wild-type), heterozygous (heterozygous mutant-type) and homozygous for minor allele (homozygous mutant-type) (Brooks, 2003; Guerra and Yu, 2005). The dominant allele is known as wild-type allele while rare allele recognised as mutant allele.

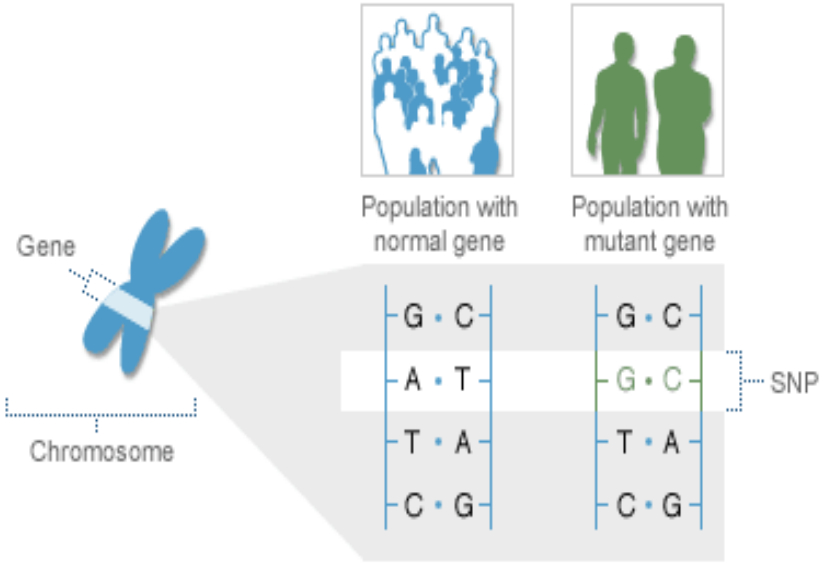

Figure 1.2: An example of SNPs. The polymorphism is denoted by substitution of nucleotide A to nucleotide G. A, adenosine; C, cytosine; G, guanine; T, thymine. (Adapted from https://www.celera.com/celera/cdx_discover).

1.3.2 SNPs and human diseases

The association of SNPs and human disease is complex. The genetic pathogenesis of certain common diseases is still not well understood and further research is warranted to identify the genetic architecture of these diseases. Approximately 6 million human SNPs are listed in the database of single nucleotide polymorphisms (dbSNP) (Wheeler *et al.*, 2004). It has been estimated that SNPs manifest about once every 1000 bases in human DNA sequence (Guerra and Yu, 2005). Identification of large number of SNPs across human genome has brought the attention of the genetic association studies with common human diseases in a population because common population shares similar genetic variances. This database has contributed to a very useful information in the field of research.

Perhaps common diseases that exist in an observed population are resulting from interaction of multiple genes and polymorphisms eventhough most of them exert little effects (Li *et al.*, 2012). Unlike single-gene disorders, SNPs result from the complex interplay between genetic and environmental factors (Brooks, 2003). The studies of SNPs have taken into account the genotype distributions and allele frequencies between cases (people who carry the desired disease to be studied) and controls (people who do not have the disease) (Fareed and Afzal, 2013).

In the past, family-based study is more popular in genetic association study. Nowadays, researchers are more interested in SNPs. However, this case-controlled study needs large number of population to determine the optimal association of SNPs and diseases in the population. It is also difficult to differentiate the association of SNPs with specific diseases because many SNPs are also related with other diseases. Apart from that, the researchers need to choose which gene or promoter region that are related to the disease (Bell, 2002; Crawford and Nickerson, 2005).

Despite the disadvantages mentioned above, SNPs still have value in genetic association studies of common diseases in certain population. This owes to the discovery of vast number of potential SNPs across human genome and it is more cost efficient as compared to other genetic studies (Cordell and Clayton, 2005). The alteration in genetic sequence are inherited from one generation to subsequent generations, thus studies on SNPs could be easier to be identified in the population. SNPs has become an effective tools in the genetic field because polymorphisms are likely to be involved in disease pathogenesis as well as targets for therapeutic interventions (Sripichai and Fucharoen, 2007).

1.4 Tumour necrosis factor (TNF) gene polymorphisms

1.4.1 TNF gene

The position of TNF gene has been established in the short arm of chromosome 6 which occupying the highly polymorphic domain of Class III major histocompatibility complex (MHC). Several polymorphic microsatellites have been recognized along this region, namely TNF a, b, c, d, e and f (Figure 1.3) (Hajeer and Hutchinson, 2000). Perhaps the involvement of TNF gene in various immune responses is because of its proximity to HLA gene in chromosome 6. Literature has shown that there is a linkage equilibrium between TNF microsatellites and HLA gene (Hajeer and Hutchinson, 2001). There are plentiful SNPs that have been classified in the TNFα promoter region at position (relative to transcription start region) -1031, -863, -857, -851, -419, -376, -308, -238, -162 and -49 (Hajeer and Hutchinson, 2001; Elahi *et al.*, 2009).

