Molecular mechanisms underlying the pathogenesis of nasal polyposis and its response to steroid treatment

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Publications

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- Wang DY, Li CW. **Control of nasal obstruction in patients with persistent allergic rhinitis.** *Zhonghua Er Bi Yan Hou Tou Jing Wai Ke Za Zhi.* 2006 Sep;41(9):716-20.

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Molecular mechanisms underlying the pathogenesis of nasal polyposis and its response to steroid treatment

Content of the Thesis

Chapter 2. Objectives and Significance

Chapter 3. Materials and Methods

Summary

Nasal polyposis (NP) is a chronic inflammatory airway disease, which represents severe infiltration of inflammatory cells (e.g. eosinophils and neutrophils), epithelial damage, and stromal edema. Although glucocorticosteroid (GC) treatment is effective in relieving NP inflammation, the high recurrence rate makes the etiology and pathogenesis of NP complicated. The results from our research group reported profiles of cellular infiltration in Asian NP. In this respect, this thesis focuses on the molecular mechanisms underlying the pathogenesis of Asian (especially Chinese) NP and its response to GC treatment.

At first, we started to test the hypothesis of *Staphylococcus aureus* (*S. aureus*) and its superantigens in Asian NP. A low incidence rate of *S. aureus* was found in the studied NP and superantigens could not be found in all NP tissues, indicating no significant effects of *S.aureus* related superantigens in Asian NP.

Secondly, we tried to find if some cancer related mechanism (methylation) would be involved in NP pathogenesis. This is based on the assumption that NP pathological features are somewhat similar to tumor growth, such as tissue hyperplasia and high recurrence rate. Although methylation of common tumor suppressor genes (TSGs) (*CDH1*, *TSLC1*, *DAPK1*, and *PTPN6*) was detected in NP, the frequency of gene methylation did not differ between NP and nasal mucosal controls, indicating the role of methylation of these TSGs appears to be minimal in NP.

The first two studies came out with negative results which were not anticipated initially. For this reason, a systemic microarray analysis was used to identify novel

gene markers and molecular pathways which underlie the NP pathogenesis and its response to GC treatment. Two sets of NP biopsies, *i.e.,* before the initiation and after oral GC treatment, were taken from the same patient with bilateral NP. The inferior turbinate from patients with nasal septal deviation served as a nasal mucosal control. All subjects were Chinese. Histological results demonstrated that GCs had potent effects on epithelial repair and suppression of eosinophils. Pathway analysis revealed that alteration of AP-1 network, anti-inflammatory gene network, apoptosis signaling, complement system, EGF/EGFR signaling, Leukotriene signaling, PGE_2 signaling, ERK/MAPK signaling, IL-6 signaling, and NF-kappaB signaling would be involved in the NP pathogenesis. AP-1/AP-1 related genes and their interactive networks were considered to be the central molecular evidence for the epithelial healing effect by GCs. GCs also regulated the expression of several important pro-/anti-inflammatory genes (e.g., MMPs, DUSPs, and SPRYs) and then performed the anti-inflammatory effects to control the inflammatory responses in NP. In addition, eosinophil- and neutrophil-associated genes were reviewed in array data based on literature reports and they were able to differentiate eosinophilia and neutrophilia in nasal samples. The pathological features of NP were also attributed to the change of other genes/gene families in NP, such as oxidant/antioxidant related genes, edema related genes, and mucin genes.

In conclusion, we demonstrate the molecular profiles underlying the beneficial effects of GCs on NP and the histopathological patterns of NP. Identification of these genes and gene networks ultimately contributes to the knowledge of NP pathogenesis and improvement of NP therapy.

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List of Abbreviations

AA	arachidonic acid
ADAM8	ADAM metallopeptidase domain 8
AIF	apoptosis-inducing factor
ALOX5AP	arachidonate 5-lipoxygenase-activating protein
ANGPT1	angiopoietin 1
ANGPT2	angiopoietin 2
ANXA1	annexin A1
AP-1	activation protein 1
APCs	antigen presenting cells
AREG	Amphiregulin
ATP1A2	ATPase, Na+/K+ transporting, alpha 2 (+) polypeptide
BGS	bisulfite genomic sequencing
Bid	BH3 interacting domain death agonist
C1QB	complement component 1, q subcomponent, B chain
C3	complement component 3
C4A	complement component 4A
CASP3	caspase 3
CASP7	caspase 7
CCL11	chemokine (C-C motif) ligand 11,(known as eotaxin)
CCL15	chemokine (C-C motif) ligand 15
CCL28	chemokine (C-C motif) ligand 28
CCRs	chemokine receptors
CDH1	cadherin 1, type 1,(known as E-cadherin)
CDKN2A	cyclin-dependent kinase inhibitor 2A (known as p16)
CEACAM1	carcinoembryonic antigen-related cell adhesion molecule 1
CEACAM6	carcinoembryonic antigen-related cell adhesion molecule 6
CF	cystic fibrosis
CFH	complement factor H, (known as HF1)
c-Fos	v-fos FBJ murine osteosarcoma viral oncogene homolog
c-Jun	jun oncogene
CLIC3	chloride intracellular channel 3
CLIC5	chloride intracellular channel 5
CLIC6	chloride intracellular channel 6
CRISP3	cysteine-rich secretory protein 3
CRS	chronic rhinosinusitis
CT	computed tomography
Ct	threshold cycle
CXCL11	chemokine (C-X-C motif) ligand 11
CXCL12	chemokine (C-X-C motif) ligand 12,(known as SDF-1)
CXCL2	chemokine (C-X-C motif) ligand 2
CXCL6	chemokine (C-X-C motif) ligand 6, (known as GCP2)
CXCL9	chemokine (C-X-C motif) ligand 9

CYSLTR1	cysteinyl leukotriene receptor 1
CYSLTs	cystinyl-leukotrienes
DAPK1	death-associated protein kinase 1
DEFB1	defensin, beta 1
DUOX1	dual oxidase 1
DUSP1	dual specificity phosphatase 1
DUSP2	dual specificity phosphatase 2
DUSP4	dual specificity phosphatase 4
DUSP5	dual specificity phosphatase 5
DUSP6	dual specificity phosphatase 6
ECM	extracellular matrix
ECP	eosinophilic cationic protein
EGF	epidermal growth factor
EGR1	early growth response 1
EPO	eosinophil peroxidase
ERBB4	v-erb-a erythroblastic leukemia viral oncogene homolog 4
ERK	extracellular-signal-regulated kinase
FDR	false discovery rate
FGF	fibroblast growth factors
FosB	FBJ murine osteosarcoma viral oncogene homolog B
GC	Glucocorticosteroid
GCLM	glutamate-cysteine ligase, modifier subunit
GM-CSF	granulocyte-macrophage-colony stimulating factor
GPX3	glutathione peroxidase 3
GR	glucocorticoid receptor
GRE	glucocorticoid response element
GRα	glucocorticoid receptor alpha
GRβ	glucocorticoid receptor beta
HBEGF	heparin-binding EGF-like growth factor
HETEs	hydroxyeicosatetraenoic acids
ICAMs	intercellular adhesion molecules
IFNAR1	interferon alpha receptor 1
IL	Interleukins
IL13RA2	interleukin 13 receptor, alpha 2
IL18	interleukin 18
IL5Ra	interleukin 5 receptor, alpha
IL-6	interleukin 6
IL6ST	interleukin 6 signal transducer (known as gp130)
IP	inverted papilloma
IPA	Ingenuity Pathways Analysis
IPKB	Ingenuity Pathway Knowledge Base
IT	inferior turbinate
ITGB2	integrin, beta 2
JAK	Janus Kinase

JNK	Jun N-terminal kinase
JunB	jun B proto-oncogene
LGALS8	lectin, galactoside-binding, soluble, 8 (known as galectin 8)
LGALS9	lectin, galactoside-binding, soluble, 9 (known as galectin 9)
LPO	Lactoperoxidase
LT	Leukotriene
LTA4H	leukotriene A4 hydrolase
LTB4R	leukotriene B4 receptor
LX	Lipoxins
LYN	v-yes-1 Yamaguchi sarcoma viral related oncogene homolog
MAPKs	mitogen-activated protein kinases
MEKs	mitogen-activated protein kinase kinase
MBP	major basic protein
MHC II	major histocompatibility complex class II
MIF	macrophage migration inhibitory factor
MMP7	matrix metallopeptidase 7
MMP9	matrix metallopeptidase 9
MSP	methylation specific PCR
MUC16	mucin 16
MUC20	mucin 20
MUC4	mucin 4
MUC7	mucin 7
NFKBIA	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
NFKBIZ	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta
NO	nitric oxide
NOS2A	nitric oxide synthase 2A
NOX4	NADPH oxidase 4
NP	nasal polyposis
NPC	nasopharyngeal carcinoma
NR4A1	nuclear receptor subfamily 4, group A, member 1
NR4A2	nuclear receptor subfamily 4, group A, member 2
NR4A3	nuclear receptor subfamily 4, group A, member 3
NRG3	neuregulin 3
NUSE	normalized unscaled standard errors
OMC	ostiomeatal complex
OXR1	oxidation resistance 1
PBMC	peripheral blood mononuclear cell
PCA	principal component analysis
PGs	Prostaglandins
PLA2	phospholipase A2
PLA2G10	phospholipase A2, group X
PLA2G4A	phospholipase A2, group IVA

PLM	probe-level model
PM	perfect match
PRDX1	peroxiredoxin 1
PRDX5	peroxiredoxin 5
PTGER2	prostaglandin E receptor 2 (subtype EP2)
PTGER3	prostaglandin E receptor 3 (subtype EP3)
PTGIS	prostaglandin I2 synthase
PTGS2	prostaglandin-endoperoxide synthase 2,(known as COX-2)
PTPN6	protein tyrosine phosphatase, non-receptor type 6 (known as SHP-1)
PTX3	pentraxin-related gene, rapidly induced by IL-1 beta
QC	quality control
RASSF1A	Ras association domain family member 1
RBC	red blood cell
RLE	relative log expression
RMA	robust multichip average
RNS	reactive nitrogen species
ROS	reactive oxygen
RT PCR	reverse transcription PCR
RTK	receptor tyrosine kinases
S.aureus	*Staphylococcus aureus*
SAM	significant analysis of microrarray
SCGB1A1	secretoglobin, family 1A, member 1 (known as uteroglobin)
SCNN1A	sodium channel, nonvoltage-gated 1 alpha
SCNN1B	sodium channel, nonvoltage-gated 1 beta
SCNN1G	sodium channel, nonvoltage-gated 1 gamma
SEA	*Staphylococcus aureus* enterotoxin A
SEB	*Staphylococcus aureus* enterotoxin B
SEC	*Staphylococcus aureus* enterotoxin C
SED	*Staphylococcus aureus* enterotoxin D
SEE	*Staphylococcus aureus* enterotoxin E
SEG	*Staphylococcus aureus* enterotoxin G
SEI	*Staphylococcus aureus* enterotoxin I
SELPLG	selectin P ligand
SERPINA1	serpin peptidase inhibitor, clade A, member 1
SHE	*Staphylococcus aureus* enterotoxin H
sIgE	specific IgE
SOCS3	suppressor of cytokine signaling 3
SOD3	superoxide dismutase 3, extracellular
SPRY1	sprouty homolog 1
SPRY2	sprouty homolog 2
SPRY4	sprouty homolog 4
STAT3	signal transducer and activator of transcription 3
TAE	tris-acetate EDTA
TEK	TEK tyrosine kinase, endothelial

TGF	transforming growth factor
THBD	thrombomodulin
TSGs	tumor suppressor genes
TSLC1	tumor suppressor in lung cancer 1
TSTT-1	toxic shock syndrome toxin-1
TXN	thioredoxin
VCAMs	vascular cell adhesion molecules
VEGF	vascular endothelial growth factor
ZFP36	zinc finger protein 36, C3H type, homolog

Chapter 1. Nasal Polyposis – a Multifactorial Chronic Inflammatory Disease (Literature review)

1.1 Histopathology

Nasal polyposis (NP) is a common inflammatory disease in upper airway. Nasal polyps are generally regarded as a benign mucosal swelling that arises from the middle meatus and ethmoid sinus and prolepses into the nasal cavity. In some cases, polyps also arise from the maxillary sinuses and from the middle and superior turbinates.

In macroscopical appearance (**Figure 1.1**), nasal polyps are usually soft, lobular and mobile swellings, and have a smooth and shiny surface with a bluish-grey or pink translucent color. The cut surface is moist and pale but appears more pink or red if the polyp is more vascular. The polyp often has an elongated stalk and the polyp size varies from 2 to 3 cm in diameter.

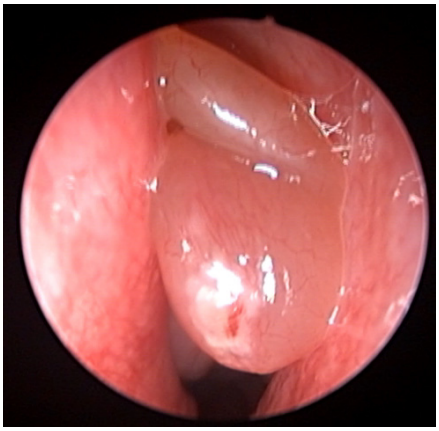


Figure 1.1 Gross view of nasal polyps. Picture was taken from the patient with NP under endoscope examination.

The characteristic features of nasal polyps are large quantities of extracellular edema

and an inflammatory cell infiltrate consisting of mast cells, eosinophils, lymphocytes, neutrophils and plasma cells, with eosinophils often dominant. The epithelium of polyps is often damaged followed by aberrant remodeling (such as squamous metaplasia). Other characteristics of nasal polyps include proliferation of stromal elements, a thickening of the basement membrane, sparse blood vessels and few mucous glands lacking normal innervation.

NP is categorized into four types based on the different histological patterns [Hellquist, 1997]. The most common one is the edematous, eosinophilic polyp, which is characterized by edema, goblet cell hyperplasia of the epithelium, thickening of the basement membrane, and infiltration of numerous leukocytes, predominantly eosinophils. The second common type is the fibro-inflammatory polyp, which is characterized by squamous metaplasia of epithelium and intensive infiltration of lymphocytes, but lack of stromal edema and goblet cell hyperplasia. The less common polyp presents with pronounced hyperplasia of seromucinous glands but also shows extensive edema. The rarest type is a polyp with stromal atypia which contains atypical fibroblast-like cells without mitoses.

1.2 Epidemiology

In the general population, the prevalence of nasal polyposis (NP) ranges from 0.2% to 4.3%, making it one of the most common chronic diseases of the upper respiratory system [Falliers, 1974; Hedman et al., 1999; Larsen and Tos, 1991; Mygind et al., 2000]. A far higher prevalence of NP was found at 32% from an autopsy study [Larsen & Tos, 2004]. The incidence of NP is higher in men than in women and increases with age [Larsen & Tos, 2002], while the frequency of NP is rare (about 0.1%) in children [Triglia & Nicollas,

1997]. There is lack of epidemiology data in Asian populations, and only one Korean group reported that the incidence of NP in Korea was 0.5%, based on a nationwide survey of 10,054 subjects [Min et al., 1996]. Whether there is any difference in the prevalence among various population groups is not clear.

NP is usually associated with chronic rhinosinusitis, aspirin intolerance, asthma, and cystic fibrosis:

(1) NP and chronic rhinosinusitis (CRS)

Chronic rhinosinusitis (CRS) is a common disease closely associated with NP. The percentage of CRS in patients with NP has been reported to range from 65% to 90% [Bunnag et al., 1983; Slavin, 1988]. In addition, a higher incidence rate of CRS in patients with NP was reported in Asians, compared to Caucasians [Tan et al., 1998]. Although CRS almost always coexists with NP, only about 20% of the patients with CRS develop NP [Settipane, 1996]. Accumulated evidence has shown that CRS with NP and CRS without NP actually are two different disease entities [Polzehl et al., 2006; Van Zele et al., 2006], while it is still not clear whether CRS predisposes for NP or results from it.

(2) NP and aspirin intolerance

NP is commonly found in aspirin intolerant patients, who are manifested by acute bronchospasm and rhinorrhea within 3 hours after injection of aspirin. The reported incidence rate of NP in patients with aspirin intolerance varies from 36% to 95% [Larsen, 1996]. Samter described the triad of NP, aspirin intolerance and asthma, which was so called “Samter’s syndrome” [Samter & Beers, 1968]. The triad seems to develop in a time sequence: asthma usually occurs first followed by aspirin intolerance within

one year, while NP occurs within the next 10 years of asthma onset [Settipane, 1986].

(3) NP and asthma

Asthma is a chronic respiratory disease which is characterized by bronchoconstriction in response to various stimuli, including allergen, cold air, moist air, exercise and emotional stress. NP was found in 13% of non allergic asthma and only 5% of allergic asthma [Settipane, 1977], suggesting that non allergic asthma was most commonly associated with NP. In addition, one French study reported that the prevalence of asthma in patients with NP was as high as 45% in 224 cases without relevant sex difference [Rugina et al., 2002].

(4) NP and cystic fibrosis (CF)

Cystic fibrosis (CF) is a hereditary disease that mainly affects the respiratory and digestive system, causing progressive disability and early death. CF is one of the most common life-shortening, childhood-onset inherited diseases, especially in Caucasians. Patients with CF have a high frequency of NP, ranging from 20% to 37% [Settipane, 1996; Hadfield et al., 2000]. In addition, it has been reported that 50% of the patients with nasal polyps aged 16 or younger had CF [Schramm, 1980], indicating children with nasal polyps need to be evaluated for CF.

1.3 Anatomy

The nasal cavity and nasal sinuses have important physiological functions: airflow ventilation, olfaction, sensation, filtration, warming and humidifying, and immunity [Jones, 2001]. The nasal cavity is divided sagittally into left and right halves by the nasal septum. The roof of the nasal cavity is the cribriform plate, separating it from

the anterior cranial cavity. The inferior wall is the palate which separates the nasal cavity from the oral cavity. The superior, middle, and inferior turbinates (also called concha) form the lateral wall as horizontal projections, where the superior, middle and inferior meatus line below the respective turbinate (**Figure 1.2**). They are considered to be the main nasal passages.

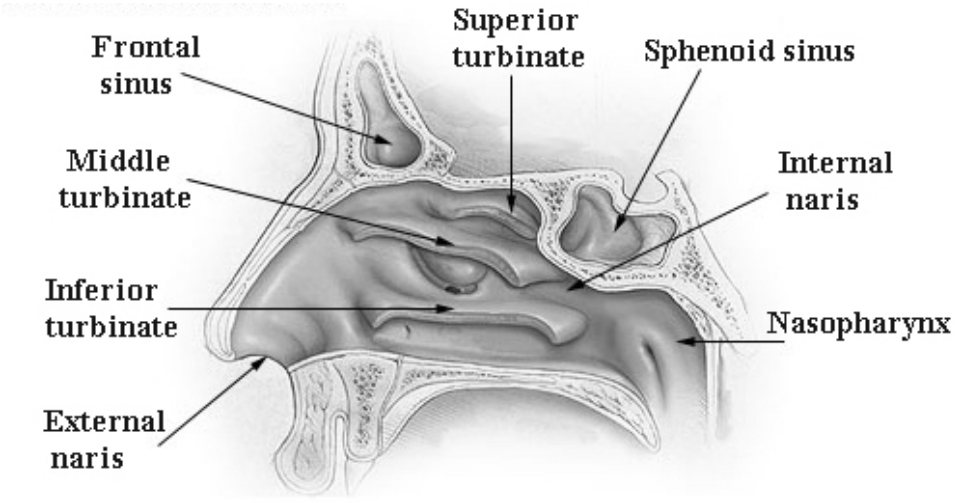


Figure 1.2 Lateral wall of the nose. Superior, middle and inferior turbinates are shown. (Picture source: http://training.seer.cancer.gov/ module_anatomy/ images/ illu_nose_nasal_cavities.jpg)

There are four nasal sinuses: the frontal, sphenoidal, maxillary and ethmoidal sinuses. The maxillary sinus, anterior ethmoidal and frontal sinuses all drain into the middle meatus via the ostiomeatal complex (OMC). OMC is important, because obstruction here by inflammation and swelling due to some pathological conditions (e.g. allergy, infection, anatomical variants and nasal polyps) will interfere with the drainage and aeration of these three sinuses.

The middle meatus and ethmoids have been considered the important region where most NP and sinusitis develop. Messerklinger described his nasal endoscopic findings on the pathophysiologic roles of this area: when the mucosal surfaces from middle

meatus and ethmoids contact directly, localized disruption of the mucociliary clearance occurs, resulting in retention of secretions in the surface contact, preventing or slowing drainage, predisposing the patient to infection and leading to inflammation and edema [Messerklinger, 1978].

From the ultrastructure view: (i) the nasal lining consists of a pseudostratified columnar ciliated mucous membrane which is continuous with the sinuses and pharynx; (ii) one third of the anterior nasal cavity is covered by epithelium which has a typical airway structure. The normal nasal epithelium comprises the columnar ciliated cells, goblet cells and basal cells. Under the epithelium is the basement membrane which is a layer of collagen fibrils. The nasal submucosa (lamina propria) is a loose connective tissue, containing blood vessels, submucosal glands and various cell types, such as macrophage, fibroblast, lymphocyte and plasma cell. In the pathological condition, the number and status of the host cells in nasal mucosa/submucosa may change, and increase of the infiltration of some inflammatory cells (e.g. neutrophils and eosinophils) will occur.

1.4 Pathogenesis

Although the pathogenesis of NP is poorly understood, several hypotheses underlying the mechanisms of NP have been proposed in recent decades, including environmental factors, genetic predisposition, allergy, local nasal allergy, microorganisms, chemical mediators, deregulation of fluid and electrolyte transport, and epithelial rupture theory.

1.4.1 Environmental factors

Since nasal mucosa is exposed to a variety of environmental allergen, pollutants, and microbes, the role of environmental factors in the etiology of NP have been proposed. NP has been suggested to be associated with aeroallergen hypersensitivity [Asero & Bottazzi, 2001]. NP patients exposed to noxious inhalant pollutants were significantly associated with NP occurrence [Pimentel, 1995]. Moreover, an association between the use of a woodstove as a primary source of heating and the development of NP was also reported [Hanley and Kim, 2002].

1.4.2 Genetic predisposition

Some epidemiological evidence supports the genetic factors on NP: (i) Drake-Lee reported the development of NP in identical twins [Drake-Lee, 1992]; (ii) high rates of NP (52.6%) and asthma (43.6%) have been reported in the family history of patients affected with NP [Rugina, 2002]. However, evidence is still lacking for a genetic basis for this.

Human leukocyte antigen (HLA) genetic patterns have been reported in NP: (i) Moloney et al. reported a higher incidence of HLA-A1/B8 in 29 patients with NP and asthma, but not the patients with NP alone [Moloney & Oliver, 1980]; (ii) a significant association was found between HLA-A74 and NP [Luxenberger et al., 2000]; (iii) Molnar-Gabor et al. showed that HLA-DR7-DQAI*0201 and –DQBI*0202 haplotype had two to three times higher odd ratios in patients with NP compared to controls [Molnar-Gabor et al., 2000]. Moreover, the mutation of the cystic fibrosis transmembrane regulator (CFTR) gene was reported in few patients with NP but without cystic fibrosis, however the vast majority of patients with NP do not have inactivation of the CFTR gene [Irving, 1997].

In the recent literature, some studies were able to show linkage of certain phenotypes of NP to candidate gene polymorphisms. Karjalainen et al. reported that subjects with a single G-to-T polymorphism in exon 5 at +4845 of the gene encoding IL-1alpha (IL-1A) were found to have less risk of developing NP as compared to subjects with common G/G genotype [Karjalainen et al., 2003]. In another study, polymorphism of IL-4 (IL-4/-590 C-T), a potential determinant of IgE mediated allergic disease, was also found to be associated with a protective mechanism against NPs in the Korean populations [Yea et al., 2006]. In addition, asthma-related Argl6gly polymorphism of the beta2-adrenoceptor gene (ADRBeta2) was found to be associated with an increased risk of nasal polyposis [Bussu et al., 2007].

1.4.3 Allergy

Allergy has been assumed to be the underlying cause of NP because of three factors: (i) presence of eosinophilia; (ii) association with asthma; (iii) allergic symptoms and signs, such as high levels of IgE, mast cell degranulation, and high recurrence rate.

However, there is still much evidence to support the association between allergy and NP. Park et al. found that allergen-induced *in vitro* release of granulocyte-macrophage-colony stimulating factor (GM-CSF) and interleukin (IL)-8 from NP tissue in atopic subjects and these mediators were associated with increased eosinophil survival [Park et al., 1997]. In Thailand, Pumhirun et al. reported that the incidence rate of positive skin prick test was 40% (24 out of 40) in patients with NP, while 20% (6 out of 30) in the control group [Pumhirun et al., 1999]. Asero and Bottazzi found 40% (8 out of 20) of patients with NP showed skin reaction to *Candida*

albicans, a common commensal mold of the upper airway tract [Asero and Bottazzi, 2000]. Asero et al. also found that 70% (30 out of 43) of the patients with NP who were tested positive in the skin prick test seemed to be sensitive to perennial airborne, compared to 19% (215 out of 1128) of the controls with respiratory allergy, suggesting that perennial airborne allergens may play a relevant role in the NP [Asero and Bottazzi, 2001].

It has been suggested that food allergy may have a possible role in the pathogenesis of NP although evidence for this is limited. Pang et al. reported that 81% (65 out of 80) of NP patients showed positive intradermal test results relating to food allergy, while only 11% (4 out of 36) of control subjects were positive to food allergy test [Pang, 2000]. Another study showed that 31% of NP patients gave a history of food or drug allergy [Rugina et al., 2002]. Because the food allergy studies in NP are mostly based on clinical trials, its role need to be further investigated in molecular and cellular levels.

1.4.4 Microorganisms

Several types of microorganisms have been investigated to determine their role in NP. Some old studies have found that the nasal cavity is normally colonized with some non-pathogenic bacteria: (i) Calenoff et al. showed that 59 out of 61 NP patients exhibited positive serum IgE to at least one out of 11 bacteria tested [Calenoff et al., 1983]; (ii) Dunnette et al. reported that multiple aerobic bacterial species occurred in NP from patients with asthma more frequently than in those from patients without asthma, and the number of bacteria was related to the number of infiltrating neutrophils [Dunnette et al., 1986]; (iii) Daws et al. showed that pus cells and bacteria were found in 16% of sinus irrigations [Dawes et al., 1989].

Recent studies have suggested the role of superantigens secreted by *Staphylococcus aureus* (*S. aureus*), which may contribute to the pathogenesis of NP [Bachert et al., 2008]. Superantigens are defined as toxins of bacterial or viral origin that are able to cross-link antigen presenting cells (APCs) and T-lymphocytes by binding to the major histocompatibility complex class II (MHC II) on APCs and the TCRs on T-lymphocytes. The recognition of superantigens is generally not MHC restricted and unprocessed superantigens directly bind to the conserved amino acid residues that are outside the peptide-antigen binding groove. Such special cross-inking characters results in an extreme polyclonal activation of CD4 and CD8+ T cells. *S. aureus* produces a large variety of enterotoxins, including *S. aureus* enterotoxins (SEs) A to E, G to I, and TSST-1 (toxic shock syndrome toxin-1), which act as superantigen function and activate large subpopulations of T cells, B cells, and other pro-inflammatory cells.

Van Zele et al. presented some interesting results about superantigens in NP [Van Zele et al., 2004]: (i) coagulase-positive *S. aureus* colonization in the middle meatus is higher in patients with NP (64%) compared to patients with chronic sinusitis (27%) and healthy controls (33%); (ii) the potential to produce enterotoxins was also parallel with the *S. aureus* colonization rates in NP; (iii) tissue concentrations of specific IgE against *S. aureus* enterotoxins were higher in the NP patients with aspirin intolerance and asthma, compared to those with NP only; (iv) the infiltration of eosinophils and concentration of total IgE in tissues were also significantly increased in samples with the presence of specific IgE to enterotoxins. Moreover, there is also some evidence that *S. aureus* superantigens play a role in the induction of Th2 cytokines like IL-4

and IL-5 [Bachert et al., 2008]. These results indicate that NP with local overproduction of IgE, eosinophilia, and Th2 shift may represent an allergic phenomenon originated from *S. aureus* derived superantigens.

Other than bacteria, it has been suggested that viruses may be involved in the pathogenesis of NP, such as the Influenza A virus [Ginzburg et al., 1982] and the Epstein-Barr virus [Tao et al., 1996]. However, these opinions are not of any interest, mostly because viruses were found in both healthy individuals and NP patients.

1.4.5 Cellular components

Two major cell types have been determined in NP: infiltrated inflammatory cells and structure-related cells. Traditionally, the inflammatory cells including eosinophils, lymphocytes, mast cells, plasma cells and neutrophils were considered the major sources of inflammatory chemical mediators. However, there is a growing awareness that structure-related cells including epithelial cells, endothelial cells and fibroblasts have also been seen as active participants in the interaction with other inflammatory cells as well as the release of various mediators, but not just passive barrier lining. The crosstalk between these different cell populations and various mediators ultimately contribute to the complicated pathogenesis of NP.

1.4.5.1 Lymphocytes

Lymphocytes play a central role in adaptive immunity. T helper 1 (Th1) cells stimulate phagocyte-mediated defense against infections, T helper 2 (Th2) cells stimulate IgE and eosinophil/mast cell-mediated immune reactions, and cytotoxic T cells recognize and kill target cells expressing foreign peptide antigen in association

with class I MHC molecules. T lymphocytes were often prominent over B lymphocytes [Liu et al., 1994]. Bernstein et al. reported that nasal polyps were found to have more lymphocytes than the inferior turbinates [Bernstein et al., 1997]. In contrast, Linder et al. reported that the relative proportion and spatial distribution of T and B lymphocytes were similar with regards to both NP and normal mucosa from disease-free controls [Linder et al., 1993]. The findings of helper T cells (CD4+) and cytotoxic T cells (CD8+) were also controversial. Liu et al. demonstrated that staining of CD4+ T cells were present in greater numbers than CD8+ T cells in NP [Liu et al., 1994]. However, Stoop et al. reported that more CD8+ T cells than CD4+ T cells were found in the NP [Stoop et al., 1989]. In addition, our previous study showed significantly higher levels of CD8+ T cells and an inverse median ratio of CD4+/CD8+ T cells were found in nasal polyps compared to the middle turbinates from controls [Hao et al., 2006]. It has been suggested that the cytokine pattern in NP assumes neither a Th1 nor Th2 type predominance, because IL-5, IL-5 and IFN-gamma have all been shown to be up-regulated in NP, without influence of the atopic status [Bachert et al., 2002]. One most recent study has indicated that a dysfunction of T regulatory cells may contribute to severe inflammation in NP tissues due to a decreased expression of forkhead box P3 (FOXP3) [Van Bruaene et al., 2008].

1.4.5.2 Eosinophils

The activated infiltrating eosinophils produce a large amount of granule-associated toxic proteins, such as eosinophilic cationic protein (ECP), major basic protein (MBP) and eosinophil peroxidase (EPO), causing cellular injury and tissue damage. There is recognition that the accumulation of eosinophils into a tissue site involves a number of events, including (i) differentiation of bone marrow progenitors into functionally

mature cells; (ii) rolling, adhesion and migration through the endothelium; (iii) chemotaxis, activation and survival within the tissue [Rothenberg, 1998]. Several studies have indicated that cytokines (e.g. IL-3, IL-5, and IL-13) [Allen et al., 1997], chemokines (e.g. eotaxin, RANTES and CCL24) [Olze et al., 2006], growth factors (e.g. GM-CSF) [Allen et al., 1997], leukotrienes [Parnes et al., 2002], adhesion molecules (e.g. integrins, VCAM1 and ICAM1) [Kupczyk et al., 2006] and other regulatory factors may participate in the eosinophil infiltration in NP.

It is widely accepted that eosinophils are a hallmark of allergy. Bachert et al. found significantly more eosinophilic infiltration in NP containing high total IgE tissue concentrations and these high total IgE levels were more frequently found in asthmatic and aspirin-intolerant NP patients [Bachert et al., 2001]. However, in non-asthmatic and aspirin tolerant NP patients, the resulting eosinophilic infiltration appears to be the same for both atopic and non-atopic NP. Therefore, it remains unknown what causes the primary recruitment of eosinophils to the site of nasal polyps; to investigate the cellular sources of those inflammatory mediators should be helpful to clarify the mechanism of eosinophilia in NP.

1.4.5.3 Neutrophils

Increase of neutrophils has been commonly found in bacterial infectious disease. Studies of neutrophil infiltration in NP remain controversial, i.e., high infiltration in NP [Takasaka et al., 1986] as opposed to lack of difference between NP and controls [Fujisawa et al., 1990]. The correlation between neutrophil infiltration and bacteria count in NP has been reported [Dunnette et al., 1986]. Some mediators (e.g. IL-8, GM-CSF and ICAM) have been reported to regulate the recruitment and survival of neutrophils in

NP [Demoly et al., 1998; Takeuchi et al., 1995; Vancheri et al., 1991]. Furthermore, the relationship between neutrophils and other inflammatory cells in nasal polyps has also been documented. A significant correlation was identified between neutrophil elastase+ cells and activated mast cells or eosinophils [Park et al., 1997]. Neutrophil elastase may contribute to tissue inflammation and remodeling by inducing the expression of secretory leukocyte protease inhibitor [Marchand et al., 1997].

1.4.5.4 Mast cells

Mast cells are known to play a key role in IgE-mediated diseases, but they are also involved in non-IgE-mediated inflammatory diseases. Mast cells can be detected in the stroma as well as the epithelium of nasal polyps. The level of mast cells in NP was higher than that in the sinus mucosa from patients with sinusitis and the middle turbinate mucosa from patients with allergic rhinitis [Otsuka et al., 1993]. Mast cells in NP produce a variety of cytokines such as Il-4, IL-5, Il-6, Il-13, GM-CSF and IL-8 [Pawankar, 2003]. In addition, mast cell mediators like histamine and tryptase are able to up-regulate the release of RANTES and GM-CSF from epithelial cells and fibroblasts in NP, indicating a vicious cycle that further promote eosinophilia in NP [Pawankar, 2003].

1.4.5.5 Epithelial cells

In NP, epithelium is both an active player and a “passive” target in the pathology. It plays a central role in the interaction with eosinophils and myfibroblasts. Epithelial damage caused by inflammatory mediators can trigger aberrant epithelial remodeling processes in NP, causing hyperproliferation of epithelial cells and squamous metaplasia. During this process, epithelial cells would release various molecules (e.g.

TGF-beta, EGF, VEGF, GM-CSF, eotaxin and RANTES) which are related to growth, differentiation, migration, and activation, and then such anti-apoptotic microenvironment further promote the infiltration of eosinophils and survival of myofibroblasts [Devalia & Davies, 1993; Mullol et al., 1995; Shin et al., 2003].

1.4.5.6 Fibroblasts/Myofibroblasts

Myofibroblasts are an activated phenotype of fibroblasts and are involved in wound repair and tissue differentiation in non-pathological circumstances [Serpero et al., 2006]. Myofibroblasts are atypical stromal cells that play a crucial role in the pathological tissue changes seen in both NP and asthma. In NP, myofibroblasts produce large amounts of extracellular matrix molecules, such as collagens (type I, III, IV and VIII) and fibronectin, which would contribute to the stromal fibrosis [Beju et al., 2004]. The fibrosis in NP seems to represent an unchecked and deranged tissue repair since the myofibroblasts do not go into apoptosis [Zhang et al., 1999], and consequently it may promote the growth of NP. It has been suggested that some growth molecules secreted by eosinophils or epithelial cells may cause uncontrolled proliferation and survival of myofibroblasts [Elovic et al., 1994].

1.4.6 Molecular and chemical mediators

The molecular and chemical mediators (e.g., peptides, proteins, amines, or lipids) released from the inflammatory/structural cells contribute to the complicated inflammatory signaling networks and appear to be important in the development of NP. In recent decades, most of the NP studies have focused on the molecular evidence in NP.

1.4.6.1 Histamine

Histamine is released primarily by mast cells after activation by IgE or other histamine releasing factors. Recent study showed that histamine content in NP and normal nasal mucosa did not differ, but histidine decarboxylase (histamine biosynthesis enzyme) was elevated in NP tissue and histamine-N-methyltransferase (histamine degradative enzyme) activity was enhanced in NP compared to the control; hence, histamine metabolism seems to be increased in NP [Jokuti et al., 2004]. Another report showed that histamine H4 receptor was elevated in NP and associated with the eosinophil infiltration [Jokuti et al., 2007]. In addition, former study showed that the level of histamine was higher in NP patients with allergy than in those with aspirin intolerance, due to the difference in histamine-N-methyltransferase activity [Ogino et al., 1993].

1.4.6.2 Arachidonic acid metabolites

Arachidonic acid (AA) is the precursor of a family of chemical mediators. Two main pathways exist: the lipoxygenase pathway synthesizing hydroxyeicosatetraenoic acids (HETEs), lipoxins (LX), and cystinyl-leukotrienes (cysLTs); the cyclooxygenase pathway producing prostaglandins (PGs), thromboxanes, and prostacyclin.

Abnormalities of AA metabolism may be related to the chronic inflammation of NP, especially those with aspirin intolerance. The major AA metabolite in NP is 15-HETE, which was found in a higher level in NP compared to normal nasal mucosa [Jung et al., 1987]. 5-Lipoxygenase (5-LO) is the key enzyme which can convert 15-HETE to Lipoxin A_4 and Lipoxin B_4, and consequently perform vasodilation effects during inflammatory progression. Both 5-LO and Lipoxin A_4 were increased in NP

[Perez-Novo et al., 2005].

cysLTs have profound effects on airway function by inducing airway smooth muscle contraction, vasodilatation, and vascular permeability and altering the remodeling process in asthma [Funk et al., 2001; Holgate et al., 2003]. All of the cysLTs have been found in NP tissue: LTE_4 was frequently identified in nasal lavage [Salari et al., 1986]; both LTB_4 and LTC_4 was found in higher concentration in NP [Jung et al., 1987], and the level of LTB_4 is higher in NP from allergic patients than non-allergic ones [Ogino et al., 1993]; LTC_4 and LTD_4 are predominant in NP patients with aspirin intolerant asthma [Yamashita et al., 1989].

NP contains detectable levels of PGD_2 and PGE_2. PGE_2 has been proposed to reduce cysLTs synthesis [Szczeklik et al., 1997], and its production has been found lower in NP [Mullol et al., 2002], especially in aspirin intolerant patients [Picado et al., 1999]. PGD_2 has been proposed to prolong eosinophil survival [Monneret et al., 2001], and its production was increased in NP and was positively correlated with eosinophil accumulation [Hyo et al., 2007].

1.4.6.3 Granular proteins

The non-enzymatic, performed granular proteins of eosinophils include eosinophil cationic protein (ECP) and major basic protein (MBP). Two types of ECP have been identified: non-secretory form and secretory form, and both have been found in greater amounts in NP tissues than in healthy nasal mucosa [Stoop et al., 1993]. Nasal lavage from patients with NP contained more ECP than that from patients without NP, but levels did not change with seasonal allergen exposure [Keith et al., 1994]. MBP has

also been found in mast cells in NP, suggesting the sequestration of MBP in mast cells [Butterfield et al., 1990]. The amount of MBP present in NP tissues has been positively correlated with the degree of epithelial damage [Fujisawa et al., 1990].

1.4.6.4 Interleukins

Interleukins (ILs) are a family of mediators released by a number of inflammatory and non-inflammatory cells. IL-1 (alpha and beta) was found primarily in mononuclear leukocytes, but not commonly in polymorphonuclear cells [Liu et al., 1993]. IL-3 expression was elevated in NP tissues compared to controls, and the level of IL-3 was associated with eosinophil infiltration [Allen et al., 1997]. IL-4 was localized in eosinophils[Nonaka et al., 1995], and it regulated eotaxin-2/CCL24 (potent eosinophil attractant) production in a dose-dependent manner [Lezcano-Meza et al., 2003], suggesting IL-4 perform an indirect effect on eosinophil infiltration in NP.

IL-5, a key cytokine for the maturation and activation of eosinophils, was found to be significantly increased in NP compared to controls [Bachert et al., 1997]. IL-5 expression was correlated with the degree of eosinophilic inflammation in NP [Allen et al., 1997] and anti-IL-5 treatment induced eosinophil apoptosis in NP tissue homogenates *in vitro* [Simon et al., 1997]. In addition, IL-5 receptor alpha subunit, which transduces IL-5 signal to the nucleus of the target cells, was significantly up-regulated in NP versus the control [Gevaert et al., 2003].

IL-8 is a chemokine produced by macrophages and other cell types such as epithelial cells. Mullol et al. showed that epithelial cells from NP released more IL-8 than those from healthy nasal mucosa, and IL-8 was reduced in NP after dexamethasone

treatment [Mullol et al., 1995]. Furthermore, allergen-induced *in vitro* release of IL-8 from NP tissue in atopic individuals was associated with increase of eosinophil survival [Park et al., 1997]. IL-13 is an important mediator of allergic inflammation and GC treatment can reduce IL-13 expression in NP [Hamilos et al., 1999]. However, there is no evidence to support the up-regulation of IL-13 in NP.

1.4.6.5 Growth factors

Several growth factors appear to be involved in the pathogenesis of NP. Colony stimulating factors (CSFs) have been found to be important in proliferation and differentiation of granulocyte precursors. They are classified depending on different stimulated cells: macrophages (M-CSF), granulocytes (G-CSF), and both granulocyte and macrophages (GM-CSF). They are released from macrophages and lymphocytes, as well as from epithelial cells, endothelial cells, eosinophils, and fibroblasts in NP tissues.

GM-CSF is supposed to be the primary growth factor in NP. GM-CSF staining was stronger in NP subepithelium than in normal mucosa and the number of GM-CSF staining cells was correlated strongly with the number of activated eosinophils [Ohno et al., 1991]. Both non-allergic and allergic NP presented large numbers of GM-CSF immunoreactive cells, but healthy nasal mucosa did not [Hamilos et al., 1998]. Fibroblast and epithelial cells cultured from NP tissues produced significantly higher levels of GM-CSF in their supernatants compared to those from inferior turbinate tissues [Ohtoshi et al., 1991; Vancheri et al., 1991]. In addition, conditioned media from NP derived cell lines has been used to study the roles of GM-CSF. Epithelial cells from NP tissue survived and proliferated better than normal tissue *in vitro* [Otsuka et al., 1987]. The

conditioned media from NP tissues induced differentiation of monocytes and neutrophils to a greater level than media from controls [Ohtoshi et al., 1991]. Supernatants of epithelial cells and fibroblasts increased eosinophil survival and activation, and this effect was abrogated by antibody to GM-CSF [Gauldie et al., 1994; Xaubet et al., 1994].

Transforming growth factor beta (TGF-beta) is another growth factor important for inducing fibroblast proliferation, and the increased stromal fibrosis seen in NP may be due to the increased expression of TGF-beta [Elovic et al., 1994]. Eosinophils are an important source of TGF-beta, suggesting that eosinophils could enhance their infiltration via TGF-beta regulation [Elovic et al., 1994]. Vascular endothelial growth factor (VEGF) which is important for inducing angiogenesis and edema was reported to be increased in NP and was further up-regulated by TGF-beta [Coste et al., 2000]. However, other studies showed that TGF-beta could inhibit the synthesis of IL-5 and abrogate the survival of eosinophils [Alam et al., 1994], and the expression of TGF-beta was higher in chronic rhinosinusitis than in NP [Watelet et al., 2004].

Fibroblast growth factors (FGF) constitute a family of at least nine heparin-binding polypeptide growth factors, which may promote stromal fibrosis and the proliferation of endothelial and epithelial cells. Up-regulation of both acidic FGF (aFGF) and basic FGF (bFGF) was found in NP compared to nasal turbinates [Kim et al., 2006], and GCs may decrease bFGF levels in NP [Yariktas et al., 2005]. However, the mRNA level of aFGF and bFGF was lower in NP than healthy nasal mucosa, while mRNA level of keratinocyte growth factor (KGF or FGF-7) was higher in NP compared to nasal mucosa [Ishibashi et al., 1998].

Other growth factors have been reported to be involved in the cellular proliferation in NP. Insulin-like growth factor I (IGF-I) was present in high concentrations in NP tissues, but in low level in adjacent control nasal mucosa [Petruson et al., 1988]. Platelet-derived growth factor (PDGF) and proliferating cell nuclear antigen (PCNA) were also up-regulated in NP compared with controls [Coste et al., 1996].

1.4.6.6 Chemokines

Chemokines are a family of low molecular weight cytokines that stimulate leukocyte movement and regulate the migration of leukocytes from the blood to tissues. Some inflammatory chemokines are important in recruiting monocytes, neutrophils, and eosinophils into NP tissues.

The CC-chemokine eotaxin has been considered to play a key role in tissue eosinophilia in NP [Bartels et al., 1997; Shin et al., 2000]. Olze et al. showed that not only eotaxin, but also eotaxin-2 and eotaxin-3 were increased in NP tissues compared to healthy turbinate tissues, and all eotaxin family members were positively correlated with eosinophil infiltration [Olze et al., 2006]. Another CC-chemokine, RANTES was found to be stained more intensively in NP than in healthy controls [Beck et al., 1996], and was found to be increased in eosinophilia NP tissues compared to those without tissue eosinophilia [Meyer et al., 2005]. Some monocyte chemotatctic proteins (MCP-3 and MCP-4) have been also considered potent eosinophil chemoattractants [Bartels et al., 1997; Woodworth et al., 2004]. These chemokines bind to chemokine receptor 3 (CCR3) and then recruit eosinophils in NP, therefore, antagonism of CCR3 could have a therapeutic role in this disease.

1.4.6.7 Adhesion molecules

Adhesion molecules are membrane proteins which regulate cell to cell interaction. An early study demonstrated that intercellular adhesion molecule 1 (ICAM-1), E-selectin and P-selectin were expressed in NP endothelium and contributed to eosinophil adhering and rolling through the endothelium [Symon et al., 1994]. Jahnsen et al. reported that both the number of eosinophils and the proportion of vessels positive for vascular cell adhesion molecule 1 (VCAM-1) were significantly increased in NP compared with the turbinate mucosa of the same patients [Jahnsen et al., 1995]. Moreover, the ligand of VCAM-1 on the peripheral blood eosinophils is very late antigen 4 (VLA-4), and both VCAM-1 and VLA-4 were significantly increased in NP patients with aspirin intolerance than those with aspirin tolerance [Kupczyk et al., 2006].

1.4.6.8 Neurotransmitters

Neurotransmitters, released from autonomic and sensory nerves, may contribute to airway inflammation via their effects on the immune system and respiratory glands, particularly if their metabolism is impaired. Neuropeptide Y (NPY) and vasoactive intestinal peptide (VIP) were predominantly found around the thick wall vessels and in close proximity to the submucosal glands in NP tissues, respectively [Fang et al., 1994]. Substance P was not found in NP [Fang et al., 1994] and poor response of substance P to capsaicin stimulation was present in nasal secretions of the patients with NP [Gungor et al., 1999]. A recent study showed that brain derived neurotrophic factor (BDNF), a neurotrophin which up-regulates neuropeptide production, was expressed at a higher level in epithelial cell cultures from NP than those from middle turbinates and BDNF was increased in response to pro-inflammatory cytokine

stimulation [Jornot et al., 2007]. However, dipeptidyl peptidase (DPPIV), the enzyme which degrades neuropeptides, was expressed at a similar level between NP and control [Jornot et al., 2007].

1.4.7 Deregulation of fluid and electrolyte transport

The large quantity of extracellular fluid (edema) in NP may suggest the abnormal regulation of fluid and ion channel properties. This mechanism has been supported by several studies: (i) increased expression of VEGFs would promote the vascular permeability and angiogenesis in NP [Coste et al., 2000]; (ii) the CFTR protein is an active chloride pump that plays a crucial role in fluid homeostasis, and evidence has been shown that this protein was mutated in NP with cystic fibrosis (CF) [Noone et al., 2001], and it was also expressed in a low level or located abnormally in epithelium of non-CF NP [Jang et al., 2001]; (iii) increased voltage and short circuit current was found in cell cultures from NP as compared to those from turbinate tissues, indicating a significant increase in absorption of sodium ion [Bernstein et al., 1997]. These interesting findings suggest that the micro inflammatory environment in NP would cause abnormal bioelectric properties, and then result in the increased movement of water into the interstitial fluid in NP.

1.4.8 Epithelial rupture theory

Larsen et al. proposed the epithelial rupture theory which may explain the early stages of NP formation [Larsen et al., 1991; Larsen et al., 1992]: (i) polyp formation starts with epithelial damage, necrosis, and rupture due to tissue pressure by inflammatory edema and infiltrated lamina propria; (ii) lamina propria protrudes through the epithelial defect and the mucosa tends to cover it by migration of the epithelium from the edges

of the defect; (iii) the prolapse of the lamina propria undergoes epithelialization, and the vessel stalk will be established; (iv) tubulous glands are formed; (v) polyp enlarges due to gravity with elongation of the glands; (iv) the epithelium and stroma are changed (such as transformation of pseudostratified to stratified epithelium, changes of density of goblet cells, changes of cell infiltration) and polyp is well-developed.