TNFα -1031 is one of the polymorphisms identified. Numerous studies have been carried out to determine the association between this polymorphic site and various inflammatory diseases. TNFα -1031 is a thymine (T) to cytosine (C) substitution. TNFα gene is postulated to be associated with increase in transcriptional activity and elevated circulating TNFα levels (Abraham and Kroeger, 1999; Majetschak *et al.*, 1999). This finding indicates that TNFα might be a beneficial genetic marker in future research especially in the area of inflammatory diseases.

TNFβ gene locus lies within first intron at position +252 in chromosome 6. The polymorphism in this region results from substitution of adenosine (A) to guanine (G). Evidence has demonstrated that TNFβ +252G allele is related with higher production of TNFβ expression at mRNA and protein assays. Another finding showed

that TNFβ +252A allele is correlated with elevated TNFβ as well as TNFα production (Messer *et al.*, 1991).

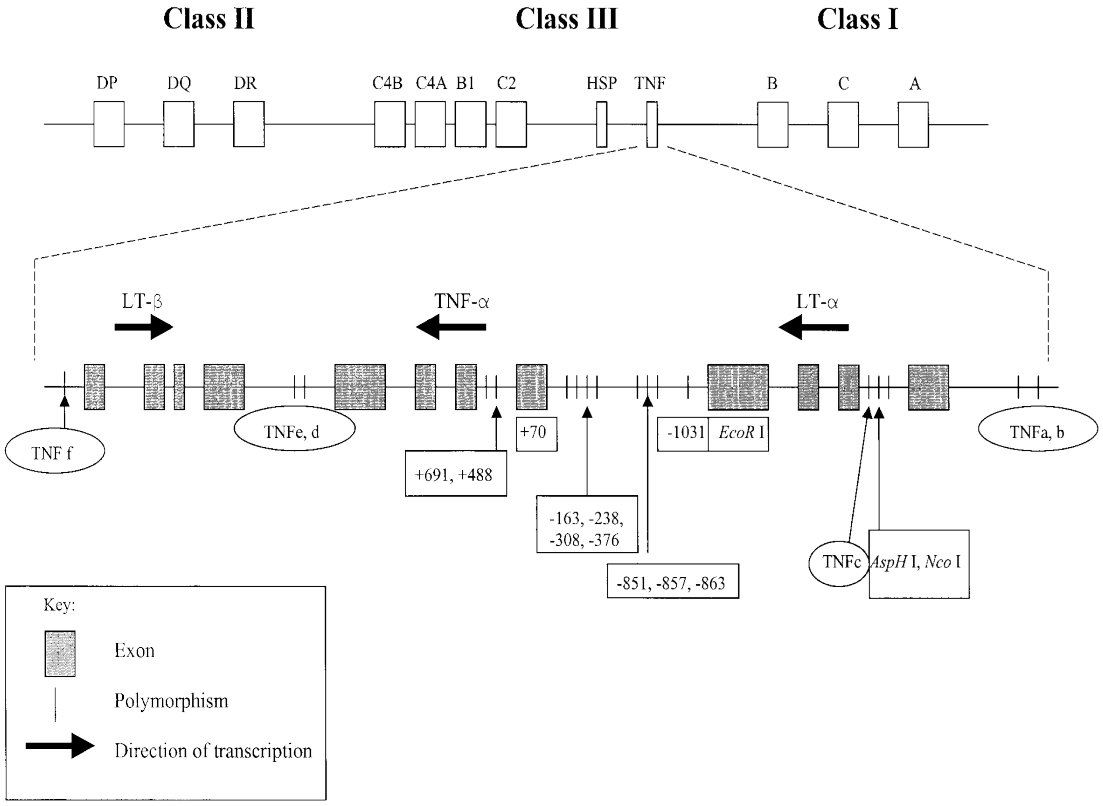

Figure 1.3: TNF gene locus on chromosome 6 depicting the position of microsatellites and SNPs.

(Adapted from Hajeer, A. H. & Hutchinson, I. V. (2001). Influence of TNF alpha gene polymorphisms on TNF alpha production and disease. *Hum Immunol*, **62**, 1191-1199).

1.4.2 SNP of TNF gene and human diseases

Numerous proinflammatory cytokine gene polymorphisms have been extensively studied in various infectious and inflammatory conditions as well as

cancer. The influence of TNF gene polymorphisms in many inflammatory diseases has been addressed in the literatures (Qidwai and Khan, 2011). Majority of the studies are focusing on whether these gene polymorphisms involve in the pathogenesis as well as prognosis of human diseases.

TNF gene composed of multiple sites of polymorphisms and these polymorphisms have shown to predispose individuals to certain diseases. Hence, the identification of such polymorphisms might suggest the role of TNF gene in establishing early diagnosis as well as treatment plan. The TNF gene polymorphisms have been associated with many chronic inflammatory conditions including rheumatoid arthritis, inflammatory bowel disease and hepatitis B virus infection (Sashio *et al.*, 2002; Cuenca *et al.*, 2003; Li *et al.*, 2006; Romero *et al.*, 2008; Al-Rayes *et al.*, 2011). It has becomes evidence that TNFα antagonists are effective in the treatment for rheumatoid arthritis. Etanercept and Infliximab demonstrated significant improvement of the symptoms in affected patients (Seymour *et al.*, 2001).