1.5 Clinical symptoms, diagnosis and treatment

1.5.1 Symptoms

The nasal symptoms of NP are described as follows: (i) constant non-periodic nasal blockage and stuffiness, which can have a valve-like sensation allowing better airflow in only one direction; (ii) nasal congestion, which can be a feeling of pressure and fullness in the nose and paranasal cavities ("full-head"); (iii) perennial clear rhinorrhea and sneezing; (iv) white or yellow postnasal drip, which represents the sinusitis symptoms; (v) facial pain and headache, which are exacerbated when infection in sinuses; (vi) hyposmia or anosmia. About one third of patients who are often accompanying asthma have chest symptoms, including wheezing and chronic cough. Diet may induce exacerbations if the patient has aspirin intolerance.

The recurrence of NP is common. About 40% of patients with surgical polypectomy have recurrences [Settpiane et al., 1987]. It was reported that those patients with positive response of allergy skin test had a higher recurrence rate compare to those with negative response [Settpiane et al., 1987].

1.5.2 Diagnosis

The diagnosis of NP is based on the patient's history, symptoms, and clinical examination. When taking the history, the typical symptoms of the patient should be recorded during spontaneous conversation and followed by questions on most important individual symptom. Some specific techniques and scoring systems have been developed to validate the symptoms: (i) validation of nasal obstruction by rhinomanometric or nasal peak flow evaluations; (ii) validation of facial pain and pressure by maxillary antral aspiration or paranasal sinus radiographs; (iii) validation of smell abnormalities by subjective scoring of olfaction.

Endoscopic examination should be the most helpful technique to assess the severity of NP in clinics. Staging systems for NP under endoscopy view have been proposed [Malm, 1997; Fokkens et al., 2007]: (i) score "0" means absence of nasal polyps; (ii) score "1" means polyps in middle meatus only; (iii) score "2" means polyps beyond middle meatus but not blocking the nose completely; (iv) score "3" means polyps completely obstructing the nose.

Computed tomography (CT) scanning is the imaging modality of choice confirming the extent of pathology and the anatomy in paranasal sinuses. It is also helpful to aid the sinus surgery by providing information on anatomy. The Lund-Mackay system relies on a score of 0-2 depending upon the absence (score = 0), partial (score = 1) and complete opacification (score = 2) of each sinus system and of the ostiomeatal complex, deriving a maximum score of 12 per side [Lund et al., 1993].

In addition, routine pathological examination of nasal biopsy is the gold standard to confirm NP diagnosis at the histopathological level and helpful to differentiate

neoplasia and vasculitides.

1.5.3 Treatment

The management of NP includes: drug treatment, which is traditionally based on the use of topical or systemic glucocorticosteroids (GCs); and surgical operation, which is trying to eradicate all polyp tissues from the nasal lumen and sinuses. The aims of treatments are to relieve nasal blockage, restore olfaction, and improve sinus drainage.

1.5.3.1 Topical and systemic glucocorticosteroids

GCs can suppress many phases of the inflammatory process, which may explain their strong effect on inflammation. As NP represents intensive infiltration of various inflammatory cells, especially eosinophils, GC treatment is the first-choice approach. This can range from topical GC sprays or drops in mild to moderate NP, to a short course of systemic GCs in severely affected patients. Systemic application affects all NP tissues within the nose and sinuses, but has the disadvantage of systemic side-effects when used for long-term treatment. Topical application significantly reduces adverse effects but does not impact nasal polyps within the sinus.

The biological effect of GCs is mediated through activation of intracellular glucocorticoid receptors (GRs). Activated GRs bind to the DNA sequence called glucocorticoid response element (GRE) and perform transactivation of target gene transcription (often anti-inflammatory molecules) [Beato, 1989]; while activated GRs also interact with transcription factors such as AP-1 and NF-kappaB to perform transrepression of some inflammatory genes [Cato et al., 1996].

With regard to the transcriptional activation, GRs can increase gene transcription through an action on chromatin remodeling and recruitment of RNA polymerase II to the site of local DNA unwinding [Hayashi et al., 2004]. GRs can also interact with other coactivator proteins such as SRC-1, TIF-2, p300/CBP that enhance local histone acetylation activity. It has been found that GRs could up-regulate the transcription of some anti-inflammatory genes such as annexin-1, inhibitors of NF kappa B, and MAP kinase phosphatase [Hayashi et al., 2004].

Except for the ability of GCs to induce gene expression, the major anti-inflammatory effects of GCs are thought to suppress the transcription of those inflammatory genes. This inhibitory effect of GCs could be attributed to the interaction between GRs and the proinflammatory transcription factors, such as NF kappa B and AP-1 [Hayashi et al., 2004]. For example, NF kappa B recruits transcriptional coactivators, such as CBP or p300/CBP associated factor which have intrinsic histone acetyltransferase (HAT) activity, leading to increased transcription of inflammatory genes. Activated GRs can then translocate to the nucleus and interfere the binding between NF kappa B and its coactivators, resulting in the inhibition of HAT activity and reduction of histone acetylation. Those pro-inflammatory genes such as chemokines, cytokines, and leucotrienes, have been found to be suppressed by GCs [Hayashi et al., 2004].

GCs also affect migration, activation and survival of inflammatory cells such as eosinophils [Burgel et al., 2004], by regulating a variety of mediators such as cytokines (e.g. IL3, IL-5 and IL-13), chemokines (e.g. eotaxin and RANTES), adhesion molecules (e.g. ICAM-1, VCAM-1 and integrins), growth factors (e.g. GM-CSF) and leukotrienes [Bachert et al., 1999].

There is a good amount of evidence that topical as well as systemic GCs are effective in reducing the size and symptoms (e.g. nasal blockage, rhinorrhoea and hyposmia) of NP [Ruhno et al., 1990; Small et al., 2005]. After surgery, high dosage of topical GC may reduce the incidence of NP recurrences or prolong the symptom-free time interval. Nevertheless, a substantial number of patients appear to be refractory to both topical and systematic GC treatments, or seem to develop a decreasing GC sensitivity during the medication period. The underlying mechanism for this glucocorticoid resistance has not been fully clarified; some reports suggested GR-beta isoform was increased in glucocorticoid insensitive patients and may interfere with GR-alpha function [Pujols et al., 2004]. Although the clinical efficacy of GCs in alleviating NP inflammation is prominent, the molecular mechanism has been poorly understood due to the pleiotropic effects of GCs on multiple signaling pathways. Therefore, to identify the candidate molecules which may contribute to beneficial or side effects of GCs will be helpful in developing GC therapy.

1.5.3.2 Antimicrobials

Several microorganisms have been investigated to indicate their role in NP. Antibacterials can control the bacterial infection effectively and their potential benefit in bilateral NP has been discussed by Bachert and Van Cauwenberge [Bachert & Van Cauwenberge, 2003]. It has been suggested that macrolide antibiotics not only decrease the colonizing bacteria, but also perform anti-inflammatory activities: (i) in a clinical study, an improvement in 52% of 20 NP patients was observed after treating with roxithromycin, one macrolide antibiotic, 150 mg/day for at least 8 weeks [Ichimura et al., 1996]; (ii) Nonaka demonstrated that roxithromycin, could directly suppress NP

fibroblast proliferation [Nonaka et al., 1999]; (iii) Yamada reported the three-month administration of macrolides in patients with NP resulted in reduced polyp size paralleled by a decrease of local IL-8 level [Yamada et al., 2000]; (iv) Iino showed that macrolides enhance CD80 positive macrophages which was negatively correlated with eosinophil infiltration in NP [Iino et al., 2001].

As discussed in **Chapter 1.4.4**, the possible role of *S.aureus* enterotoxins in the pathogenesis of NP calls for placebo-controlled clinical trials to confirm if antibiotics could be effective for this pathogen in NP. However, it should be noted that low-dose and long-term treatment with antibiotics may also induce bacterial resistance, which may limit this approach.

1.5.3.3 Antihistamines

Histamine, which be released from mast cells in NP, significantly increases the expression of intracellular adhesion molecule (ICAM)-1 and HLA-DR in epithelial cells. However, their use in patients with only polyps has not been extensively studied. Several studies have shown that *in vitro*, antihistamines significantly inhibit the leukotriene (LT) C4/D4, LTB4, prostaglandin (PG) D2, TNFα and GM-CSF in NP cell cultures in a dose-dependent manner [Carayol et al., 2002; Crampette et al., 1996]. One clinical study reported that after polypectomy, NP patients were treated with antihistamines (20mg, twice daily) for 3 months [Haye et al., 1998]. The results showed that the number and size of polyps remained unchanged, while nasal sneezing and rhinorrhea were effectively relieved, and nasal obstruction was reduced in the later part.

1.5.3.4 Possible future medical treatment approaches

Consistent with current knowledge on the pathophysiology of NP, new therapeutic approaches may focus on eosinophilic inflammation, eosinophil recruitment, the T cell as the orchestrating cell and IgE antibodies, as well as on tissue destruction and remodeling processes. Some interesting markers may act as possible drug targets, such as IL-5 antagonists, chemokine receptor 3 and eotaxin antagonists, IL-4 and IL-13 antagonists, local IgE antagonists, matrix metalloproteinase inhibitors.

1.5.3.5 Surgical operation

Surgical polypectomy is the preferred treatment for NP with many patients undergoing repeated operations. Functional endoscopic sinus surgery (FESS) is the standard surgical treatment of NP nowadays, and it has been proven to improve the quality life of the patients satisfactorily [Uri et al., 2002]. Whereas, some studies have shown that the effect of surgery had no significant difference from medical treatment [Lildholdt et al., 1988]. One recent clinical study showed the sense of smell improved after treatment with systemic and topical GCs, and surgery had additional beneficial effects on nasal obstruction and secretion [Blomqvist et al., 2001]. The study suggests that surgical treatment is applicable if nasal obstruction is the main problem which cannot be relieved by GCs, and surgery should be based on the patient's symptoms rather than the examiner's polyp score. However, to date there is too little data available to determine if there is any difference between surgery and GC therapy in the long-term outcome of patients with NP, especially their benefits on the recurrence of NP.

Chapter 2. Objectives and Significance

2.1 Research questions

The pathogenesis of NP is highly complicated due to its heterogeneity. Fundamental research on the pathogenesis of NP is hampered by two main problems: (i) various clinical phenotypes influence the nature of NP; (ii) whether NP should be considered a local disease or a local manifestation of a systemic disease. Although numerous papers have dealt with the pathogenesis of this nasal disease, the molecular/cellular alterations required for its development and progression are poorly understood. Several research questions concerning the pathogenesis and treatment of NP have been raised in this thesis.

(1) Are Staphylococcus aureus and its superantigens involved in inflammation of Asian NP?

Staphylococcus aureus (*S. aureus*) and its superantigen have been thought to result in Th2 shift, eosinophil activation, and overproduction of IgE observed in NP (**Chapter 1.4.4,** *Page 9*). Most superantigen studies have focused on Caucasian NP patients, who show eosinophil dominant polyps with less abundant neutrophils [Bachert et al., 2008]. However, NP in Asian patients has been found to show a neutrophilic pattern with a relative lack of eosinophil-dominated inflammation [Jareoncharsri et al., 2002]. In addition, our previous results demonstrated a combined cell infiltration with eosinophils, neutrophils and CD4+/CD8+ T lymphocytes in Asian patients with NP [Hao et al., 2006]. Importantly, activation of neutrophils is likely associated with innate immune defense which counteract the microbial infection. Hence, the evidence raises an issue whether *S. aureus* and its superantigens have some role in this differential

infiltration of inflammatory cells in Asian NP.

(2) Is methylation of tumor suppressor genes associated with NP inflammation?

NP has not been considered a cancer-prone lesion, but the hyperplasia feature (especially increase of squamous cells) and high recurrence rate of NP raises the question as to whether NP would share some pathological mechanisms with those malignant (e.g. nasopharyngeal carcinoma, NPC) and benign (e.g. inverted papilloma, IP) neoplasms in upper respiratory tissues. Promoter methylation of tumor suppressor genes (TSGs) is a major molecular defect in cancers and is associated with loss of protein expression in cancer cells [Jones et al., 2001]. Patients with cancer-prone chronic inflammatory diseases, such as ulcerative colitis, gastritis, and some hyperplastic polyps showed hypermethylation in several TSGs [Abraham et al., 2004; Chan et al., 2002;]. In addition, it was shown that inflammation mediated cytokine damage can alter the methylation pattern and critical gene regulation [Valinluck et al., 2007]. This evidence suggests that DNA methylation is a mechanism that could link inflammation with tumorigenesis.

In nasopharyngeal carcinoma, a high frequency of epigenetic inactivation of TSGs was confirmed [Kwong et al., 2002]. The methylation profile of TSGs in inverted papilloma has not been intensively studied, and only one report demonstrated that the cell cycle marker, *CDKN2B* was frequently methylated in inverted papilloma [Stephen et al., 2007]. In regard to the hyperplastic property of NP, it is worth studying whether methylation of some TSGs could occur in NP, although NP is generally considered a benign proliferative lesion. As far as we know, the methylation status of TSGs has not been reported in NP.

(3) What are the molecular evidences underlying the histopathologic patterns of NP as well as the GC effects on NP?

NP represents severe inflammatory cell infiltration and structure remodeling. A number of molecular/chemical mediators have been shown to be associated with NP histopathologic features (reviewed in **Chapter 1.4.6,** *Page 15*). However, most of these NP studies investigated inflammatory mediators objectively and could not elicit an overall picture of the molecular profile of NP. Hence, to explore the genes as well as their interaction network underlying the NP pathogenesis, it has been necessary to identify a large number of differentially expressed genes simultaneously and novel disease related candidates in NP.

GCs are considered the most effective pharmacological therapy for chronic upper airway inflammation including NP and asthma [Fokkens et al., 2007; Bateman et al., 2008]. The predominant effects of GCs are to suppress eosinophil infiltration and relieve tissue damage and remodeling in NP by altering the expression of inflammation related genes. However, the underlying molecular mechanism of GCs in treating NP has not been fully elucidated.

DNA microarray technology consists of a matrix with attached sequences that allow simultaneous analysis of expression of panels of human genes. This provides unique opportunities to identify the change of disease-associated gene and analyze the effects of drug treatment on a genome-wide scale. In addition, the comparison of profiles of significant genes can highlight the involvement of both expected and unsuspected biological functions and pathways by utilizing advanced bioinformatics tools (e.g.,

pathway analysis).

Therefore, it is of interest to investigate the presence of genes differentially transcribed in human NP tissues and nasal mucosal control, and to analyze the alteration of gene expression in NP in response to oral GC treatment by using DNA microarray. Furthermore, the biological function and pathways associated with the micorarray-identified genes could also be explored by using bioinformatics software. So far, no report has shown the gene expression profiles in Asian NP and its response to oral GC treatment. Moreover, no integrated functional pathway analysis has been performed in previous NP studies.

2.2 Aims of the study

The principal aims of this thesis are to investigate the molecular/cellular mechanisms underlying the pathogenesis of NP as well as the response of NP to GC treatment.

Specific aims of the thesis are as follows:

(1) To test the superantigen hypothesis in Asian NP. (Chapter 4)

(2) To study if methylation of tumor suppressor genes has some role in the hyperplasia of NP. (Chapter 5)

(3) To identify the candidate genes/gene families underlying the development of NP as well as the response of NP to GC treatment. (Chapter 6)

(4) To explore the biological functions and interactive pathway among the disease-related genes and the GC-responsive genes in NP. (Chapter 6)

2.3 Significance

Most NP studies have been performed in Caucasian patients, but Asian (especially Chinese) NP may present different histopathological features, indicating the underlying molecular mechanisms of Chinese NP may be different from those of Caucasian NP. Our studies systemically study the pathogenic mechanisms of NP from Asian/Chinese patients at molecular and cellular levels: (i) superantigen study can elicit the involvement of *Staphylococcus aureus* and its superantigens in Asian NP; (ii) methylation study can provide novel information for possible epigenetic alterations of the commonly reported TSGs in a benign outgrowth situation in upper airway tissues; (iii) microarray study is applicable to identify disease/therapeutic candidates as well as their functional networks, and then to enhance the knowledge of the pathophysiology and improve the GC management in NP.

Chapter 3. Materials and Methods

3.1 Study Subject 1 (for superantigen and methylation studies)

Fresh NP tissues were obtained from 24 patients (19 males and 5 female, aged from 21 to 58 years old, median age of 44) with unilateral/bilateral NP. They were scheduled for functional endoscopic sinus surgery at the Department of Otolaryngology, Head & Neck Surgery in the National University Hospital of Singapore. A control biopsy of inferior turbinate (IT) mucosa was obtained from 10 non-NP patients (7 males and 3 females, aged from 18 to 56 years old, mean age of 24), who were scheduled for septal plastic surgery due to septal deviation in the same hospital. All NP and control subjects did not have aspirin intolerance and asthma diagnosed by means of clinical history, and did not take either any form of antibiotics or GCs within 3 months before the study. In addition, three nasal inverted papilloma (IP) samples were obtained after being reviewed by a pathologist to confirm diagnosis, and none of the patients with IP had evidence of carcinoma. Papilloma biopsies from these IP patients were only used in methylation study. Three to five milliliters of peripheral blood were taken during surgery in all NP and IP patients as well as controls. Cases were coded to provide confidentiality. Fresh specimens were dissected into two sections: one section was quickly frozen by liquid nitrogen for DNA analysis; and the other section was wrapped with tissue freezing medium and then frozen by liquid nitrogen for histo-immunohistological examination. All samples were preserved at -80℃ until use.

A signed informed consent was obtained from the study patients before surgery. Approval to conduct this study was granted by the National Medical Research

Council of Singapore and the Institutional Review Board of the Medical Faculty of National University of Singapore.

3.2 Study Subject 2 (for gene expression study)

In this prospective study, NP patients and non-NP controls were recruited from the Otorhinolaryngology Hospital, the First Affiliated Hospital, Sun Yat-Sen University. All subjects are Chinese. Twelve patients (9 males and 3 females; median age of 36; age range, 18-55 years) with bilateral NP that have refractory nasal obstruction requiring functional endoscopic sinus surgery (FESS) were included in this study. The diagnosis of NP was based on medical history and clinical examinations. Two sets of polyp biopsies were taken from the same patient, *i.e.,* before the initiation (GC-naïve) and after (GC-treated) the oral prednisone treatment (10 mg thrice per day for 3-5 days). Biopsy of inferior turbinate was obtained from control subjects (7 males and 3 females; median age of 28 years; age range, 23-36 years) who underwent surgery for nasal septal deviation. This tissue served as nasal mucosal control. None of the patients and controls had upper respiratory infection nor undertook any forms of GCs and antibiotics for more than three months before the study. All subjects did not have history of aspirin exposure, asthma, and cystic fibrosis. Cases were coded to provide confidentiality. Fresh specimens were dissected into two sections: one section was preserved with RNA*later* (Ambion, Austin, TX) for gene expression profiling and the other section was fixed in formalin for histological evaluation. All patients were recruited through physician referrals. Approval to conduct this study was obtained from the Institutional Review Board of the First Affiliated Hospital, Sun Yet-Sen University and the National University of Singapore.

3.3 Allergy test

Serum samples from all study subjects were analyzed for total IgE levels, Phadiatop assay, and specific IgE (sIgE) to a common panel of inhalant allergens, including *Dermatophagoides pteronyssinus*, *Dermatophagoides farianae*, *Aspergillus fumigatus*, cockroach, common pollen, and ragweed mixtures. IgE test was carried out by using the ImmunoCAP system (Phadia AB, Uppsala, Sweden). The results were evaluated following manufacture's instruction: a Phadiatop PAU/l (Pharmacia Arbitrary Units/l) value ≥ 0.35 was considered as atopy.

3.4 DNA extraction

Genomic DNA was extracted from frozen solid tissues and peripheral blood by using the Gentra Puregen Kit (Gentra Systems, Suite, MN). Both solid tissues and peripheral blood were collected from study subject 1 as indicated in **Chapter 3.1**.

3.4.1 Extraction from solid tissues

Solid tissues (including NP, IT, and IP) were frozen by using liquid nitrogen and then stored at -80℃ until used. DNA extraction followed the manufacturer's protocols: (i) solid tissue was merged in Cell Lysis Solution followed by homogenizing; (ii) homogenate was treated with Proteinase K Solution and then incubated overnight; (iii) RNasae treatment (by using RNase A Solution) followed by protein precipitation (by using Protein Precipitation Solution) was done in cell lysate; (iv) DNA was precipitated was by using by using 100% Isopropanol (Sigma Aldrich, St.Louis, MO); (v) DNA hydration was used by DNA Hydration Solution. DNA concentration and purity were determined by using NanoDrop™ 1000 Spectrophotometer (Thermo

Fisher Scientific, Waltham, MA). DNA was then stored at -20℃.

3.4.2 Extraction from peripheral blood mononuclear cell (PBMC)

Clotted whole blood from both patients and controls was separated into serum and cellular components, both of which were stored at -20℃ until use. DNA extraction from PBMC was performed according to the manufacturer's protocol: (i) blood clot was dispersed using a Clotspin™ basket; (ii) red blood cells were removed using RBC Lysis solution; (iii) cell pellet was lysed using Cell Lysis Solution with Priteinase K and then incubated overnight; (iv) protein precipitation was collected using Protein Precipitation Solution in cell lysate; (v) DNA was precipitated using 100% Isopropanol (Sigma Aldrich); (vi) DNA hydration was performed using DNA Hydration Solution. DNA concentration and purity were determined by using NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific). DNA was then stored at -20℃.

3.5 Experiments for superantigen study

3.5.1 Standard polymerase chain reaction (PCR)

The DNA (both solid tissues and PBMC) from Study Subject 1 (except the patients with IP) were selected. DNA from bacterial strain Oxford Heatley NCTC 657 was used as a positive control (kindly provided by Dr. Taylor, Department of Microbiology, NUS). Primers for the *S.aureus* related superantigens and nuc gene were described in **Table 3.1**. The PCR mixture contained 1x PCR Buffer II (Applied Biosystems, Foster City, CA), 3 mM $MgCl_2$, 0.2 mM dNTP, 0.5 μM each primer (sense and antisense), 1 U Taq-Gold polymerase (Applied Biosystems), and 1μl DNA template in a final volume of 25 μl. Reactions were initially denatured at 94℃ for 5 minutes followed by

35 cycles (30 sec at 94℃, 30 sec at 55℃, and 60 sec at 72℃) and finally followed by a 5-min extension at 72℃. Negative control without DNA template was performed for each set of PCR. PCR products were separated by electrophoresis on agrose gel with proper concentration (from 1% to 1.8%), stained with ethidium bromide, and visualized under UV illumination.

Table 3.1 Primers for the detection of *S.aureus* related superantigens and *nuc* gene

Primer sets	Forward primer 5' to 3'	Reverse primer 5' to 3'	Size (bp)	Ref *
SEA	TTGGAAACGGTTAAAACGAA	GAACCTTCCCATCAAAAACA	120	1
SEB	TCGCATCAAACTGACAAACG	GCAGGTACTCTATAAGTGCC	478	1
SEC	GACATAAAAGCTAGGAATTT	AAATCGGATTAACATTATCC	257	1
SED	CTAGTTTGGTAATATCTCCT	TAATGCTATATCTTATAGGG	317	1
SEE	TAGATAAAGTTAAAACAAGC	TAACTTACCGTGGACCCTTC	170	1
TSST-1	ATGGCAGCATCAGCTTGATA	TTTCCAATAACCACCCGTTT	350	1
SEG	TGCTATCGACACACTACAACC	CCAGATTCAAATGCAGAACC	704	2
SEH	CGAAAGCAGAAGATTTACACG	GACCTTTACTTATTTCGCTGTC	495	2
SEI	GACAACAAAACTGTCGAAACTG	CCATATTCTTTGCCTTTACCAG	630	2
NUC	GCGATTGATGGTGATACGGTT	AGCCAAGCCTTGACGAACTAAAGC	279	3

*Ref:

1. Johnson et al., 1991

2. Mclauchlin et al., 2000

3. Brakstad et al., 1992

3.5.2 Direct sequencing

The PCR product amplified by primers of *nuc* gene was subjected to direct

sequencing analysis. The PCR mixture contains 1X PCR Buffer II (Applied Biosystems), 3mM $MgCl_2$, 0.2mM dNTP, 0.5μM each primer (sense and antisense), 1U AmpliTaq polymerase (Amplied Biosystems), and 100 ng DNA template in a final volume of 25μl. Reactions were initially denatured at 94℃ for 5 minutes followed by 40 cycles (30 sec at 94℃, 30 sec at 55℃, and 30 sec at 72℃), and finally followed by a 5-min extension at 72℃. PCR products were separated by electrophoresis on 1.8% agrose gel, stained with ethidium bromide, and visualized under UV illumination. The correct size band was excised from agrose gel, purified by using the GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences) following the manufacture's instruction. Purified PCR product was subjected to cycle sequencing PCR by using ABI Prism BigDye® Terminator v3.1 (Applied Biosystems) and reverse primer of *nuc* gene. The cycle sequencing PCR product was then sent to service lab for sequencing.

3.6 Experiments for methylation study

3.6.1 Bisulfite modification of DNA

Purified DNA (both solid tissues and PBMC) and CpGenome Universal Methylated DNA (Chemicon International, Temecula, CA) were subjected to bisulfite modification by using EZ DNA Methylation-Gold Kit™ (Zymo Research Corporation, Orange, CA). Briefly, 1 μg of DNA was denatured by a thermal cycle (98°C /10 min, 64°C /2.5 h) with CT Conversion Reagent (Zymo Research Corporation). Denatured DNA was applied in column for desulphonation and clean-up. Finally the DNA was eluted in M-Elution Buffer (Zymo Research Corporation) and stored at -20°C.

3.6.2 Methylation-specific PCR (MSP)

Primer were designed at the promoter regions and described in **Table 3.2**. The PCR

mixture contained 1x PCR Buffer II (Applied Biosystems), 2 mM $MgCl_2$, 0.2 mM dNTP, 0.6 μM each primer (sense and antisense), 1.875 U Taq-Gold polymerase (Applied Biosystems), and 25ng bisulfite treated DNA template in a final volume of 12.5 μl. Reactions were initially denatured at 95℃ for 8 minutes followed by 35 to 40 cycles (30 sec at 94℃, 30 sec at the annealing temperature listed in **Table 3.2**, and 30 sec at 72℃) and finally followed by a 3-min extension at 72℃ [Tao et al., 1999]. CpGenome Universal Methylated DNA was used as a methylation-positive control, while negative control without DNA template was performed for each set of PCR. PCR products were separated by electrophoresis on 1.8% agrose gel, stained with ethidium bromide, and visualized under UV illumination.

Table 3.2 MSP and BGS primers

Primer Set*	Forward primer 5' to 3'	Reverse primer 5' to 3'	Size (bp)	AT†(℃)/ cycles
MSP-*p16* M [a]	TTATTAGAGGGTGGGGCGG ATCGC	GACCCCGAACCGCGACCG TAA	150	65/35
MSP-*p16* U [a]	TTATTAGAGGGTGGGGTGG ATTGT	CAACCCCAAACCACAACC ATAA	151	60/40
MSP-*RASSF1 A* M[b]	GGTTTTTTTTAGTTTTTTTTC GTC	CTACCGTATAAAATTACA CGCG	269	57/40
MSP-*RASSF1 A* U[b]	TGGTTTTTTTTAGTTTTTTTT TGTT	ACTACCATATAAAATTAC ACACA	271	57/40
MSP-*PTPN6* M [b]	TGTAGTTTTTAGTGTCGGGT C	CCC CAA AAA ATC GAC CGC G	123	58/40
MSP-*PTPN6* U [b]	ATGTAGTTTTTAGTGTTGGG TT	TCC CCC AAA AAA TCA ACC ACA	126	58/40
MSP-*TSLC1* M [b]	TAGTATTTTATTAGTTGTTCG TTC	TATAAAAATCAATACCGCG ACG	194	58/38
MSP-*TSLC1* U [b]	TTAGTATTTTATTAGTTGTTT GTTT	CTATAAAAATCAATACCAC AACA	196	58/38
MSP-*CDH1* M [c]	GGTGAATTTTTAGTTAATTA GCGGTAC	CATAACTAACCGAAAACG CCG	204	56/37
MSP-*CDH1* U [c]	GGT AGG TGA ATT TTT AGT TAA TTA GTG GTA	ACCCATAACTAACCAAAA ACACCA	211	56/37
MSP-*DAPK1* M [d]	GGATAGTCGGATCGAGTTA ACGTC	CCCTCCCAAACGCCGA	98	58/37
MSP-*DAPK1* U [d]	GGAGGATAGTTGGATTGAG TTAATGTT	CAAATCCCTCCCAAACAC CAA	106	58/37
BGS-*PTPN6* [b]	TTAAATTTTTTGAGTTTTAG GTTTT	CCTCAAATACAACTCCCA ATA	581	58/37
BGS-*TSLC1* [b]	ATGTTATTAGTATTTTATTAG TTGTT	CACACCTACCTATAA AAATCAATA	211	57/37
BGS-*CDH1* [e]	GAATTTAGTGGAATTAGAAT YGTGT	CTCCAAAAACCCATAACT AACCR	372	57/37
BGS-*DAPK1* [e]	GAGTGTGAGGAGGATAGTY G	TAACCTTCCCAATTACTCR AAA	351	57/37

* M, methylated sequence; U, unmethylated sequence.

† AT, annealing temperature.

[a] *p16* MSP primer sequences described by Herman *et al*. [Herman et al., 1996]; Genbank accession numbers: X94154.

[b] *RASSF1A, PTPN6* and *TSLC1* MSP or BGS primers designed by us; Genbank accession numbers: *RASSF1A*, AF102770; *PTPN6*, AB079851; *TSLC1*, AP003174.

[c] *CDH1* MSP primers described by Graff *et al.* [Graff et al., 1997]. Genebank accession number: L34545.

[d] *DAPK1* MSP primers described by Katzenellenbogen *et al.* [Katzenellenbogen et al., 1999]; Genbank accession numbers: AL161787.

[e] *CDH1* and *DAPK1* BGS primers designed by using "Primo MSP 3.4" software (Chang Bioscience, Inc., USA).

3.6.3 Bisulfite genomic sequencing (BGS)

BGS primers for the indicated genes were shown in **Table 3.2**. The PCR mixture contained 1x PCR Buffer II (Applied Biosystems), 2 mM $MgCl_2$, 0.2 mM dNTP, 0.6 μM each primer (sense and antisense), 1.875 U Taq-Gold polymerase (Applied Biosystems), and 100 ng bisulfite treated DNA template in a final volume of 25 μl. PCR condition is the same as that described in MSP. The PCR products amplified with BGS primers were separated by electrophoresis on agrose gel. The band of the interest was carefully excised from the gel, and the gel slice was placed into the Spin-X® Centrifuge Tube Filters (Corning Incorporated Life Sciences, Lowell, MA) and mixed with distilled water. The DNA was eluted by centrifuging the tube at 13,000 × g for 10 minutes at room temperature.

Eluted DNA was cloned into pCR2.1-TOPO® vector by using TOPO® TA Cloning kit (Invitrogen, Carlsbad, CA). In brief, the steps include: (i) TOPO® cloning reaction – mix DNA with TOPO® vector; (ii) One Shot® Chemical transformation – mix the TOPO® cloning reaction with One Shot® Chemically Competent *E.coli*; (iii) incubation – incubate the transformants on Luria Broth (LB) plates containing ampicillin; (iv) selection of clones – randomly pick the white colonies; (v) isolation of plasmid DNA – use Wizard® Plus SV Minipreps kit (Promega, Madison, WI). Isolated plasmid DNA was subjected to cycle sequencing PCR by using ABI Prism BigDye® Terminator v3.1 (Applied Biosystems) and M13 reverse primers supplied by TOPO® TA Cloning kit. The PCR product was then sent to service lab for sequencing.

3.7 Experiments for gene expression study

3.7.1 RNA extraction from nasal tissues

Total RNA was extracted from solid tissues (NP and IT) by using RiboPure kit (Ambion), according to the manufacture protocol. Briefly, the procedures were described as follows: (i) tissues were removed from RNA*later* and submerged in TRI Reagent® (Molecular Research Center Inc, Cincinnati, OH), followed by homogenizing; (ii) chloroform was added in homogenate and centrifuge at 12,000 × g to separate the mixture into a lower, red, organic phase, an interphase, and a colorless, upper aqueous phase; (iii) RNA remains in the aqueous phase while DNA and proteins are in the interphase and organic phase; (iv) aqueous phase was transferred into a filter cartridge-collection tube and RNA was binding to the filter; (v) after washing steps, the RNA was eluted through the filter.

3.7.2 Quantification and gel electrophoresis of RNA

Total RNA was quantified by NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific). The overall quality of total RNA can be assessed by electrophoresis on a denaturing agarose gel. To prepare the gel, 0.5 g agarose was dissolved in 1×MOPS running buffer (Ambion) mixed with 37% formaldehyde (12.3 M). 1 μg of total RNA was mixed with 1×volume Formaldehyde Load Dye (Ambion) and ethidium bromide (10 μg/ml), and then RNA samples were heated to denature at 65℃. Load the gel and electrophoresis at 5-6 V/cm. Visualize the gel on a UV transilluminator (UVP, Upland, CA). The RNA samples with good integrity which showed the ratio of 28S rRNA: 18S rRNA at least 1.2:1 in gel picture were selected for downstream work (microarray and real-time RT PCR). This quality assessment led to exclude 2 pairs of NP samples and 4 controls.

3.7.3 Microarray experiment

Ten pairs of NP samples (before and after GC treatment) and six controls were recruited in microarray experiment. Each subject was analyzed with a single gene chip. Preparation of cDNA, labeled cRNA, hybridization, staining, and scanning of Human Genome U133 Plus 2.0 (HG_U133 plus 2.0) arrays (Affymetrix, Santa, Clara, CA) was performed according to the technical manual outlined by Affymetrix (http://www.affymetrix.com/ Auth/ support/ downloads /manuals/ expression_ever_manual.zip)". **Figure 3.1** shows the flowchart of the microarray experiment procedures. Briefly, 1 μg of total RNA and diluted poly-A RNA controls were reverse transcribed to double-stranded cDNA using an T7-oligo (dT) primer (Affymetrix). *In vitro* transcription from the double-stranded cDNA was carried out using IVT Labeling Kit (Affymetrix). The biotinylated cRNA (20 μg) was fragmented by using 5× Fragmentation Buffer (Affymetrix). The fragmented cRNA was hybridized for 16 h at 45°C in a Hybridization Oven 640 (Affymetrix) to a HG-U133 plus 2.0 chip (Affymetrix). Washing and staining of the arrays with phycoerythrin-conjugated streptavidin (Molecular Probes, Eugene, OR) was completed in a Fluidics Station 450 (Affymetrix). The arrays were then scanned using a confocal laser GeneChip Scanner 3000 and GeneChip Operating Software (Affymetrix).

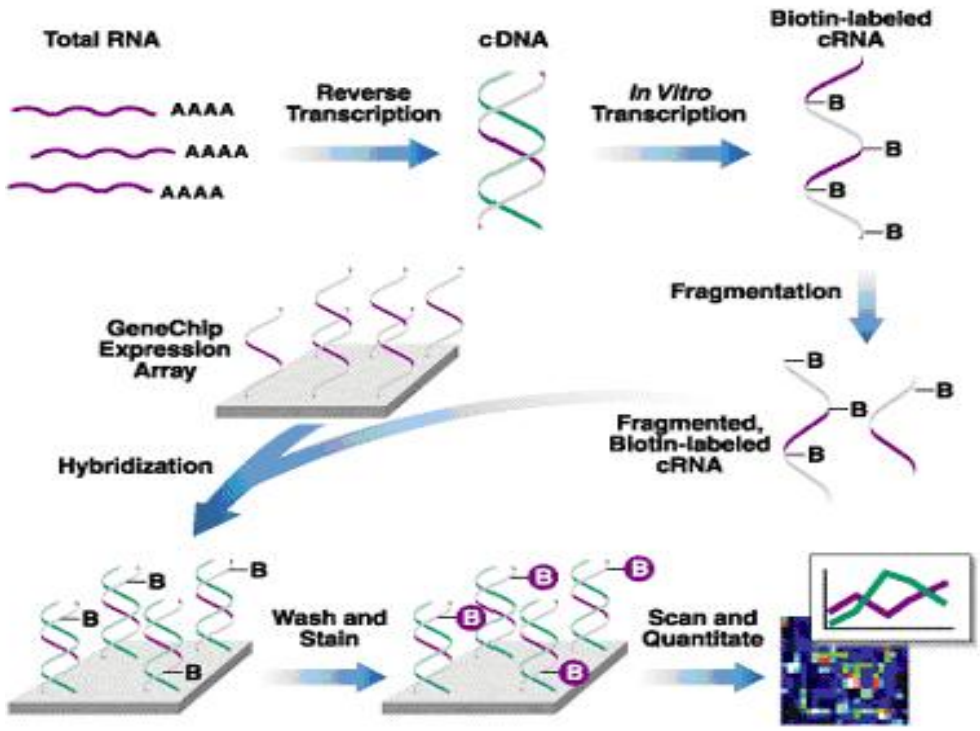


Figure 3.1 Flowchart of affymetrix gene chip experiment. Labeled cDNA or cRNA targets derived from the mRNA of a tissue are hybridized to nucleic acid probes attached to the gene chips. This picture is cited from Paszek, E. Introduction, Connexions Web site. http: // cnx.org/ content/ m12370/ 1.3/, Sep 13, 2004.

3.7.4 Quality control (QC) assessment for microarray experiment and data

3.7.4.1 QC for fragmentation

The unfragmented cRNA and fragmented cRNA were loaded into agarose gel with tris-acetate EDTA (TAE) buffer. cRNA with no more than 300 bp size was considered to be successfully fragmented and can be used in hybridization step.

3.7.4.2 QC for assay performance

To achieve the quality assessment for assay/hybridization performance, several control parameters were evaluated in data report (.rpt file) following the criteria outlined in “data analysis fundamentals” (https://support/downloads/manuals/ data_ analysis_ fundamentals_ manual.pdf). The control parameters include: (i) average

background and noise values; (ii) Poly-A controls; (iii). hybridization controls; (iv) internal control genes; (v) percent present; (vi) scaling factor.

The average background and noise values represent the electrical noise of the scanner and sample quality. Affymetrix guideline recommends that typical average background values range from 20 to 100 for arrays scanned with the GeneChip Scanner 3000. Although each scanner has a unique inherent electrical noise associated with its operation, array data acquired from the same scanner should ideally have comparable noise values.

Poly-A RNA controls can be used to monitor the entire target labeling process, and all of the Poly-A controls should be called "Present" with increasing signal values in the order of *lys, phe, thr,* and *dap*. Hybridization controls can be used to evaluate sample hybridization efficiency, and all of the controls should be called "Present" with increasing signal values in the order of *bioB, bioC, bioD,* and *cre*, reflecting their relative concentration.

The 3' to 5' ratio of the internal control genes (β-actin and GAPDH) in each sample should not be more than 3, which indicate intact RNA and efficient transcription of double-strand cDNA. Compared array samples should have similar percent present values, and extremely low percent present values are a possible indication of poor sample quality.

Differences in overall intensity are most likely due to assay variables including pipetting error, hybridization, washing, and staining efficiencies, which are all

independent of relative transcript concentration. Scaling/normalization method can correct these variables. Therefore, larger discrepancies among scaling/normalization factors of the studied arrays (e.g. 3-fold or greater) may indicate significant assay/biological variability or sample degradation, which leads to noisier data.

3.7.4.3 QC for raw array data

In Affymetrix GeneChip (HG-U133 Plus 2.0™), oligonucleotides of 25 base pairs in length are used to probe genes. There are two types of probes: reference probes that match a target sequence exactly, called the perfect match (PM), and partner probes which differ from the reference probes only by a single base in the center of sequence, so called mismatch (MM) probes. Typically 11 of these probe pairs, each interrogating a different part of the 3'-end sequence for a gene, make up what is known as a probeset. The intensity information from the values of each of the probes in probeset are combined together to get an expression measure.

RMAexpress software version 1.0 Release (Free version available in http://rmaexpress.bmbolstad.com/) can analyze the unadjusted PM intensities of Affymetrix array data. The undjusted PM intensity of raw array data could be visualized by the "RMAExpress Raw Data Visualizer" window. Two output plots available: boxplots and density plots. Potential low quality data often present higher/lower PM intensity than the other arrays in the dataset in boxplots; while in density plots, array data with low quality are often shifted away from the main set of density curves.

3.7.4.4 Data normalization by Robust Multichip Average method

The need for normalization arises naturally when dealing with experiments involving multiple arrays. To reduce the variation that is introduced during the process of carrying out the experiment, a total of 54,000 probesets, representing approximately 38,500 genes on the HG-U133 Plus 2.0™ arrays from three Affymetrix probe-level datasets were underwent normalization. The three datasets are as follows: (i). GC-naïve NP (8 chips) versus controls (5 chips); (ii). GC-treated NP (8 chips) versus controls (5 chips); (iii). GC-treated NP and GC-naïve NP from the same patient (8 pairs of chips). Each set was normalized by the Robust Multichip Average (RMA) method [Irizarry et al. 2003], available in RMAexpress software version 1.0 Release (Free version available in http://rmaexpress.bmbolstad.com/). RMA method does not depend on the choice of a baseline array, but rather treat PM and MM all as intensities that need to be normalized. The processing steps of RMA consist of a background adjustment, quantile normalization, and the median-polish summarization method, producing a single normalized expression set for each comparison. Therefore, RMAExpress computed expression value for each probeset and exported the results in log scale. Detail instruction of RMAexpress software can be referred to its Users Guide (http://rmaexpress.bmbolstad.com/RMAExpress_UsersGuide.pdf).

3.7.4.5 QC for normalized array data

In addition to normalizing array data, RMAExpress software can also be used to carry quality assessment for normalized array data. Two main QC options were available in RMAExpress software: chip pseudo-images of residues and probe-level model (PLM) based quality statistics. The detailed instruction and algorithm of these two options are available in the published book [Bolstad et al., 2005] and the software document

(http://bioconductor.org/packages/2.1/bioc/vignettes/affyPLM/inst/doc/QualityAssess.pdf.)

When selecting the "Store Residuals" option from the software dialog box, chip pseudo-images are generated. Poor quality data typically has large intense patches of a single color in distinct regions. The typical images, both of good and poor quality, are listed in the online resource http://PLMImageGallery.bmbolstad.com.

When using "PLM" as summarization method instead of "median-polishing" in the software dialog box, the QC statistics can be examined. Two PLM methods are used in RMAExpress: Relative Log Expression (RLE) and Normalized Unscaled Standard Errors (NUSE). Specifically, RLE values are computed for each probeset by comparing the expression value on each array against the median expression value for that probeset across all arrays. On the other hand, NUSE estimates the standard error for each gene on each array across arrays so that the median standard error for that gene is 1 across all arrays. Both RLE and NUSE processes account for differences in the variability between genes. Typically arrays with poor quality show up with boxes that are not centered about 0 (for RLE) / 1(for NUSE) and are more spread out; hence, RLE and NUSE are useful in determining outliers among the normalized data.

3.7.5 Statistical analysis by Significant Analysis of Microrarray (SAM)

Three RMA normalized data sets were then imported to Microsoft Excel spreadsheets, respectively, and formatted for analysis by the Significant Analysis of Microrarray (SAM) software, version 3.00 (Free version available in http://www-stat.stanford.edu/~tibs/SAM/) [Tusher et al., 2001]. Differentially expressed genes in each comparison

were identified by using SAM. SAM computes a statistic measuring the strength of the relationship between gene expression and a response variable, while taking into account the multiple testing nature of a microarray experiment. SAM method accepts normalized expression data sets and identifies statistically significant changes in gene expression by assigning each gene a score (called "*d* score") based on its change in expression relative to the standard deviation of repeated measurements. Genes with score greater than a threshold, as determined by a tuning variable Δ, are deemed potentially significant. The percentage of such genes identified by chance is the false discovery rate (FDR). SAM uses permutations of empirical measurements to estimate the FDR for the called list in the form of a 90% confidence interval. FDR controls the expected proportion of incorrectly rejected null hypotheses (type I errors). In addition, fold change has also been indicated in each probeset ID in SAM generated gene list. The options selected for the SAM analysis: (i) response type: two-class, unpaired data (comparison of GC-naïve/GC-treated NP versus control); two-class, paired data (comparison of NP without versus with GC treatment); (ii) data logged: logged (base 2); (iii) number of permutations: 5000. Hence, genes identified with FDR of 0.8%/0.7% in the datasets of GC-naïve/GC-treated NP vs. control and with a FDR of 6% in the dataset of GC-treated vs. GC-naïve NP were deemed significant if they passed a 1.5-fold change filter [Liu et al., 2004].

How to define and control the FDR has been hotly debated, since each dataset is particular and must be treated as a special case. Based on the literature, we could not find the any widely accepted FDR bench mark. Therefore, to estimate a proper FDR for the given dataset, we have compared different gene lists based on different FDR value issued to the dataset.

With regard to the gene lists generated from the datasets of GC-naïve NP vs. control and GC-treated NP vs. control, the cut-off of FDR was defined strigenently low, otherwise there would be huge amount of significant genes. In contrast, the threshold of FDR for the dataset of GC-treated vs. GC-naïve NP was defined a little bit higher since the number of GC-responsive genes was not large. Then we have tried to use FDR 0.005, 0.006, 0.007, 0.008, 0.009, and 0.01 to the datasets of GC-naïve NP vs. control and GC-treated NP vs. control; while used FDR 0.05, 0.06, 0.07, 0.08, 0.09, and 0.10 to the dataset of GC-treated vs. GC-naïve NP.

To compare the gene lists generated based on different FDR values, we always looked for those genes assigned with the cut-off FDR value (here we called them "marginal genes"), i.e., examining the genes with FDR 0.005 in the gene list generated from FDR 0.005. Some parameters of these "marginal genes" were examined in order to evaluate the suitability of the prescribed FDR: (i). the SAM score; (ii). the expression level (i.e., extent of detection signal); (iii). the biological sense. For example, if the "marginal genes" with FDR 0.005 matched the criteria of high SAM score (> 2.0 or < -2.0), expression with detection signal, and biological sense, FDR 0.005 is proper for the dataset. Then we went to check the "marginal genes" from gene list with FDR 0.006, if it also passed the criteria, we looked for the FDR 0.007; in such sequence, we chose the next to last FDR which cannot pass the criteria. Finaly, FDR 0.008, FDR 0.007, and FDR 0.06 were considered to be suitable for the datasets of GC-naïve NP vs. control, GC-treated NP vs. control, and GC-treated vs. GC-naïve NP, respectively.

After generating the gene list based on proper FDR, we filtered these genes by fold

change. From the literature, 1.5- or 2-fold appears to be commonly used as bench mark. Hence, we also compared the gene lists filtered by 1.5-fold and 2-fold. In this case, we considered that 1.5-fold should be a suitable cut-off since it can guarantee most genes with biological sense.

3.7.6 Annotation analysis

Gene annotations of the analyzed transcripts were verified by means of web-based program Netaffx™ Analysis Center (http://www.affymetric.com/analysis/index.affx). This resource contains probe sequences and up-to-date gene annotations and allows researchers to quickly search for information of gene annotation, compare and refine results, and export data into Excel-friendly formats. Two filtration steps were performed: firstly, those transcripts which did not contain identified gene symbols were filtered; secondly, those genes with redundant probe identities and those transcripts with unknown gene ontology (such as those annotated with “chromosome open reading frame”, “hypothetical protein”, “KIAAs” and “family with sequence similarity”) were filtered.

3.7.7 Class predictor analysis

The SAM-generated significant genes from two datasets (GC-naïve NP vs. control and GC-treated vs. GC-naïve NP) with their identifiers were imported in GeneSpring software version 7.3 (Agilent Technologies, Santa Clara, CA) and subjected to class predictor analysis, including hierarchical clustering analysis and principal component analysis (PCA).

3.7.7.1 Cluster analysis

Tree view cluster, which is composed of gene tree and condition tree, is useful to explore the similarity among the genes and conditions (or say "samples", such as NP and control samples) [Eisen et al., 1998]. Gene trees are dendrograms used as a method of showing relationships among the expression levels of genes over a series of conditions. Condition trees like gene trees, instead of showing the relationships between genes, they show the relationships among the expression levels of conditions. Gene trees and condition trees were built up by selecting "Clustering>Gene Tree/Condition Tree" option from the Tools menu of the software. In both of these two "trees", spearman correlation was used in similarity measure, while average linkage was used in clustering algorithm. In addition, similar branches were merged by default and confidence levels of each branch were calculated by bootstrapping. Cluster analysis was also conducted in those 31 eosinophil associated genes by using the same algorithm.

3.7.7.2 Principal component analysis

PCA is a decomposition technique that produces a set of expression patterns known as principal component [Raychaudhuri et al., 2000]. It is helpful to find out how samples can be separated after the analysis. Liner combinations of these patterns can be assembled to represent the behavior of all of the conditions in a given dataset. PCA scores (or called eigenvalues, between -1 and 1) are calculated by computing the standard correlation between each condition's expression profile vector and each principal component vector (eigenvector). Eigenvalues can be representative of the level of explained variance as a percentage of total variance (reported under "Percent Variance" in the results window). The percent of variance explained is dependent on how well all the components summarize the data. To run PCA, just select "Principal

Components Analysis" from the Tools menu of the software and then choose "PCA on Conditions" tab. The output results can be chosen as two-dimensional (2D) plot.