1.4.3 Association of TNF gene polymorphisms with CRSsNP and CRSwNP

At present, studies on association of TNF gene polymorphisms and CRS render rather ambiguous results. Nonetheless, ongoing studies on this gene are still of interest because it becomes increasingly apparent that the presence of these gene polymorphisms has a potential role in the therapeutic strategies as well as preventive measures.

Erbek *et al.* (2007) detected the role of TNFα (-238 and -308) as well as interleukin 1α (IL1α) and interleukin 1β (IL1β) gene polymorphisms in the

development of CRSwNP among Turkish population. Similar study in Canada identified the association of IL1α gene polymorphism with CRS. However, they unable to replicate the associations between TNFα and IL1β gene polymorphisms towards CRS susceptibility (Endam *et al.*, 2010).

Bernstein *et al.* (2009) genotyped 14 cytokines gene polymorphisms in CRSwNP patients and controls. TNFα polymorphisms showed statistically significant relationships among Caucasian population. In this study, no significant difference between CRSwNP and controls were reported for these cytokine polymorphisms except TNFα gene. SNPs in TNFα were also investigated in CRS and control among Turkish and Hungarian populations. They found that the presence of polymorphisms in TNFα gene might increase the risk of CRS development (Batikhan *et al.*, 2010; Szabo *et al.*, 2013).

The studies of SNPs have given different associations in different populations. TNFα and TNFβ gene polymorphisms were investigated among Japanese with CRS. TNFα polymorphisms have demonstrated no significant difference between CRS patients and healthy individuals. In contrast, significant association was established between allele frequency for TNFβ polymorphism (Takeuchi *et al.*, 2000). Another study was carried out among patient who suffered from CRSwNP in Mexico. The results of this study showed that there was no significant association between TNFα polymorphisms and susceptibility towards CRSwNP (Fajardo-Dolci *et al.*, 2006).

The exact mechanism on how TNF affects the inflammatory reaction in CRS is still controversial. It is still unknown whether TNF causes severe inflammatory reaction or the severity of the inflammatory reaction produce more TNF in CRS. With respect to CRSwNP, the expression of TNFα gene was detected in nasal polyp tissue

but not in normal mucosa suggesting the role of this proinflammatory cytokines in the pathogenesis of this disease (Finotto *et al.*, 1994). In addition, literature did demonstrate the improvement of inflammatory reaction and mucus production after administration of TNFα antagonist in lipopolysaccharide-induced rhinosinusitis mice (Kim *et al.*, 2011a). Given the evidence mentioned above, we hypothesised that there might be association between TNF gene polymorphisms and chronic rhinosinusitis with and without nasal polyps.

CHAPTER 2: OBJECTIVES

2.1 General objective

- To determine the association of TNF gene polymorphism in chronic rhinosinusitis with and without nasal polyps

2.2 Specific objectives

i. To identify the presence of TNF gene polymorphism in chronic rhinosinusitis and control

ii. To identify the presence of TNF gene polymorphism in chronic rhinosinusitis patient with and without nasal polyps

iii. To determine the association of TNF gene polymorphism among chronic rhinosinusitis and healthy control

iv. To determine the association of TNF gene polymorphism among chronic rhinosinusitis with and without nasal polyps

v. To determine the association between TNF gene polymorphism with factors related to CRS

CHAPTER 3: METHODOLOGY

3.1 Hypothesis

Alternative hypothesis: There is association of TNF gene polymorphisms in chronic rhinosinusitis with and without nasal polyps.

3.2 Study design

This is a case-controlled study to determine the association between TNF gene polymorphisms in chronic rhinosinusitis with and without nasal polyps.

3.3 Study population

All participants were above 18 years old. Cases were recruited from patients who attended Otorhinolaryngology - Head and Neck (ORL-HNS) Clinic, Hospital Universiti Sains Malaysia (USM) while controls were recruited from healthy individual that came from same district as cases.

3.4 Inclusion and exclusion criteria

3.4.1 Inclusion criteria

3.4.1.1 Inclusion criteria for cases

- Participants who were confirmed to have chronic rhinosinusitis with or without nasal polyps based on history and physical examination
- Nasal polyps were confirmed by nasoendoscopy

3.4.1.2 Inclusion criteria for controls

- Healthy individuals and not known to have sinonasal disease
- Selected from the same district area as cases

3.4.2 Exclusion criteria

3.4.2.1 Exclusion criteria for cases

- Participants whom were known to have chronic rhinosinusitis secondary to other diseases such as cystic fibrosis, Kartagener's syndrome or Young syndrome
- Patient with antrochoanal polyps, inverted papilloma or sinonasal malignancy

3.4.2.2 Exclusion criteria for controls

- Participants who are blood-related with the cases
- Participants who are known to have allergy