3.7.8 Ingenuity Pathways Analysis (IPA)

The gene lists identified by SAM, containing affymetrix proset ID as transcript identifiers and corresponding expression values (*d* scores), were uploaded into the Ingenuity Pathway Analysis (IPA) tool (version 6.0, Ingenuity® Systems, www.ingenuity.com), a web-delivered application that enables the discovery, visualization, and exploration of molecular interaction networks in gene expression data. Each gene identifier was mapped to its corresponding gene object in the Ingenuity Pathway Knowledge Base (IPKB), which provided up-to-date high quality knowledge of pathway interactions in broad-genome-wide coverage. Functional analysis, network analysis, and Canonical Pathway analysis were executed by IPA.

3.7.8.1 Functional analysis

The database of IPA functional analysis currently has three primary categories: (i) Diseases & Disorders; (ii) Physiological System Development & Function; (iii) Molecular & Cellular Functions. There are 85 high level functional categories that are classified under these primary categories. Lower level functions are classified within the high level categories. Specific functions are the lowest level functions found in IPA. Each lowest level function has a population of associated molecules. The example for this hierarchical classification is: Molecular & Cellular Functions (primary category) > Cellular Movement (high level function) > Migration (lower level function) > Migration of Eukaryotic Cells (specific function). Three types of functional analysis can be performed in IPA: functional analysis for a dataset,

comparison of functional analysis, and functional analysis for a network. For more details, see the Ingenuity Functional Analysis *p*-value whitepaper (https:// analysis.ingenuity.com/ pa/ info/ help/ help.htm#ipa_help.htm).

(1) Functional analysis for a dataset

Genes from the dataset that were associated with biophysiological functions and/or diseases in the IPKB were considered for the functional analysis. Fischer's exact test was used to calculate a *p*-value determining the probability that each biophysiological function and/or disease assigned to that dataset is due to chance alone. In this method, the *p*-value for a given function is calculated by considering (i) the number of functional analysis molecules that participate in that function; (ii) the total number of molecules that are known to be associated with that function in IPKB. In general, *p*-value less than 0.05 indicates a statistically significant, non random association.

IPA then generated a series of significant high level functions in each primary category for each dataset. In the category of Molecular & Cellular Functions, top-10 high level functions were selected based on the rank of the significant level (*p*-value) assigned to function (**Appendix I,** *Page 265*). However, in the other two primary categories, some respiratory irrelevant diseases/disorders (e.g., reproductive system disease, ophthalmic disease and metabolic disease) and some respiratory irrelevant physiological development/functions (e.g. embryonic development, behavior and reproductive system development and function), no matter how significant or not, were filtered out first; consequently, the top significant functions of these two categories were selected according to the significant level assigned to the high level function (**Appendix I,** *Page 265*).

(2) Comparisons of functional analysis

To determine whether and to what extent a given high level function is affected from one observation to another within a comparison we can start by comparing the extent to which the significances change from one observation to another, i.e. if the significance of a function changes from one treatment (e.g., GC treatment) to the next, then it is likely that the treatment had an impact on the function under investigation.

The strategy to compare the GC treatment effects on the high level functions among these three datasets are described here: (i) Step 1, the functions in dataset of GC-naïve NP vs. control were set as baselines and these selected functions in other two datasets were automatically arranged side-by-side with the baselines by IPA software; (ii) Step 2, if the significant levels of the relevant functions in the dataset of GC-treated NP vs. control were much lower than the baselines, it means that GCs may have effects on the genes associated with these functions; (iii) Step 3, the functions found to be associated with GC effects in step 2 were referred to dataset of GC-treated vs. GC-naïve NP, if these functions showed similar or higher significant levels in dataset of GC-treated vs. GC-naïve NP as compared to dataset of GC-treated NP vs. control, it double confirms the GC effects on these functions in NP.

(3) Functional analysis for a network

The functional analysis of a network identifies the biophysiological functions and/or diseases that are most significant to the genes in the network. The network genes associated with biophysiological functions and/or diseases in the IPKB were considered for the analysis. Fisher's exact test was used to calculate a *p*-value

determining the probability that each biophysiological function and/or disease assigned to that network is due to chance alone. In general, *p*-values less than 0.05 indicate a statistically significant, non random association.

3.7.8.2 Network analysis

The uploaded significant gene lists were also subjected to network analysis by IPA. The molecules of interest which interact with other molecules in IPKB are identified as Network Eligible molecules. These so-called focus genes were then used as a starting point for generating biological networks. To start building networks, the application queries the IPKB for interactions between focus genes and all other gene objects stored in the knowledge base, and generates a set of networks. Every resulting gene interaction has supporting literature findings available online. Networks are limited to 35 molecules each to keep them to a usable size. IPA then computes a score for each network according to the fit of the user's set of significant genes. The score is derived from *p*-value and indicates the likelihood of the focus genes in a network being found together as a result of random chance. A score of 2 indicates that there is a 1-in-100 chance that the focus genes are together in a network as a result of random chance. Therefore, scores of 2 or higher have at least a 99% confidence of not being generated by random chance alone. The score is not an indication of the quality or biological relevance of the network; it simply calculates the approximate "fit" between each network and the Network Eligible molecules. For more details, see the Ingenuity Pathways Analysis Network Generation Algorithm whitepaper (https:// analysis.ingenuity.com/ pa/ info/ help/help.htm#ipa_help.htm).

3.7.8.3 Canonical Pathway Analysis

IPA provides the Canonical Pathways, which are well-characterized cell signaling pathways that have been curated and hand-drawn by Ph.D. level scientists. The information contained in Canonical Pathways comes from specific journal articles, review articles, text books, and KEGG Ligand. The diagram displayed in the Canonical Pathway view is representative of the canonical pathway at the cellular level. The significance of the association between the dataset and the Canonical Pathway was measured in 2 ways: (i) a ratio of the number of genes from the dataset that map to the pathway divided by the total number of genes that map to the Canonical Pathway is displayed; (ii) Fischer's exact test was used to calculate a *p*-value determining the probability that the association between the genes in the dataset and the Canonical Pathway is explained by chance alone. In general, *p*-value less than 0.05 indicate a statistically significant, non random association.

Since most canonical pathways are specifically made up of limited well-known genes, the involvement of just one key gene within a Canonical Pathway may be still biologically interesting, even if this pathway is not statistically significant. So that we prefer to look for the ratio of the number of genes rather than the *p*-value, and focus on those pathways related to the results of functional analysis (e.g., inflammation, cellular growth, proliferation, movement and death) and the NP pathological patterns. In addition, in order to narrow down the selection of the Canonical Pathways, some "redundant" pathways (e.g., apoptosis signaling vs. death receptor signaling, EGF/EGFR signaling vs. neuregulin signaling, and ERK/MAPK signaling vs. p38/MAPK signaling), and the pathways without alteration of key genes (e.g., no alteration of IL-10 in IL-10 signaling pathway) were filtered. Finally, top-7 relevant Canonical Pathways were selected, including apoptosis signaling, complement system,

EGF/EGFR signaling, eicosanoid signaling, ERK/MAPK signaling, IL-6 signaling, and NF-kappaB signaling.

3.7.9 Real-time reverse transcription (RT) PCR

One microgram of the total RNA was reversely transcribed using TaqMan Reverse Transcription Reagents kit (Applied Biosystems) based on the manufacturer's protocol. Briefly, the reverse transcription PCR (RT-PCR) mixture contained 1X TaqMan RT buffer, 5.5 mM $MgCl_2$, 500 μM dNTP, 2.5 μM Random Hexamer, 0.4U/μl RNase inhibitor and 1.25U/μl MultiScribe Reverse Transcripase in a final volume of 50 μl. RT-PCR reactions were performed in thermal cycler with the following conditions: 25℃/10 min, 48℃/30 min, 95℃/5 min. cDNA samples were stored in aliquots at -20℃ until use.

Real-time RT PCR analysis was performed to validate the expression of selected target genes which showed significant differences in microarray analysis. In addition, the expression of two GR gene products (GRα and GRβ) whose probe IDs were not available in affymetrix arrays was also determined. The TaqMan assays (Applied Biosystems) of target genes were described in **Table 3.3**. Both target and reference (GAPDH) genes were amplified in separate wells in triplicate. The PCR amplification was performed in a final volume of 25 μl, containing 10 ng of cDNA, 1× TaqMan Gene Expression Assay (Applied Biosystems), and 1× TaqMan Universal PCR Master Mix (Applied Biosystems). The cycling conditions for ABI Prism 7300 Sequence Detection System (Applied Biosystems) were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Briefly, each gene was amplified in separate tubes, and the increase in fluorescence was measured in

real-time. The threshold cycle (Ct), which is defined as the fractional cycle number at which the fluorescence reaches 10× the standard deviation of the baseline that was calculated. Relative gene expression was calculated using the comparative $2^{-\Delta\Delta Ct}$ method as previously described [Livak et al. 2001].

Table 3.3 Identity for human Taqman Gene Expression Assays-On-Demand™

Gene Symbol	Description	Assay ID
AP-1 genes		
c-Fos	v-fos FBJ murine osteosarcoma viral oncogene homolog	Hs00170630_m1
c-Jun	Jun oncogene	Hs00277190_s1
FosB	FBJ murine osteosarcoma viral oncogene homolog B	Hs00171851_m1
JunB	Jun B proto-oncogene	Hs00357891_s1
AP-1 related genes		
COX-2	Cyclooxygenase-2	Hs00153133_m1
IL-6	Interleukin 6	Hs00174131_m1
HBEGF	Heparin-binding EGF-like growth factor	Hs00181813_m1
AREG	Amphiregulin	Hs00155832_m1
EGR1	Early growth response 1	Hs00152928_m1
Pro-inflammatory genes in NP in response to GCs		
CXCL9	Chemokine (C-X-C motif) ligend 9	Hs00171065_m1
CXCL11	Chemokine (C-X-C motif) ligend 11	Hs00171138_m1
MMP7	Matrix metallopeptidase 7	Hs00159163_m1
MMP9	Matrix metallopeptidase 9	Hs00234579_m1
Anti-inflammatory genes in NP in response to GCs		
DUSP1	Dual specificity phosphatase 1	Hs00610256_g1
DUSP2	Dual specificity phosphatase 2	Hs00358879_m1
DUSP6	Dual specificity phosphatase 6	Hs00169257_m1
SPRY1	Sprouty homolog 1	Hs00398096_m1
SPRY2	Sprouty homolog 2	Hs00183386_m1
SPRY4	Sprouty homolog 4	Hs00540086_m1
NFKBIZ	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	Hs00230071_m1
SOCS3	Suppressor of cytokine signaling 3	Hs00269575_s1
ANXA1	ANNEXIN A1	Hs00167549_m1
SCGB1A1	Secretoglobin, family 1A, member 1 (uteroglobin)	Hs00171092_m1
ZFP36	Zinc finger protein 36	Hs00185658_m1
THBD	Thrombomodulin	Hs00264920_s1
Eosinophil related genes		
CD69	CD69 molecule	Hs00156399_m1
NR4A1	Nuclear receptor subfamily 4, group A, member 1	Hs00374230_m1

Gene Symbol	Description	Assay ID
NR4A2	Nuclear receptor subfamily 4, group A, member 2	Hs00428691_m1
CXCL2	Chemokine (C-X-C motif) ligand 2	Hs00236966_m1
CCL11	Chemokine (C-C motif) ligand 11 (eotaxin)	Hs00237013_m1
IL5Ra	Interleukin 5 receptor, alpha	Hs00602482_m1
C3	Complement component 3	Hs00163811_m1
IL13RA2	Interleukin 13 receptor, alpha 2	Hs00152924_m1
IL18	Interleukin 18	Hs00155517_m1
NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	Hs00230071_m1
Glucocorticoid receptor genes		
GRα	Glucocorticoid receptor alpha isoform	Hs01005211_m1
GRβ	Glucocorticoid receptor beta isoform	Hs00354508_m1
House keeping gene		
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	4333764T

3.8 Histo-immunohistochemical examination

Evaluation of the Study Subject 1 was used by frozen tissues, while evaluation of the Study Subject 2 was used by paraffin embedded tissues. The frozen tissue specimens were sectioned into a thickness of 4 μm by using Leica CM 1850 Cryostat (Leica, Wetzlar, Germany); while the paraffin embedded tissues were sectioned at 4 μm with Leica RM2125 Microtome (Leica). To obtain a general impression of the histopathological features of the examined specimens, slides were stained with hematoxylin and eosin (H&E). In addition, several interesting cellular markers and target genes were evaluated by immunohistochemical staining.

3.8.1 Staining procedures for frozen tissues

Frozen sections were fixed in cold methanol:acetone (1:1) and then blocked by 3% hydrogen peroxide, and 2% normal goat serum (Dako A/S, Glostrup, Denmark). Slides were stained with anti-CDH1 monoclonal antibody [mouse IgG2a; clone, 36] (BD Biosciences, San Jose, CA), anti-CD8 monoclonal antibody [mouse IgG1 kappa; clone, C8/144B] (Dako A/S), and anti-neutrophil elastase monoclonal antibody

[mouse IgG1 kappa; clone, NP57] (Dako A/S) at dilutions of 1:100, and then were incubated at room temperature for 1 h. Species- and subtype-matched antibodies were used as negative controls [N-Universal Negative Control for mouse IgG and N-Universal Negative Control for rabbit IgG] (Dako A/S) and were performed in parallel with the specific staining. The sections were blocked with 2% normal goat serum (Dako A/S) again. The slides were then incubated with DAKO EnVision System Peroxidase kit (Dako A/S) for 30 minutes at room temperature. Diaminobenzidine (DAB) was used as substrate for color development. In the end, sections were counterstained with hematoxylin, dehydrated with serial concentration of ethanol, hydrated with xylene and mounted with mounting medium (Dako A/S).

3.8.2 Staining procedures for paraffin embedded tissues

Deparaffinization and rehydration were performed in prior to the staining of paraffin embedded sections. Slides were processed with Target Retrieval Buffer (Dako A/S). Endogenous peroxidase activity was blocked with 3% H_2O_2. Slides were stained with anti-c-Jun monoclonal antibody [mouse IgG2a; clone, 3/Jun] (BD Biosciences) and anti-c-Fos polyclonal antibody [Rabbit IgG; clone, K-25] (Santa Cruz Biotechnology, Santa Cruz, CA) at dilutions of 1:100 and 1:300, respectively, and then were incubated at room temperature for 1 hour. Species- and subtype-matched antibodies were used as negative controls [N-Universal Negative Control for mouse IgG and N-Universal Negative Control for rabbit IgG] (Dako A/S) and were performed in parallel with the specific staining. The slides were then incubated with DAKO EnVision$^+$System-HRP (Dako A/S) at room temperature for 30 min. Diaminobenzidine was used as substrate for color development. All slides were counterstained with hematoxylin (Sigma Aldrich), dehydrated with serial

concentration of ethanol, hydrated with xylene and mounted with mounting medium (Dako A/S).

3.8.3 Evaluation of histo-immunohistochemical patterns

Several histo-immunohistochemical patterns were examined with a light microscope including infiltration of inflammatory cells, epithelial integrity, edema status, and target gene expression. To have a standardized histological evaluation of the staining (including both H&E staining and immunostaining), the pathologist independently assessed all cases in a blind fashion.

3.8.3.1 Evaluation of infiltrated inflammatory cells

Infiltration of eosinophils was evaluated based on H&E staining, while infiltration of neutrophils and CD8+ cells was examined based on immuno-staining. The results of cell counting were showed in percentage, which were calculated by positive staining cells per 200 cells at 400× magnification in every five fields with high-intensity positive cells. The percentage of these inflammatory cells was categorized into four grades: Grade 0, none; Grade 1, less than 10%; Grade 2, 10% to 40%; Grade 3, more than 40%.

3.8.3.2 Evaluation of epithelial integrity and edema status in the nasal tissues

Since the tissue morphology was preserved well in formalin fixed specimens, only the paraffin embedded sections were examined for assessing epithelial integrity as described [Wladislavosky-Waserman et al., 1984]. Grade 0 indicated intact epithelium which all layers of epithelial cells were present; grade 1 indicated moderately damaged epithelium which 2 or more layers of cells were present; grade 2 indicated

severely damaged epithelium which only 1 layer of cells or no epithelial cells (naked basement membrane) remained. In this study, grade 0 was defined as "intact" epithelium, while grade 1 and 2 were defined as "damaged" epithelium. The status of edema is also evaluated and classified as: grade 0, no edematous stoma; grade 1, mild or moderate edematous stoma and grade 2, severe edematous stoma.

3.8.3.3 Evaluation of target gene expression

In methylation study, protein level of CDH1 was evaluated from frozen sections; while in gene expression study, protein level of c-Jun and c-Fos was examined from paraffin embedded sections.

Differential CDH1 expression was defined in three categories as described [Zheng et al., 1999]: high level of expression (>75% cells were positive), intermediate level of expression (35% to 75% cells were positive), and low level of expression (<35% cells were positive). The counting was performed blindly without the investigator knowing the identity of the sample.

For slides stained with antibodies to c-Jun and c-Fos, a semi-quantitative scoring system considering the extent of immunoreactivity and staining intensity was performed [Putti et al., 2002]. The immunoreactivity of c-Jun or c-Fos within the epithelium region was graded as "0 point" for negative staining, "1 point" for < 15% positive cells, "2 points" for 15-60% positive cells, and "3 points" for > 60%. The intensity of c-Jun or c-Fos staining was graded as "1 point" for weak staining, "2 points" for moderate staining, and "3 points" for strong staining. The overall score was calculated by multiplying the score of immunoreactivity and intensity of staining

in each case. Hence, the maximum score for individual case is 9. In this study, the overall score of ≥ 6 was defined as "strong" expression while the overall score < 6 was defined as "weak" expression.

3.9 Statistical analysis

All data in methylation study and gene expression study were analyzed using the SPSS statistical software V14.0 (SPSS Inc., Chicago, IL).

3.9.1 Statistics in methylation study

Fisher's exact test and continuity correction were used to compare the methylation frequency in different study groups (NP versus control) and in different sample types (solid tissues versus PBMC samples) in NP and control group, respectively. Fisher's exact test was used to analyze the methylation status of *CDH1* in relation to the expression of this gene. The Mann-Whiney two-tailed test was used to compare the gene methylation status in clinical parameters (such as atopic, eosinophil/neutrophil/CD8+ cell infiltration). Values were considered significant if $p < 0.05$.

3.9.2 Statistics in gene expression study

Power and sample size estimation suggested that a minimum of four biopsies in each study group are required to detect a 1.5-fold difference in gene expression with a 1% significance level and a 90% power. Hence, we would expect to detect a 2.0-fold difference in gene expression with $p < 0.01$ and with a 90% chance of avoiding a type II error.

Wilcoxon matched pairs sign rank test was used to compare eosinophils and neutrophils count, epithelial integrity, edema status and gene expression levels, in GC-treated versus GC-naïve NP samples. Mann-Whitney two-tailed test was performed to compare gene expression levels between NP tissues, either GC-treated or GC-naïve, versus nasal mucosal controls. Spearman rank analysis was used to analyze the correlation between the gene expression levels assessed by microarray and those measured by quantitative PCR; and between fold change of c-Jun and other AP-1 related genes. Fisher's exact test was used to assess the relationship between protein expression of c-Jun and epithelium integrity in NP tissues. Values were considered significant if $p < 0.05$.

Chapter 4. Role of *Staphylococcus Aureus* and Superantigens in Nasal Polyposis

To test the superantigen hypothesis in Asian NP, we carried out a pilot study performed in 24 cases of NP samples and 10 inferior turbinate (IT) samples as controls. Standard PCR was used to detect *S. aureus* specific gene (*nuc*) and 9 *S. aureus* derived enterotoxins (superantigens) (listed in **Table 3.1,** *Page 40*) by using DNA samples isolated from NP and IT tissues.

Part I Results

4.1.1 Patient characteristics and histological evaluation

The NP and control subjects selected here are the same as the subjects selected in the methylation study, so that their clinical characteristics were summarized in **Table 5.1** (*page 76*). The majority of the NP patients are Chinese ethnics (about 70%). All NP patients had sinusitis, which was confirmed by CT scan of the sinuses. Atopy, a positive serum specific IgE antibody to common local allergens, was founded in 33% (8 out of 24) of NP patients and 50% (5 out of 10) of controls, respectively; but the IgE results were not significantly different between NP and controls (data not shown here). An increase of eosinophils, neutrophils, and CD8+ cells was evident in NP samples, but not in control turbinate samples.

4.1.2 Detection of *S. aureus* and superantigens

Nuc gene which encodes the thermostable nuclease of *S. aureus* has been considered a diagnostic marker for *S. aureus* infection [Brakstad et al., 1992]. Positive PCR results of *nuc* gene with bands of the expected size were obtained for only 2 out of 24 NP

tissues and 1 out of 10 IT tissues (**Figure 4.1**). No detection signal of *nuc* gene was found in all PBMC samples. Interestingly, there was one confounding PCR band close to the expected size of *nuc* gene in some NP tissues (e.g., NP1, NP3, and NP4 in **Figure 4.1**). To evaluate the positive PCR results, direct sequencing was performed and then the sequencing results were uploaded to Blast (online source: http://blast.ncbi.nlm.nih.gov/Blast.cgi). The blast results confirmed that the sequence from the *nuc* positive PCR band was amplified from the whole mRNA sequence of the *nuc* gene; while the sequence from the PCR band close to the correct size of *nuc* gene was confirmed to not be related to the *nuc* gene sequence (**Figure 4.2**). As far as the superantigens, there was no evidence to support that the 9 *S. aureus* enterotoxins DNA existed in any of the nasal mucosa samples and PBMC samples by PCR detection (Data not shown here).

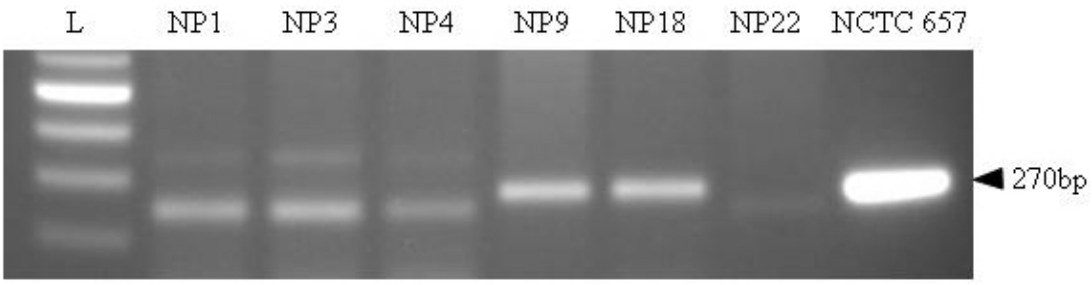


Figure 4.1 PCR product of *S.aureus* specific *nuc* gene. Gel picture shows the *nuc* gene in representative NP samples and NCTC 657 *S.aureus* bacteria chain (positive control). Only the band from NP9 and NP18 is at the same size as positive control. L, size marker (100-bp DNA ladder).

Figure 4.2

(A)

>gi|46623|emb|V01281.1|SANUCX *S.aureus mRNA for nuclease*

```
 Length = 966
 Score = 416 bits (210), Expect = e-114
 Identities = 224/228 (98%), Gaps = 1/228 (0%)

Query: 16  gactattattggttgatacacctgaancnaagcatcctaaaaaaggtgtagagaaatatg 75
           |||||||||||||||||||||||||| | ||||||||||||||||||||||||||||||
Sbjct: 566 gactattattggttgatacacctgaaacaaagcatcctaaaaaaggtgtagagaaatatg 625

Query: 76  gtcctgaagcaagtgcatttacgaaaaagatggtagaaaatgcaaagaaaattgaagtcg 135
           |||||||||||||||||||||||||| ||||||||||||||||||||||||||||||
Sbjct: 626 gtcctgaagcaagtgcatttacgaaaaaaatggtagaaaatgcaaagaaaattgaagtcg 685

Query: 136 agtttgacaaaggtcaaagaactgataaatatggacgtggcttagcgtatatttatgctg 195
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 686 agtttgacaaaggtcaaagaactgataaatatggacgtggcttagcgtatatttatgctg 745

Query: 196 atggaaaaatggtaaacgaagctttagttcgtcaaggccttggctaaa 243
           |||||||||||||||||||||||||||||||||||| ||||||||||
Sbjct: 746 atggaaaaatggtaaacgaagctttagttcgtcaagg-cttggctaaa 792
```

(B)

>gi|46623|emb|V01281.1|SANUCX *S.aureus mRNA for nuclease*

```
 Length = 966
 Score = 396 bits (200), Expect = e-108
 Identities = 229/242 (94%)

Query: 14  aaccaatgactttccnactattattggttgatacacctgaancnaagcatcctaaaaaag 73
           |||||||||| |||  |||||||||||||||||||||| | ||||||||||||||||
Sbjct: 551 aaccaatgacattcagactattattggttgatacacctgaaacaaagcatcctaaaaaag 610

Query: 74  gtgtagagaaatatggccctgaagcaagtgcatttacgnnnnnnnntggtagaaaatgcaa 133
           ||||||||||||||| ||||||||||||||||||||       ||||||||||||||||
Sbjct: 611 gtgtagagaaatatggtcctgaagcaagtgcatttacgaaaaaaaatggtagaaaatgcaa 670

Query: 134 agaaaattgaagtcgagtttgacaaaggtcaaagaactgataaatatggacgtggcttag 193
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 671 agaaaattgaagtcgagtttgacaaaggtcaaagaactgataaatatggacgtggcttag 730

Query: 194 cgtatatttatgctgatggaaaaatggtaaacgaagctttagttcgtcaaggcttggcta 253
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 731 cgtatatttatgctgatggaaaaatggtaaacgaagctttagttcgtcaaggcttggcta 790

Query: 254 aa 255
           ||
Sbjct: 791 aa 792
```

(C)

>gi|13398718|emb|AL160255.14|

```
Human DNA sequence from clone RP11-60N6 on chromosome 13, complete sequence
Length = 172923
Score = 125 bits (63), Expect = 6e-26
Identities = 112/129 (86%), Gaps = 2/129 (1%)

Query: 79    atantgggggcgggttncttgaatggctncncaccatccccnttggtatgnccttgtgat 138
              ||| ||||||||| || ||| ||||||| | |||||||||| |||||||| |||||||||
Sbjct: 97564 ataatgggggcggttttcttaaatggcttcgcaccatccccttggtatgtccttgtgat 97623

Query: 139   atcaagtnagtgtcccccaaggctctggtcatttcaaaatgagtgacacctccccntgtc 198
              | ||||| ||| |||||| ||| |||||||||||||||| |||||||||||| ||||
Sbjct: 97624 agcaagtgagt-tcccccgagg-tctggtcatttcaaaatgtgtgacacctcccccctgtc 97681

Query: 199   nctcntatg 207
               ||| ||||
Sbjct: 97682 tctcttatg 97690
```

Figure 4.2 Direct sequencing results of *nuc* gene. (A) Blast results of direct sequencing for positive control. (B) Blast results of direct sequencing for NP18, which has PCR product with correct size. (C) Blast results of direct sequencing for NP1, which has PCR product with confounding size.

Part II Discussion

S. aureus and its superantigens have been proposed as elemental causes for the Th2 shift and increase of eosinophils in NP (reviewed in **Chapter 1.4.4,** *Page 9*). We assumed the potential roles of *S. aureus* and its superantigens in NP in this prospective study. However, we found that *S. aureus* was only detected in 8% (2 out of 24) of the NP tissues and in 10% (1 out of 10) of the control IT tissues, indicating that the prevalence rate of *S. aureus* in NP is low and existence of *S. aureus* is not specific to NP. *S. aureus* was not found in any of the PBMC samples, indicating no systemic infection of *S. aureus*. Moreover, we could not find the presence of superantigens in NP. The results of no detection signal in superantigens were consistent with the low prevalence rate of *S. aureus* in NP.

Although the prevalence rate of *S. aureus* in NP has not been summarized in the

literature, based on the current results and some published reports, it may range from 8% to 64% [Fan et al., 2006; Niederfuhr et al., 2008; Van Zele et al., 2004]. Possible reasons for this wide range include the heterogeneities of NP (eosinophilic vs. neutrophilic), different studied populations (Asian vs. Caucasian), method and location of tissue sampling (nasal swab vs. mucosa biopsy; middle meatus vs. sinus mucosa), and different microbiological detection techniques (IgE test vs. PCR assays). Our study used PCR to directly detect *S. aureus* specific nuclease genes (*nuc*) in NP biopsy in Asian patients. The advantages of PCR are that it is a fast method that detects DNA of living as well as dead bacteria; and the PCR detection of *nuc* gene has shown a greater specificity (> 90%) in *S. aureus* measurement than the other methods [Brakstad et al., 1992; Niederfuhr et al., 2008]. However, *nuc*-PCR assays may have low sensitivities (about 58%) if small amounts of DNA are used [Niederfuhr et al., 2008]. Therefore, whether the lack of detection signal for *nuc* gene is attributed to the little inputed DNA copy numbers need to be carefully examined in the future study.

With regard to the superantigens, their prevalence rates appear to be different from the incidence of *S. aureus* in NP without asthma. For example, Van Zele et al. showed that only about 43% of *S. aureus* positive NP demonstrated an IgE response to superantigens [Van Zele et al., 2004]. In addition, there was no major difference in the presence of enterotoxin genes in *S. aureus* strains derived from NP or control patients [Van Zele et al., 2008]. Similarly, another study also showed no difference in the incidence of *S. aureus* among chronic sinusitis patients with or without NP and controls. *S. aureus* did not intensify the Th2 shift in NP patients [Niederfuhr et al., 2008]. Our results were in line with the recent reports which showed no detection of specific IgE of anti- *S. aureus* enterotoxins in Chinese NP [Fan et al., 2006]. In contrast, one

study showed that about one-third of the Chinese patients with NP presented an IgE response to superantigens, but the relationship between eosinophil inflammation and superantigens was low [Zhang et al., 2006]. The discrepancy in these results may be due to the different detection methods used in these studies and different histopathological features in the studied NP.

Part III Conclusion

In summary, the roles of *S. aureus* superantigens may not be evident in Asian NP, at least in our study population. Although there is increasing evidence that *S. aureus* superantigens may participate in the chronic inflammation of NP, our results and some published reports suggest that of *S. aureus* and its superantigens may not have a substantial role in Asian NP. The evidence for the role of *S. aureus* and its superantigens in NP is thought to be circumstantial and not present in all types of NP. Nevertheless, the potential role of *S. aureus* superantigens in NP cannot be ruled out. Some confounding factors (e.g., NP characteristics, studied population, and detection methods) in future superantigen studies in NP need to be carefully categorized and standardized. In addition, further studies are necessary to answer the question whether the presence of *S. aureus* and its superantigens in both NP and nasal mucosal controls have a different pathophysiologic impact in nasal mucosa.

Chapter 5 Methylation of Tumor Suppressor Genes in Nasal Polyposis

To evaluate the promoter methylation status of some common tumor suppressor genes (TSGs) in NP, six commonly reported TSGs (*p16*, *RASSF1A*, *CDH1*, *TSLC1*, *DAPK1*, and *PTPN6*) whose expressions were frequently silenced by promoter methylation in multiple cancers were studied. In this study, we recruited three groups of subjects: three inverted papilloma (IP) served as controls representing benign tumors; 24 NP were the sample group; and 10 inferior turbinates (IT) from patients with septal deviation served as healthy controls. Methylation of the TSGs was evaluated in solid tissues and PBMC from all studied subjects by using methylation specific PCR (MSP).

Part I Results

5.1.1 Patient characteristics and histological evaluation

The clinical and histological characteristics of the subjects (IP, NP, and controls) are summarized in **Table 5.1**. H&E staining was used in IP, NP, and healthy controls; while, immunostaining (for neutrophils and CD8+ T cells) was only applied in NP and controls. Atopy was evident in 33% of the NP patients and 50% of the healthy controls (allergy test was not performed in IP patients).

Table 5.1 Patient clinical and histological characters

Sample ID	Sex	Age	Atopy*	Eosinophil†	Neutrophil†	CD8+ Cell†	Squamous metaplasia
NP1	M	47	-	1	1	1	-
NP2	M	58	-	1	3	3	-
NP3	F	50	-	3	1	2	-
NP4	M	50	+	2	1	2	-
NP5	M	53	-	3	2	2	-
NP6	M	38	-	N.A	N.A	N.A	N.A
NP7	F	23	-	2	3	3	+
NP8	M	47	-	N.A	N.A	N.A	N.A
NP9	M	54	-	2	2	2	-
NP10	M	49	+	3	1	1	-
NP11	M	34	-	N.A	N.A	N.A	N.A
NP12	M	29	+	3	1	1	-
NP13	F	40	-	N.A	N.A	N.A	N.A
NP14	M	21	+	1	1	1	+
NP15	M	42	-	2	2	2	-
NP16	M	58	-	1	2	3	-
NP17	F	46	-	1	3	1	-
NP18	M	33	-	3	2	3	-
NP19	M	29	+	1	1	1	-
NP20	M	48	+	3	3	2	+
NP21	M	49	-	1	2	3	-
NP22	F	28	+	3	2	3	-
NP23	M	42	+	2	3	2	+
NP24	M	32	-	3	3	3	+
IT1	M	23	-	0	1	1	-
IT2	F	20	+	1	1	2	-

Sample ID	Sex	Age	Atopy*	Eosinophil†	Neutrophil†	CD8+ Cell†	Squamous metaplasia
IT3	M	25	+	1	1	1	-
IT4	F	40	-	0	1	2	-
IT5	M	29	-	1	1	1	-
IT6	M	29	+	1	2	2	-
IT7	F	56	+	1	1	2	-
IT8	M	18	-	1	1	1	-
IT9	M	21	+	1	2	2	-
IT10	M	23	-	1	1	1	-
IP1	M	45	N.A	1	N.A	N.A	+
IP2	F	36	N.A	1	N.A	N.A	+
IP3	M	52	N.A	1	N.A	N.A	+

* A Phadiatop PAU/L (Pharmacia Arbitrary Units/L) value ≥ 0.35 was considered as atopy. "+", atopy; "-", non-atopy.

† The percentage of eosinophil/neutrophil/CD8+ cell was categorized into four grades: Grade 0, none; Grade 1, less than 10%; Grade 2, 10% to 40%; Grade 3, more than 40%. Grade 2 and Grade 3 were considered increased infiltration of the indicated cell type.

Squamous metaplasia of epithelium was evaluated qualitatively in H&E staining sections, i.e., "+" presence of sqamous metaplasia; "-", absence of squamous metaplasia.

Abbreviations: NP, Nasal polyposis; IT, inferior turbinate; IP, Inverted papilloma; N.A., not applicable.

H&E staining revealed the patterns of eosinophil infiltration and epithelium structure in IP, NP, and controls. An increase cell count of eosinophils, neutrophils, and CD8+ cells was significant in NP, but not in IP or controls. With regard to the epithelium (**Figure 5.1**), IP showed an inversion of the neoplastic squamous epithelium with minimal mitosis and atypia, and rarely presented a respiratory epithelial structure. The surface epithelium of NP was typically respiratory mucosa with areas of transitional and squamous epithelium; moreover, squamous epithelium was not found in IT tissues.

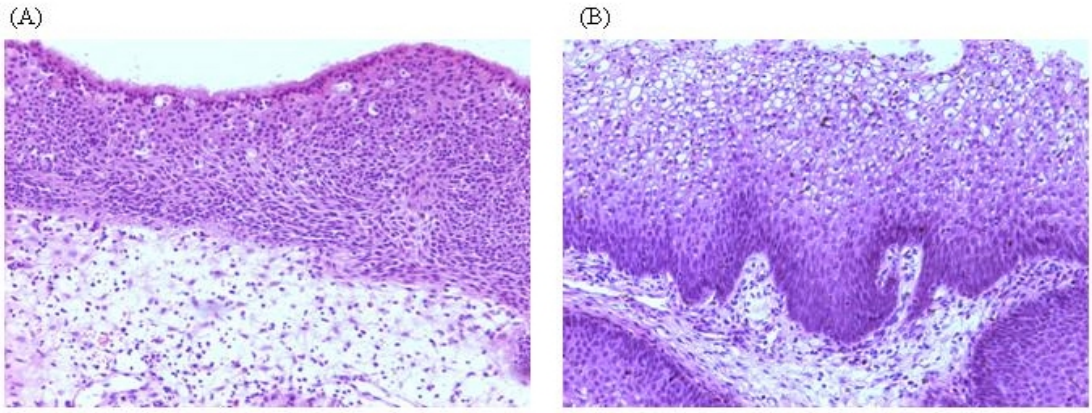


Figure 5.1 Histological patterns of epithelium with squamous metaplasia. (A) Squamous metaplasia from representative NP sample; (B) Squamous metaplasia from representative IP sample. All sections were stained with H&E staining and evaluated at ×100 magnification.

5.1.2 Detection of methylation status by methylation-specific PCR (MSP)

The DNA of solid tissues (IP, NP, and control IT) and the PBMC from the subjects were examined for promoter methylation in *p16, RASSF1A, CDH1, TSLC1, DAPK1* and *PTPN6* genes by MSP. Representative MSP results of these genes are presented in **Figure 5.2**. The presence of a methylated signal always occurs with the presence of unmethylated signal, but not vice versa, indicating that these tissues consist of mixed cell populations in terms of methylation of these genes. In all samples, MSP results were confirmed by repeated tests in order to avoid any technical bias. The summary of the promoter methylation status of all six genes are shown in **Table 5.2**. The frequency of methylation of these TSGs in NPC was cited from the published results. For *p16* and *RASSF1A*, only unmethylated alleles were detected in all of the studied samples. Methylation of *CDH1, TSLC1,* and *DAPK1* was found in some samples from IP, NP and control groups; furthermore, methylation of *PTPN6* was detected in all solid tissues. The methylation rate was significantly higher in solid tissues than in corresponding PBMC. However, no significant difference was detected in the

methylation frequencies of *CDH1, TSLC1, DAPK1,* and *PTPN6* between NP tissues and control IT tissues (data not shown). Note that since the sample size of IP was too small, statistical comparison was not performed in this sample group.

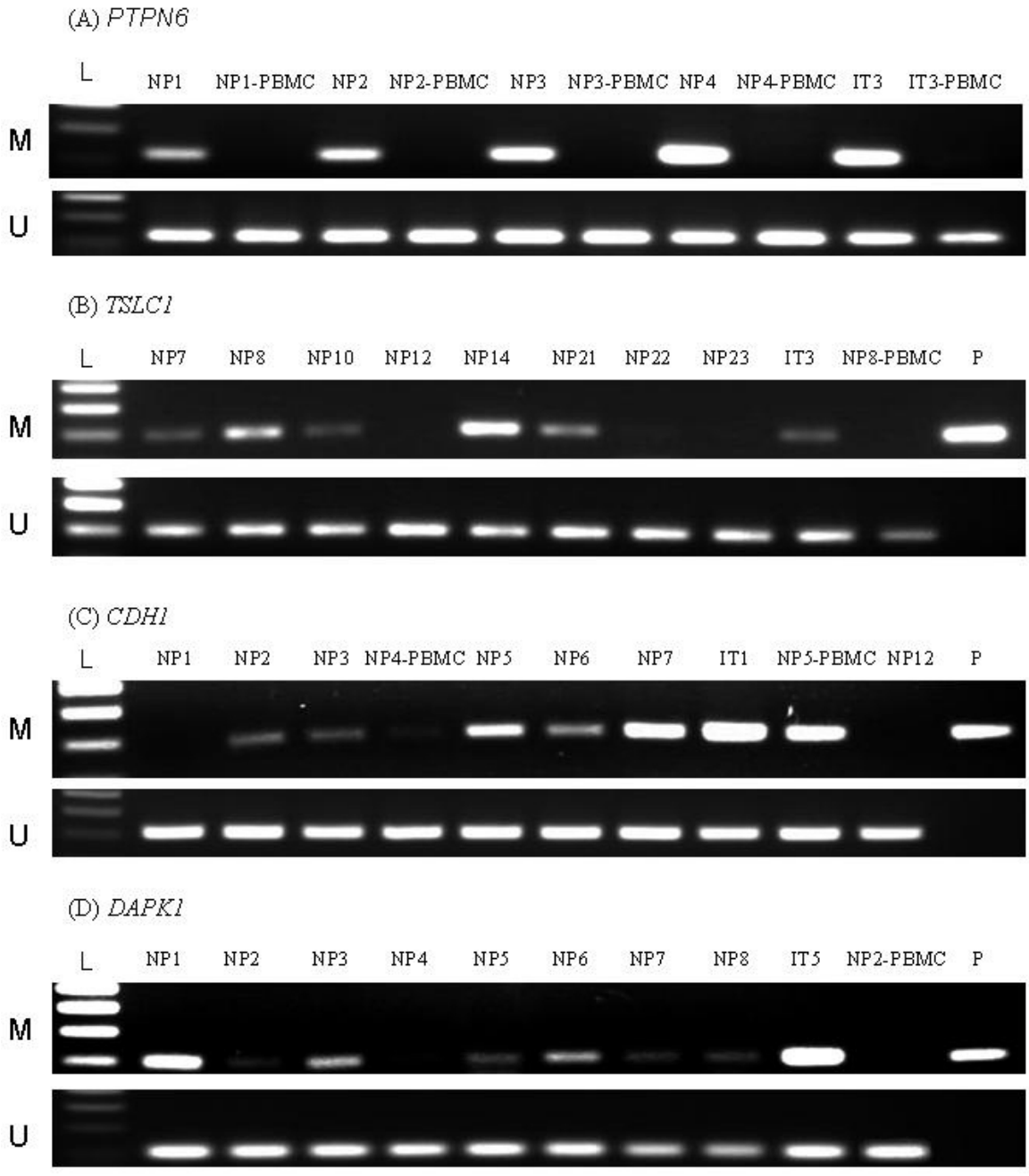


Figure 5.2 Representative samples of MSP analyses of DNA samples from NP, IT, and PBMC. Methylation (M) and unmethylation (U) primers sets were used to amplify the methylated and unmethylated sequences, respectively. L, size marker (100-bp DNA ladder); P, positive control. CpGenome Universal Methylated DNA was used as a methylation-positive control.

Table 5.2 Summary of TSGs methylation status (by MSP analysis) of different groups

Genes	NP [a] (n=24)	IT [a] (n=10)	NP-PBMC [a] (n=20)	IT-PBMC [a] (n=10)	IP [b] (n=3)	NPC [c]
p16	0	0	0	0	0	46% [d]
RASSF1A	0	0	0	0	0	84% [d]
CDH1	46%	70%	30%	20%	2	52% [e]
TSLC1	29%	30%	0	0	1	68% [f]
DAPK1	38%	50%	5%	0	1	76% [d]
PTPN6	100%	100%	0	0	3	N.A.

NP, Nasal Polyp; IT, Inferior Turbinate; IP, inverted papilloma; NPC, Nasopharyngeal carcinoma
N.A., not applicable.

[a] Methylation frequency of each gene in different sample type from NP patients and IT controls.

[b] Only counted the number of IP samples with methylated status, due to the small sample size.

[c] Methylation frequency of NPC is referred to published reports.

[d] Kwong et al., 2002

[e] Tsao et al., 2003

[f] Zhou et al., 2005

5.1.3 Confirmation of MSP results by bisulfite genomic sequencing (BGS)

For *CDH1, TSLC1, DAPK1*, and *PTPN6,* BGS analysis was further performed to check their detailed methylation status (**Figure 5.3**). BGS showed that all the cytosines at non-CpG sites were converted to thymines, validating the adequacy of the bisulfite modification. BGS results for *CDH1*, *TSLC1,* and *DAPK1* showed that only low levels of methylation (1 out of 10-15 alleles sequenced was fully methylated) were detected in samples with methylation detected by MSP; while BGS of *PTPN6* showed extensive methylation of CpG sites in most alleles (3 out of 5). Hence, the methylation signals of the genes (*CDH1*, *TSLC1,* and *DAPK1*) detected by MSP were mainly derived from a small percentage of cells (< 10%) in NP and control mucosa.

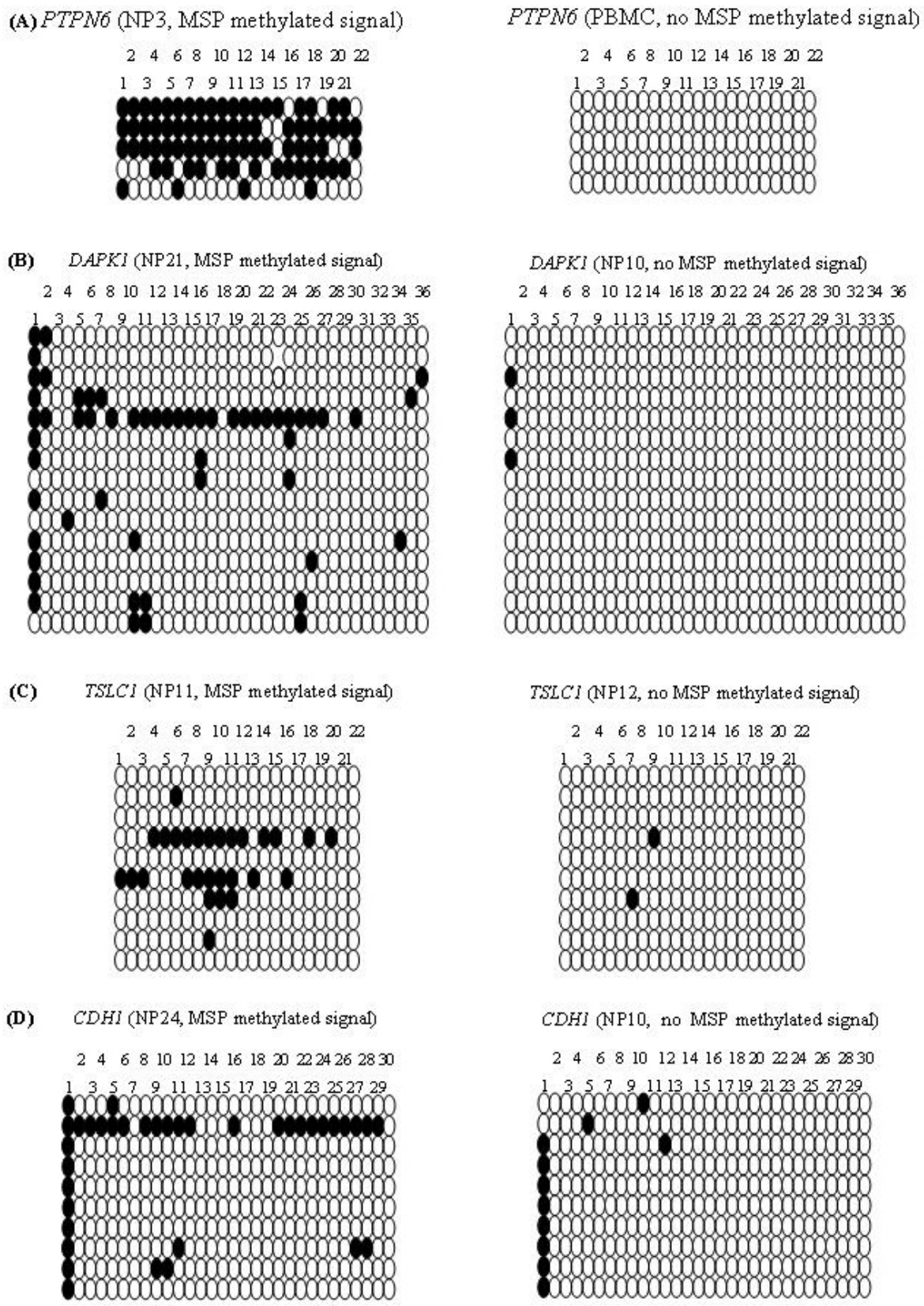


Figure 5.3 Detailed methylation analysis in promoter of selected genes (*SHP-1, DAPK1, TSLC1,* and *CDH1*) by using BGS. CpG sites are shown on the top row as numbers. Each row of circles represents a single allele of the respective gene promoter analysed. Open circles: unmethylated CpG sites; filled

circles: methylated CpG sites. For BGS, at least 10 bacterial colonies were analysed for each DNA sample (except *SHP-1*). In all of the four genes, methylated alleles are present in respective NP solid tissue with MSP methylated signal, while unmethylated alleles are shown in the NP samples (except BGS of *SHP-1* was analysed in PBMC) with no MSP methylated signal. (A) BGS primers amplify a 589-bp region with 22 CpG sites in the *SHP-1* promoter. (B) BGS primers amplify a 351-bp region with 36 CpG sites in the *DAPK1* promoter. (C) BGS primers amplify a 211-bp region with 22 CpG sites in the *TSLC1* promoter. (D) BGS primers amplify a 372-bp region with 30 CpG sites in the *CDH1* promoter.

5.1.4 Correlation between methylation status versus histopathological patterns of NP and protein expression

Methylation of *CDH1*, *TSLC1*, and *DAPK1* was not significantly associated with the appearance of squamous metaplasia in IP and NP samples (data not shown). In addition, there is no significant correlation between promoter methylation status of these three genes (*CDH1*, *TSLC1*, and *DAPK1*) and cell counts for eosinophils/neutrophils/CD8+ T cells in NP tissues (data not shown). In the above analysis, all known confounding variables, such as age, sex and atopic status were not found to be associated with methylation status of individual genes (data not shown).

Due to the importance of CDH1 in epithelial development, we attempted to investigate the relationship between methylation status of *CDH1*, and its protein expression in the NP and control mucosa. Our results showed that CDH1 expression was found in all NP and IT specimens, which was mainly in the area of epithelium and endothelium (**Figure 5.4**). However, we did not find the anticipated correlation between *CDH1* promoter hypermethylation and down-regulation of CDH1 expression in these nasal tissues (**Table 5.3**).

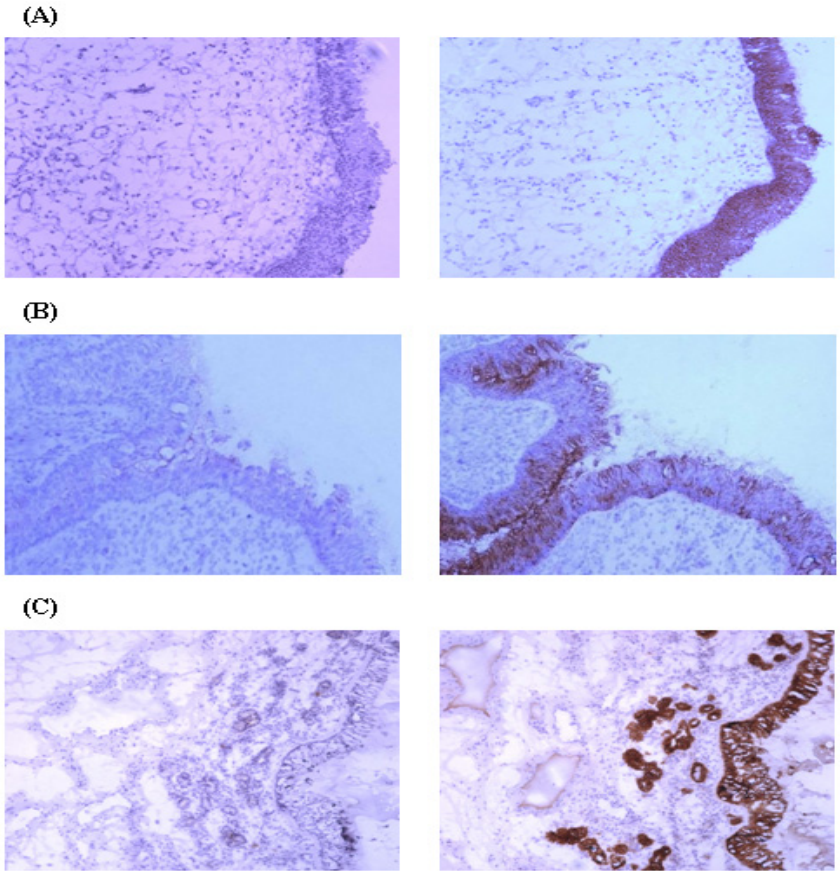


Figure 5.4 Immunohistochemical staining of CDH1 in NP samples. CDH staining is shown in right column, while the corresponding negative control is shown in left column. (A) High level of expression (>75%) of CDH1 in sample NP12 which does not have methylation of *CDH1*. (B) Intermediate level of expression (35% to 75%) of CDH1 in sample NP15 which has methylation of *CDH1*. (C) High level of expression of CDH1 in sample IT8 which has methylation of *CDH1*. A, B, and C ×100 magnification.

Table 5.3 Correlation between CDH1 expression and methylation status of *CDH1* in nasal tissues (both NP and IT)

Expression of CDH1[a]	Methylation Status of *CDH1*[b]		*p*- value[c]
	No/weak methylation	Methylation	
Low/intermediate level	3	7	0.123
High level expression	12	8	

[a] High level of expression (>75% cells are positive), intermediate level of expression (35% to 75% cells are positive), and low level of expression (<35% cells are positive)

[b] Methylation status was determined by methylation-specific PCR analysis.

[c] *p*-value was determined by two-sided Fischer's exact test, and $p < 0.05$ was regarded as statistically significant.

Part II Discussion

In the head and neck region, the most common lesion encountered by the otolaryngologist relates to alterations of the surface squamous epithelium. The epithelium of NPC represents dysplastic epithelial changes (e.g., poorly differentiated squamous cells). IP is generally regarded as a benign neoplasm of the sinonasal cavity, although the metaplastic epithelium may exhibit dyplasia, and up to 10% of the IP transform into squamous cell carcinoma [Dictor et al., 2000]. There is increasing evidence suggesting that IP may arise from a background of sinonasal mucosal inflammation, and clinically IP is frequently associated with NP [Robinson et al., 2006]. Promoter methylation-mediated gene silencing is a hallmark of many squamous cell carcinomas, including NPC [Hasegawa et al., 2002], and methylation of TSGs in IP was also implied in the association between IP and squamous cell cancers [Stephen et al., 2007].

NP represents a damaged respiratory epithelium with areas of squamous metaplasia after abnormal remodeling. The gross view of NP is similar to IP (**Figure 5.5**), while in histological observation, the severity of squamous cell remodeling in NP is much lesser than that in IP (**Figure 5.1,** *Page 78*). Although NP has not been regarded as a cancer-prone lesion, the high recurrence rate of NP after surgical polypectomy and the abnormal remodeling of epithelium raise the question as to whether NP could share some pathological mechanisms with those malignant (e.g., NPC) and benign (e.g., IP) neoplasms in upper respiratory tissues. Indeed, some hyperplastic polyps from gastric and colorectal tissues have been considered to present malignant potential even though they are non-neoplastic lesions in nature [Hawkins et al., 2001; Jass, 2004].

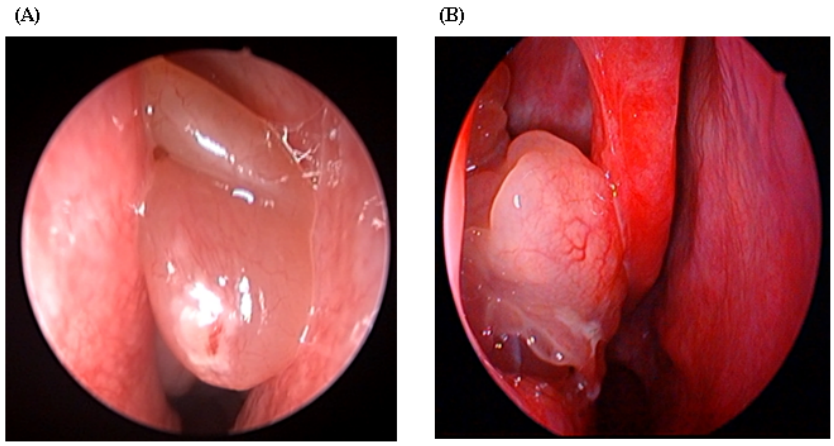


Figure 5.5 Gross view of nasal polyps and nasal inverted papilloma. (A) Nasal polyps; (B) inverted papilloma. Both are under endoscope examination. Picture was taken from patients with NP or papilloma under endoscope examination.

The six selected genes are regarded as common TSGs which control and prevent cancer development. *P16* and *RASSF1A* are cell cycle inhibitors which can prevent the cancer cell from going through the G1/S phase transaction. *CDH1* and *TSLC1* are adhesion molecules which can control the tumor cell invasions. *DAPK1* is a pro-apoptotic factor which can mediate death of cancer cells. *PTPN6* is a negative regulator of cellular signaling in immune cells which antagonize the growth-promoting tyrosine kinases in leukemia/lymphoma. Inactivation of these TSGs contributes to the aberrant proliferation and differentiation of squamous cancer cells. In addition, methylation of these genes was also observed in the chronic inflammation of gastric and intestinal epithelial lesions which have been considered to predispose to cancers.

Methylation of *CDH1*, *TSLC1*, and *DAPK1* was observed in IP and NP, confirming that the methylation of some TSGs could also be detected in benign growths and inflammatory conditions. In addition, the methylation rate of these three genes was significantly higher in solid tissues than the corresponding PBMC, indicating it is

more likely a local event. However, methylation of *CDH1*, *TSLC1*, and *DAPK1* was also found in control nasal mucosa and the methylation rate of these three genes was not significantly different between NP and controls. Indeed, sequencing results of *CDH1*, *TSLC1*, and *DAPK1* in NP and IP tissues demonstrated that the CpG sites between the MSP primer sequences were not completely methylated and the percentage of methylated alleles was low. Furthermore, significant negative correlation between *CDH1* promoter methylation and CDH1 expression was not found in the nasal tissues and some clinical characteristics of the patients (including age, appearance of squamous metaplasia, and infiltration of eosinophils) were not associated with methylation status of *CDH1*, *TSLC1*, and *DAPK1*. Hence, all this evidence would suggest that a low level of methylation of *CDH1*, *TSLC1*, and *DAPK1* may have a minimal contribution to the pathogenesis of the nasal benign lesions (IP and NP), and indicate that IP and NP should still be considered non-malignant.

Methylation of TSGs is commonly negative in PBMC from non-cancer subjects [Gutierrez et al., 2003] and has been used as molecular detection strategies in tumors. However, methylation of *CDH1* and *DAPK1* was detected in PBMC from some of the IP, NP, and control subjects, and was in line with the previous studies which showed methylation of *DAPK1* in PBMC from healthy subjects [Reddy et al., 2003]. Therefore, the methylation status of *CDH1* and *DAPK1* in PBMC may act as a confounding factor in tumor detection.

Interestingly, it is obvious that methylation of *PTPN6* was found in all solid tissues (IP, NP, and healthy control), but not at all in PBMC from all study patients. BGS results confirmed that methylation of *PTPN6* in nasal solid tissues occurred in all CpG sites

of the amplified regions in most alleles. PTPN6 is primarily expressed in hematopoietic cells, and considered a putative tumor suppressor gene in lymphoma and leukemia. Most of the methylation studies of PTPN6 focused on the malignancy of the hematologic system [Oka et al., 2002]. Only one report showed a high frequency of methylation of *PTPN6* in gallbladder carcinoma (GBC) and chronic cholecystitis. It was postulated that methylation of *PTPN6* was an early event in GBC pathogenesis [Takahashi et al., 2004], however this study did not obtain normal gallbladder epithelium as control. Based on our data, we suggest that methylation of *PTPN6* is only a tissue-specific phenomenon at least in upper airway mucosa and it may not contribute to any pathogenic mechanism in epithelial alteration on cellular infiltration. In addition, the lack of *PTPN6* methylation in normal PBMC confirms that it should be a specific serum tumor marker for the detection of lymphoid/hematopoietic cancers, but *PTPN6* is not a suitable molecular marker in detection of carcinoma (at least in airway tissues) based on *PTPN6* methylation.

It should be noted that there are limitations in this study. Because NP and IT are infiltrated by various cell populations, it is hard to identify which cell types contribute to the gene methylation. One report showed that infiltrating leukoctyes might account for the methylated CpGs in breast cancer tissues and confound the methylation detection [Lombaerts et al., 2004]. So the impurity of cell types may be a reason that normal control tissues also showed methylation signals, and that nasal samples with normal CDH1 expression showed a strong MSP product. Therefore, in future studies, it is recommended that different components of NP (especially epithelial area) could be isolated by laser capture microdissection and the gene methylation could be determined in a cell-specific manner.

Part III Conclusion

In conclusion, this study compared the methylation status of six commonly reported TSGs in IP, NP and healthy nasal mucosa. Methylation of *p16* and *RASSF1A,* were considered tumor-specific. Methylation of *CDH1, TSLC1,* and *DAPK1* was found at low level in IP, NP, and control nasal mucosa with no difference in the detection rates. Methylation of *PTPN6* is tissue-specific and should only be studied in hematopoietic disorders. The role of methylation of these genes in nasal mucosal inflammation appears to be minimal, and IP and NP are still regarded as benign lesions. Nonetheless, our study indicates that when using *CDH1*, *TSLC1*, and *DAPK1* in studies of tumors, one need to bear in mind that weak methylation of these genes could still be detected in inflammatory or benign conditions, or even normal PBMCs, hence they may not be specific molecular markers in tumor detection based on methylation.

Chapter 6. Gene Expression Profiles in Nasal Polyposis and its Response to GC Treatment

In this gene expression study, of 12 patients with untreated (GC-naïve) bilateral NP, two sets of NP biopsies were taken from each patient, *i.e.,* before the initiation and after GC treatment. Biopsies of the inferior turbinate from 10 patients who underwent surgery for nasal septal deviation served as nasal mucosal controls. DNA microarrays containing 38,500 genes were used to characterize the global gene expression profile. Integrated microarray analyses, including functional analysis, network analysis, and canonical pathway analysis as well as the comparison of literature reviews were applied to identify key molecular pathways and genes underlying the pathogenesis of NP and the response of NP to GC treatment. Selected genes were validated by means of quantitative RT-PCR and immunohistochemistry in the polyps and control samples.

Part I Results

[Note that in the following content, the prefix of GC-naïve NP sample ID is indicated as "NP", while the prefix of GC-treated NP sample ID is indicated as "NPR"; i.e., NP1 means the sample is from NP patient 1 before GC treatment, and NP1R means the sample is from NP patient 1 after GC treatment.]

6.1.1 Patient characteristics and histological evaluation

The demographic characteristics of the studied subjects are listed in **Table 6.1**. Atopy status was present in 8 patients and 2 controls, respectively. GC-naïve NP tissues revealed distinct histopathological patterns compared to control turbinates and the comparison is summarized in **Table 6.2**. The representative pictures for these histopathological profiles of NP (including infiltration of eosinophils, neutrophils, and

lymphocytes, epithelial damage and squamous remodeling, status of edema and fibrosis, density of glands) and the histological pictures of controls were shown in **Figure 6.1**.

Table 6.1 Clinical and histological characteristics of NP patients and control

ID	Sex	Age	Atopic[a]	Eosinophil[b]		Neutrophil[b]		Edema[c]		Epithelial Integrity[d]	
				Before GC	After GC	Before GC	After GC	Before GC	After GC	Before GC	After GC
NP patients											
NP1	F	37	-	3	2	3	3	2	0	2	0
NP2	M	52	-	2	1	2	1	2	0	1	1
NP3	M	18	+	3	1	2	2	2	1	0	0
NP4	M	32	+	3	2	2	2	0	0	2	0
NP5	F	48	+	3	2	2	2	2	0	2	0
NP6	M	34	+	1	1	1	1	1	0	1	0
NP7	M	30	-	1	1	1	1	0	0	1	0
NP8	M	24	+	2	1	1	1	0	0	1	1
NP9	F	55	+	3	1	1	1	2	0	2	0
NP10	M	55	+	3	1	2	1	2	0	0	0
NP11	M	25	+	3	1	2	1	2	0	0	0
NP12	M	38	-	3	1	2	1	0	0	1	0
p-value[e]				0.004		0.05		0.008		0.015	
Control subjects											
IT1	M	26	-	0	N.A.	1	N.A.	0	N.A.	0	N.A.
IT2	M	29	-	0	N.A.	1	N.A.	0	N.A.	0	N.A.
IT3	M	23	+	1	N.A.	1	N.A.	0	N.A.	0	N.A.
IT4	M	33	-	0	N.A.	1	N.A.	0	N.A.	0	N.A.
IT5	F	27	+	1	N.A.	1	N.A.	0	N.A.	0	N.A.
IT6	M	36	-	1	N.A.	1	N.A.	0	N.A.	0	N.A.
IT7	F	38	-	0	N.A.	1	N.A.	0	N.A.	0	N.A.
IT8	M	29	-	1	N.A.	1	N.A.	0	N.A.	0	N.A.
IT9	M	62	-	0	N.A.	1	N.A.	0	N.A.	0	N.A.
IT10	F	35	-	0	N.A.	1	N.A.	0	N.A.	0	N.A.

Abbreviations: NP, Nasal polyposis; IT, inferior turbinate; GC, glucocorticoidsteroid; N.A., not applicable.

[a] "+", atopy, and "-", non-atopy.

[b] Eosinophil and neutrophil counting were showed in percentage, which were calculated by positive staining cells per 200 cells at 400× magnification in every five fields with high-intensity positive cells.

Grading of cell count: Grade 0 = none; Grade 1 = less than 10%; Grade 2 = 10% to 40%; Grade 3 = more than 40%.

[c] Edema status: Grade 0 = absent; Grade 1 = mild or moderate; Grade 2 = severe.

[d] Epithelium integrity: 0, intact epithelium; 1, moderately damaged epithelium; 2, severely damaged epithelium.

[e] *p*-value obtained by Wilcoxon matched pairs sign rank test; values were considered significant if $p < 0.05$.

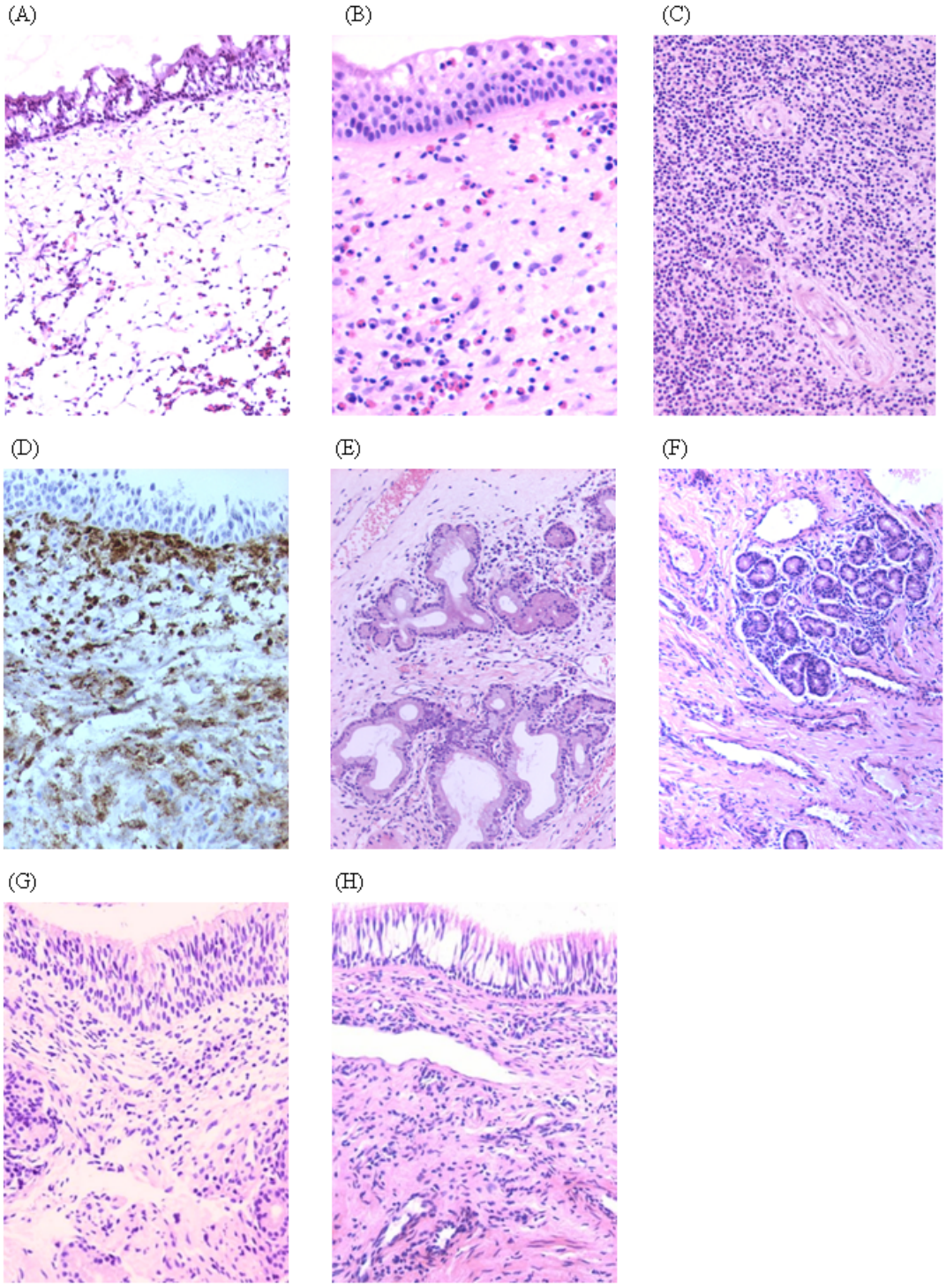


Figure 6.1 Representative staining pictures of nasal tissues. In GC-naïve NP tissues, some histopathological features are prominent: (A) epithelial damage and edema (grade 2); (B) eosinophil infiltration (grade 3); (C) neutrophil infiltration (grade 3); (D) high infiltration of lymphocytes; (E) dilation of glands. In GC-treated NP tissues, (G) absence of eosinophils and significant reduction of edema in GC-treated NP tissue. In control turbinate, (H) absence of eosinophils and edema, and presence of typical intact epithelium; (F) non-distensible glands in control turbinate. Pictures (A), (E), (C), (F), (G), and (H) were taken at magnification ×100, while pictures (B) and (D), were taken at magnification ×200.

Table 6.2 Comparison of histopathological patterns between GC-naïve NP and control

Histopathological components	GC-naïve NP	Nasal mucosal control
Epithelium	Damaged epithelium; Parts of squamous metaplasia; Thickening of basement membrane.	Typical intact respiratory epithelium containing columnar ciliated cells.
Stroma	Severe edema in most NP tissues.	No edema but with significant connective tissues
Glands	Low density, uneven distribution, and dilation with varying shape/size.	High density, even distribution, and small tubulo-alveolar shape
Infiltration cells	High infiltration of eosinophils, neutrophils, and lymphocytes.	Low infiltration of leukocytes.

Significant correlation between the infiltration of eosinophils and neutrophils among subjects of GC-naïve NP and control was evident ($r = 0.846$, $p < 0.001$) (**Figure 6.2**). However, among these GC-naïve and control subjects, infiltration of both eosinophils and neutrophils was not correlated with atopic status; and infiltration of these two cell types was also not correlated with epithelial damage and edema status (data not shown here).

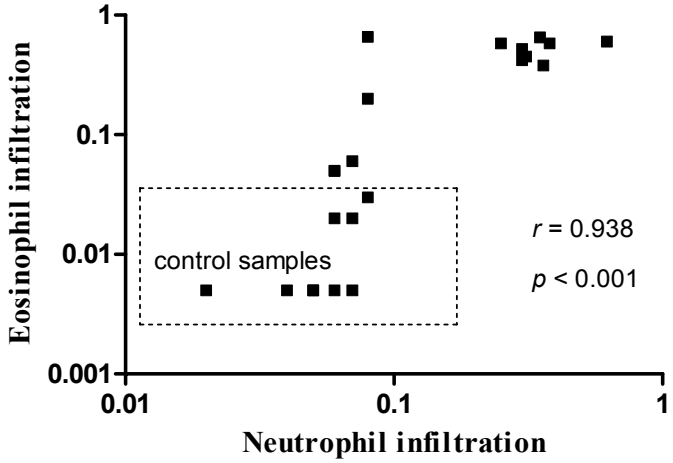


Figure 6.2 Correlation between infiltration of eosinophils and neutrophils in nasal tissues (GC-naïve NP and control). Eosinophil and neutrophil counting were calculated in percentage, which were calculated by positive staining cells per 200 cells at 400× magnification in every five fields with high-intensity positive cells. Square shapes in the dash line rectangle indicate control samples; while

the square shapes outside the rectangle indicate GC-naïve NP samples.

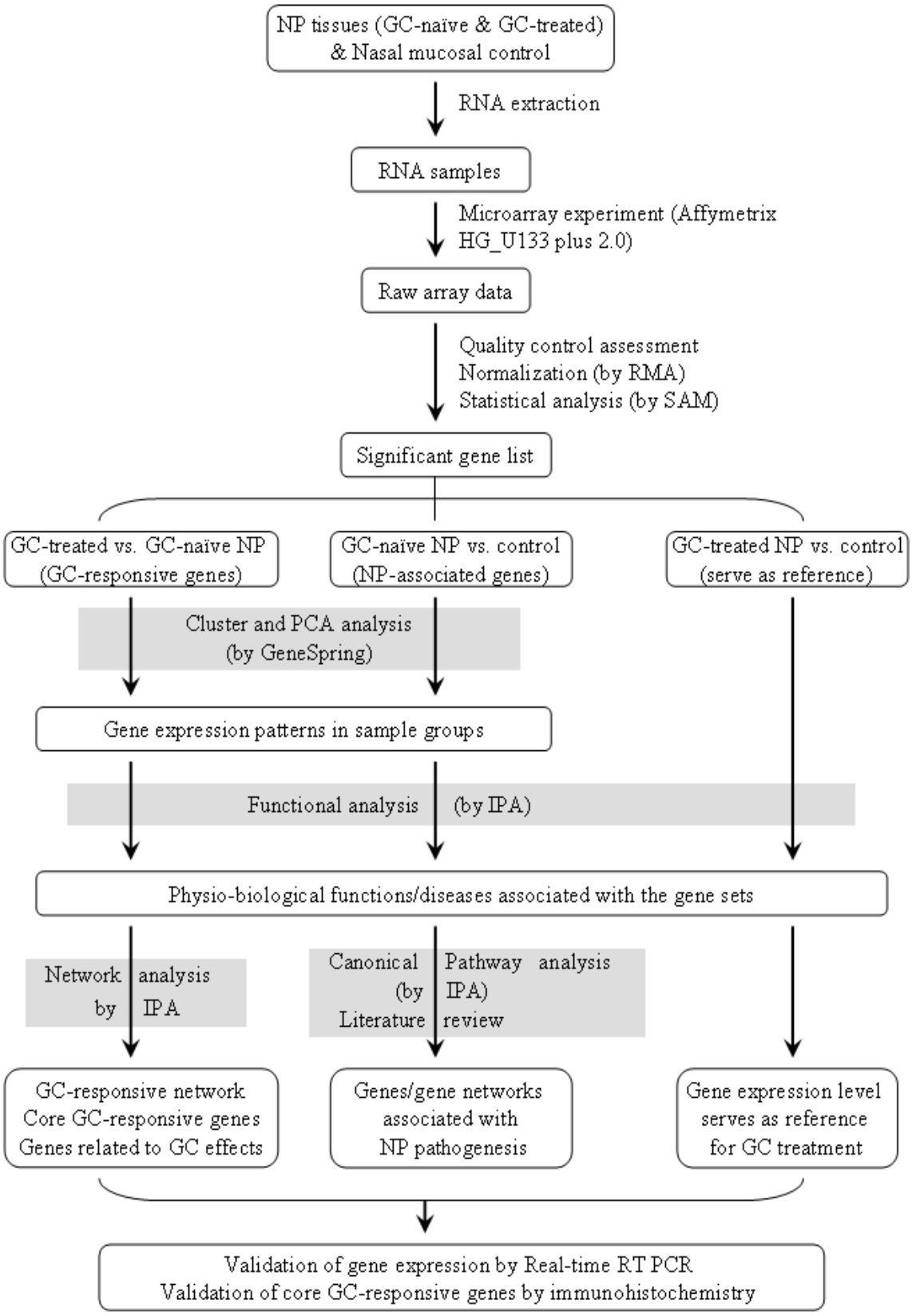
Most of the pathological findings in GC-naïve NP were reduced in response to GC treatment. Before GC treatment, epithelial damage (9 out of 12), high infiltration (grade ≥ 2) of eosinophils (10 out of 12) and neutrophils (8 out of 12), and severe edema (7 out of 12) were found in NP patients (**Table 6.1**). A short course of oral prednisone was effective in epithelial restitution, decrease of nasal eosinophil recruitment and tissue edema (**Table 6.1**). Among those 8 NP patients with increase of neutrophil infiltration, a reduction of neutrophil count following GC treatment was observed in 4 NP patients, but it did not reach statistical significance (p = 0.05). Interestingly, with regard to those 2 GC-naïve NP samples (NP3 and NP10) without significant epithelial damage, squamous metaplasia was observed in some parts of the epithelium; while improvement (i.e., no squamous metaplasia) in epithelium structure was evident after GC therapy, suggesting the effect of GCs on NP epithelial remodeling.

6.1.2 Strategy for identifying candidate genes by microarray analyses

After evaluating the histopathological patterns of NP as well as its response to GCs, we would like to study the molecular profiles underlying these histological changes in NP. The integrated microarray data analyses were therefore carried out. These analyses include the quality control evaluation of the samples/array data, statistical analysis of the genome-wide transcriptional alterations, class predictor analysis of gene expression patterns, functional and network analyses of the significant genes, and identification of candidate genes.

There are three comparison groups in the current microarray study: (i) the genes in the dataset of GC-naïve NP vs. control are considered to be involved in NP pathogenesis, i.e., NP disease associated genes; (ii) the genes in the dataset of GC-treated vs. GC-naïve NP are considered to underlie the molecular mechanism of the GC effect on NP, i.e., GC-responsive genes; while (iii) the genes in the dataset of GC-treated NP vs. control would more likely serve as references for the expression levels of NP associated genes which are regulated by GCs, i.e., whether the indicated NP relative gene is normalized by GCs or not. Therefore, the results from datasets of GC-naïve NP vs. control and GC-treated vs. GC-naïve NP are of great interest. **Figure 6.3** summarizes the strategy to identify NP and GC candidate genes by these integrated microarray analyses.

Figure 6.3 Flow chart for identification of GC-responsive genes and NP associated genes



6.1.3 Quality of samples and array data

Quality assessments, which can potentially flag outlier samples, should be carried out in the initial analysis of Affymetrix GeneChip dataset. Serials of quality control (QC) procedures have been done before analyzing the significant genes. These steps include the evaluation of RNA quality, QC for microarray experiment, QC for assay performance, QC for raw array data, data normalization, and QC for normalized data.

6.1.3.1 RNA quality

Quality evaluation was performed in extracted RNA by determining RNA concentration, purity, and integrity. Since the least amount of RNA loaded in a gene chip is 1 μg and the volume of RNA cannot be more than 8 μl, the concentration of the selected RNA samples must be more than 150 μg/ml. The A260/A280 ratio was confirmed between 1.9 and 2.1 for pure RNA.

The integrity of RNA was assessed by electrophoresis on a denaturing agarose gel. **Figure 6.4A** shows that the studied RNA have sharp 28S and 18S rRNA bands, and the 28S rRNA band is approximately 1.8 times as intense as the 18S rRNA band, indicating that these RNA are intact and acceptable for downstream work (micorarray and real-time PCR). Only the samples NP11, NP12, NP12R, IT7, IT8, IT9 and IT10 showed faint 28S:18S bands (**Figure 6.4B**) and were considered partially degraded. Since the NP samples are compared pair-wise (before versus after GC treatment), this assessment led to the exclusion of 2 pairs of NP samples and 4 control samples for downstream RNA work.

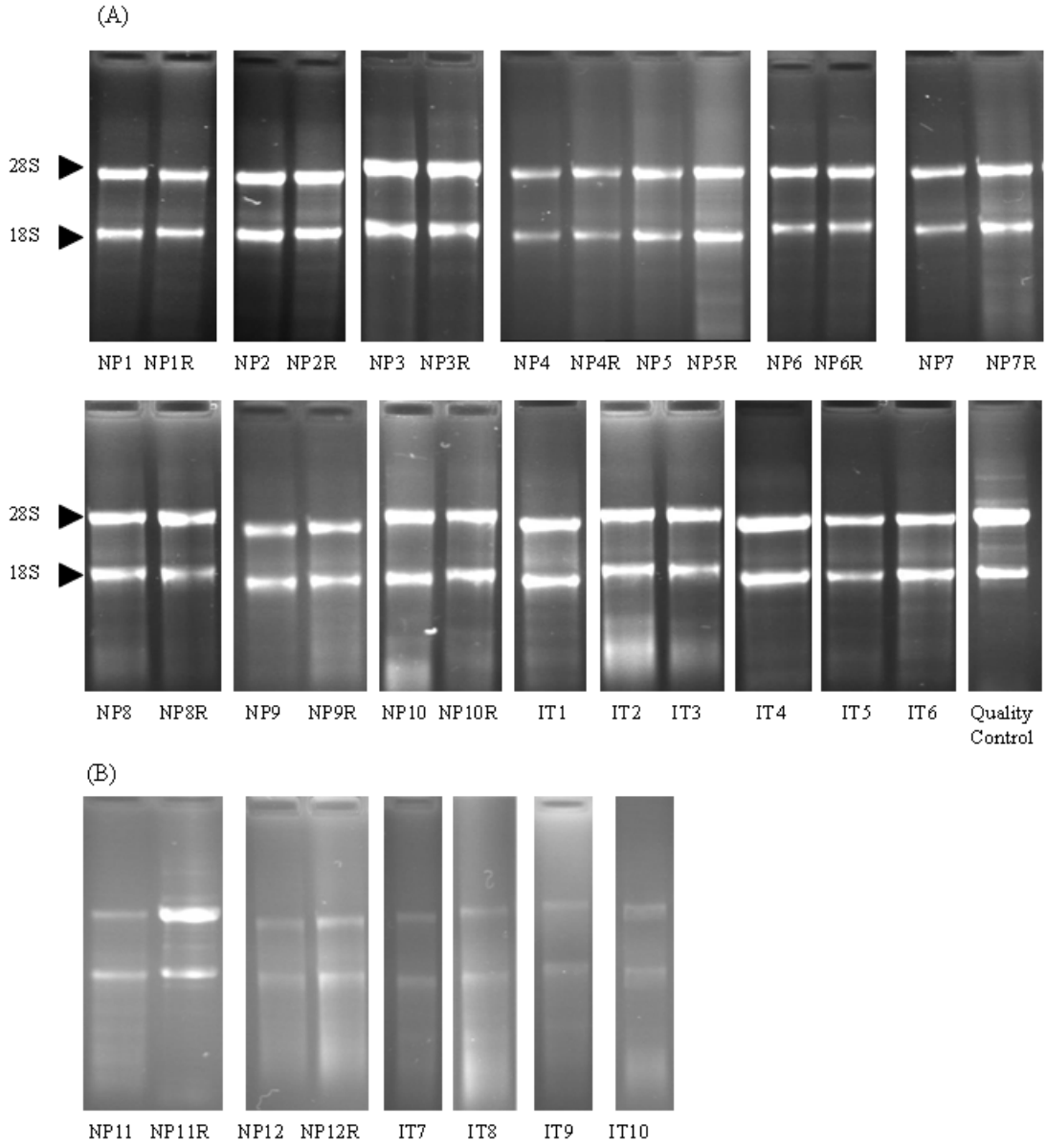


Figure 6.4 Gel electrophoresis of RNA samples. One μg of total RNA from nasal tissues (both NP and IT) were run on a 1% denaturing agarose gel. The 18S and 28S ribosomal RNA bands are clearly visible in the all RNA samples from plate (A), but are smear and faint in samples NP11, NP12, NP12R, IT7, IT8, IT9 and IT10 from plate (B). Human Lung Total RNA (Ambion) was served as quality control.

6.1.3.2 Quality of fragmented cRNA

During the microarray experiment, fragmentation of cRNA target before hybridization onto GeneChip probe arrays has been shown to be critical in obtaining optimal assay sensitivity. The size of fragmented cRNA run on the gel picture should not be more

than 300 bp. All of the cRNA ready to load in a gene chip were confirmed successfully fragmented and representative samples were shown in **Figure 6.5**.

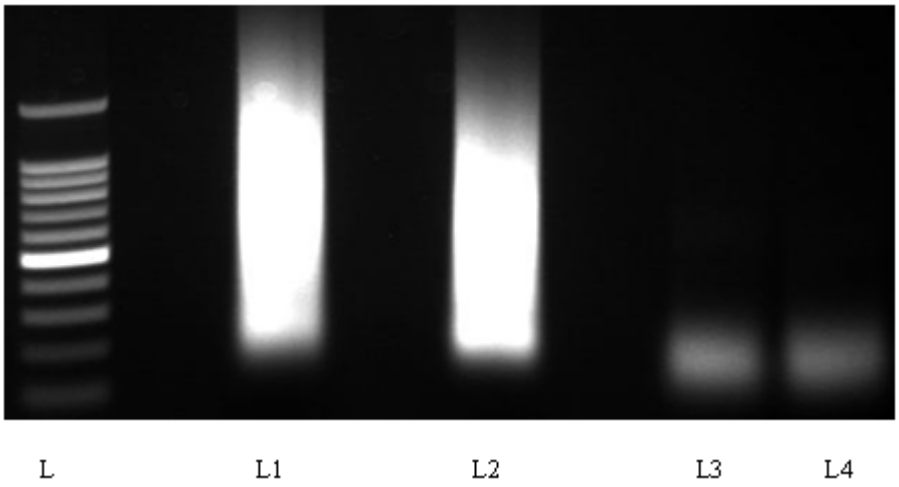


Figure 6.5 Gel electrophoresis of fragmented/unfragmented cRNA. Lane 1 and 2 represent unfragmented cRNA from NP6 and NP6R; L3 and L4 represent corresponding fragmented cRNA from sample NP6 and NP6R. L,size marker (100-bp DNA ladder)

6.1.3.3 Quality of assay performance

Once the microarray experiment is completed, the operating software generates an Expression Analysis report file (.rpt) for each array and allows the researcher to review the assay/hybridization performance. Following the Affymetrix guideline (*see* **Chapter 3.7.4,** *Page 47*), several QC parameters were assessed (**Table 6.3**): (i) comparable average background values and noise were within the arrays; (ii) poly-A controls were called 'Present' with increasing signal values in the order of *lys, phe, thr and dap*; (iii) hybridization controls were called 'Present' with increasing signal values in the order of *bioB, bioC, bioD* and *cre*; (iv) 3' to 5' ratio for GAPDH or β-actin was no more than 3; (v) present value is more than 40%; (vi) consistency of array data was validated by confirming that the range of Present Calls is 40% to 50% and the difference in scaling factor is less than 3-fold within the compared arrays. Most of the arrays match these criteria, only arrays of NP9, NP10, and IT6 show high

value (> 3) of 3' to 5' ratio for β-actin and GAPDH. Since the NP samples are compared pair-wise (before versus after GC treatment), this assessment lead to exclude two pairs of NP arrays (NP9/9R and NP10/10R) and one control array (IT6) for downstream microarray analysis.

Table 6.3 Parameters for assessing assay performance (part 1)

Sample ID	Background	Noise	Internal control genes (Signal 3'/5')		Percent present (%)		Scale factor
			β-actin	GAPDH	Present	Absent	
NP1	32.18	0.79	2.73	1.03	45.80	52.70	4.639
NP1R	32.03	0.74	2.52	0.99	43.90	54.60	5.004
NP2	26.91	0.72	2.38	1.10	47.10	51.10	4.594
NP2R	30.75	0.73	2.47	1.13	44.50	54.00	5.723
NP3	31.81	0.80	2.65	1.11	45.50	52.90	4.363
NP3R	30.81	0.75	2.30	1.02	45.10	53.40	4.759
NP4	33.28	0.82	2.80	1.17	45.80	52.70	4.330
NP4R	32.03	0.73	2.60	1.20	42.70	55.60	5.701
NP5	32.01	0.80	2.85	1.56	43.90	54.60	5.626
NP5R	32.06	0.76	2.75	1.45	44.80	53.70	5.305
NP6	30.05	0.70	2.52	1.04	41.40	57.00	6.709
NP6R	31.66	0.70	2.85	1.69	40.70	58.50	7.021
NP7	33.59	0.73	2.60	1.20	44.00	54.40	5.694
NP7R	31.26	0.71	2.69	1.69	41.40	57.10	5.809
NP8	29.47	0.68	2.68	1.12	45.20	53.10	6.160
NP8R	31.23	0.71	2.68	1.27	44.40	54.10	6.381
IT1	28.17	0.69	2.35	1.14	42.10	56.10	7.313
IT2	33.21	0.77	2.37	1.33	42.90	55.50	5.476
IT3	30.57	0.78	2.50	1.24	44.40	54.10	6.205
IT4	32.13	0.69	2.61	1.45	41.20	57.30	7.519
IT5	31.52	0.70	2.45	1.33	43.50	54.80	6.904

Table 6.3 Parameters for assessing assay performance (part 2)

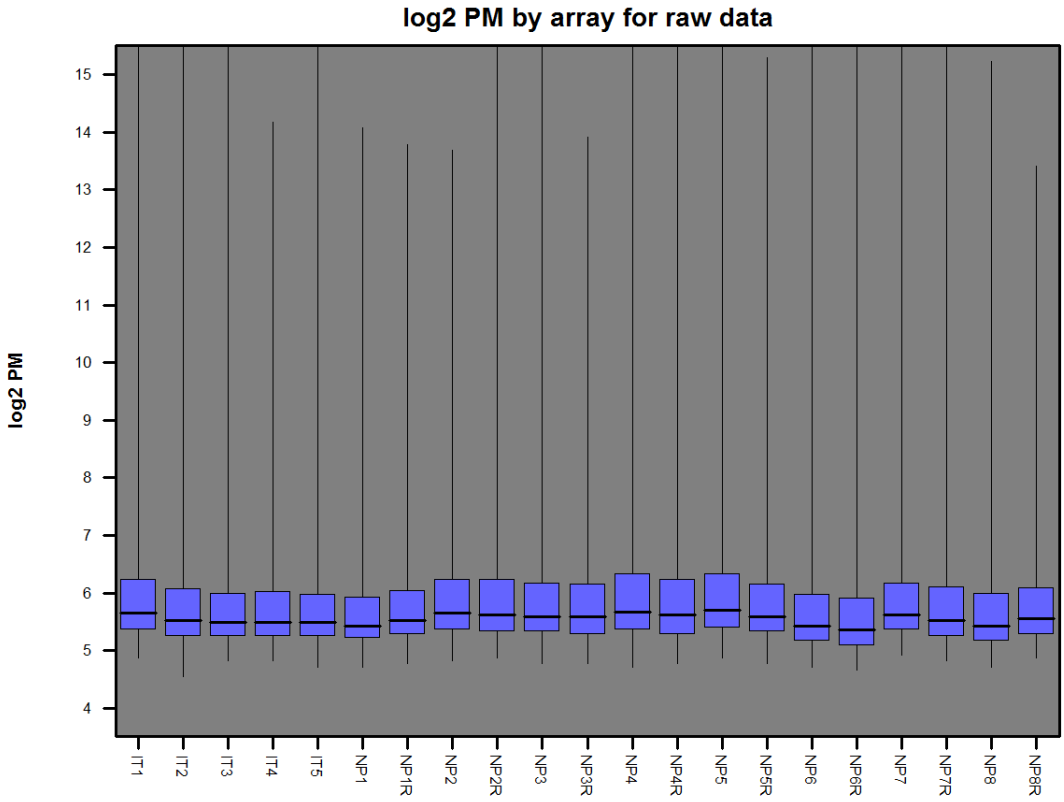
Sample	Poly-A controls (Signal)				Hybridization controls (Signal)			
ID	Lys	Phe	Thr	Dap	bioB	bioC	bioD	cre
NP1	96.35	113.42	145.26	751.87	223.75	576.33	1809.19	7810.37
NP1R	96.71	99.82	123.47	671.58	246.48	573.04	1747.9	7679.04
NP2	72.73	80.13	100.12	541.26	229.04	633.64	1987.55	7877.54
NP2R	81.45	85.22	106.98	560.37	274.42	694.89	2027.72	8108.03
NP3	67.58	80.97	102.41	565.63	207.29	501.68	1594.34	6632.25
NP3R	78.35	85.65	128.46	616.36	197.07	591.29	1692.26	7439.61
NP4	80.78	98.76	144.56	675.00	248.97	606.55	1932.21	7564.7
NP4R	107.85	117.54	165.16	760.38	237.4	644.57	2000.11	8171.95
NP5	79.65	99.36	112.60	553.63	290.54	730.56	2064.21	8796.98
NP5R	82.04	98.97	114.85	612.37	235.95	618.88	1817.48	7838.46
NP6	97.94	105.13	179.4	772.2	277.1	735.69	2017.83	9237.74
NP6R	131.2	177.73	180.24	853.78	371.4	995.56	2660.38	12707.16
NP7	113.83	126.09	181.24	821.08	231.99	582.43	1549.17	7789.59
NP7R	102.97	143.96	198.7	759.92	222.37	559.17	1875.27	7825
NP8	104.85	116.26	152.29	705.3	239.05	643.26	1977.44	8901.95
NP8R	141.75	150.27	212.34	940.76	239.1	662.81	1906.72	9271.03
IT1	122.01	131.97	185.09	798.05	262.46	686.88	1949.58	9822.6
IT2	140.13	155.36	197.78	903.21	268.99	706.06	2154.29	9134.91
IT3	114.84	124.97	163.84	777.20	259.28	717.81	1983.03	9057.46
IT4	126.48	164.26	200.02	841.56	335.15	822.44	2159.50	11559.7
IT5	148.34	186.3	239.63	1108.41	275.69	787.52	2282.75	10793.5

6.1.3.4 Quality of raw array data

Unlike the Affymetrix's array data report which only gives the quality information of individual array, RMAExpress can assess the quality of array data within a whole set of compared arrays before data normalization. The arrays from eight pairs of NP samples (before and after GC treatment) and 5 controls, which passed the QC for assay performance, were recruited for analyzing perfect match (PM) intensity. Two optional plots (boxplots and density plots) generated by RMAExpress show the PM intensities for each array (**Figure 6.6**). Both of the two plots show that all the selected arrays have similar shape and trend, and comparable PM intensities, i.e., no outliers among the datasets.

Figure 6.6

(A)



(B)

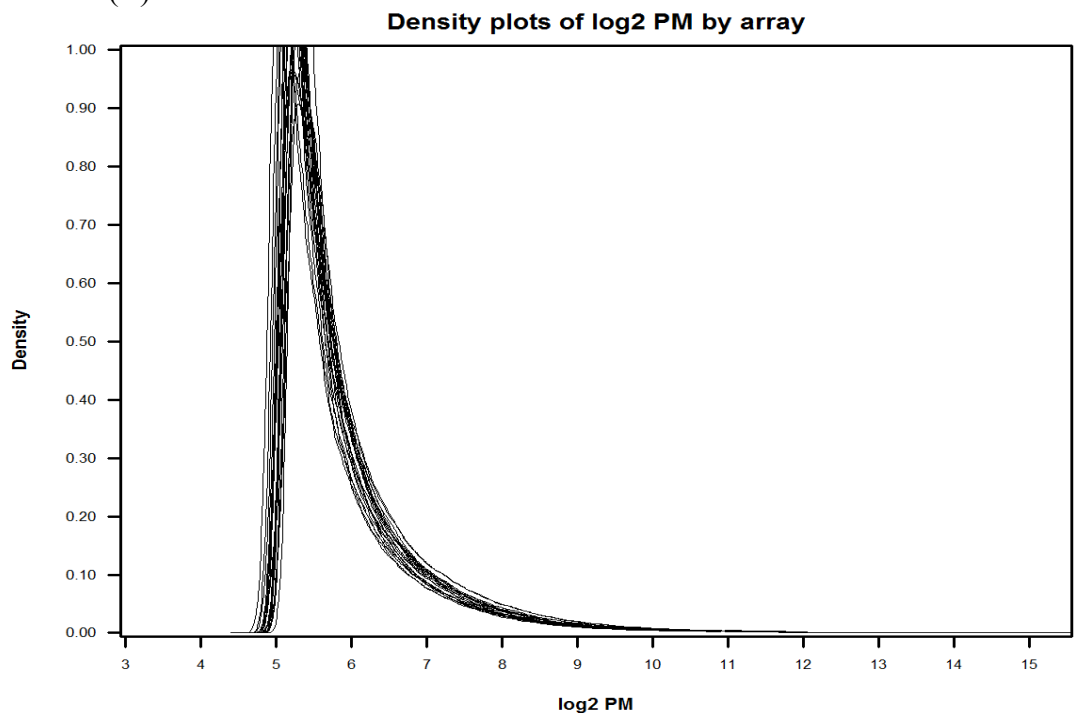


Figure 6.6 Visualization of array raw data. (A) Boxplots and (B) Density plots for visualization of the array data. For better visualization, perfect match intensities are log2 transformed.

6.1.3.5 Normalization of array data

Normalization is a process for reducing variation among the multiple high density arrays of non-biological origin. RMAExpress software performs a Robust Multichip Average (RMA) method to compute normalized expression values of arrays by doing background correction, quantile normalization, and median-polish summarization. RMA normalization is carried out at probe level for all the probes on an array. The arrays from three datasets (GC-naïve NP vs. control, GC-treated NP vs. control, and GC-treated vs. GC-naïve NP) were normalized. An expression value for each probeset was generated.

6.1.3.6 Quality of normalized data

The quality of normalized arrays could also be assessed by several tools in the RMAExpress software.

(1) Chip pseudo-images

Chip pseudo-images of residuals are very useful for detecting artifacts on arrays that could pose potential quality problems. RMAExpress software can produce residual images on a chip-by-chip basis when computing normalization expression values. **Figure 6.7** shows that the residual image of each array presented homogenized color throughout the whole region, implying no significant artifacts in these arrays.

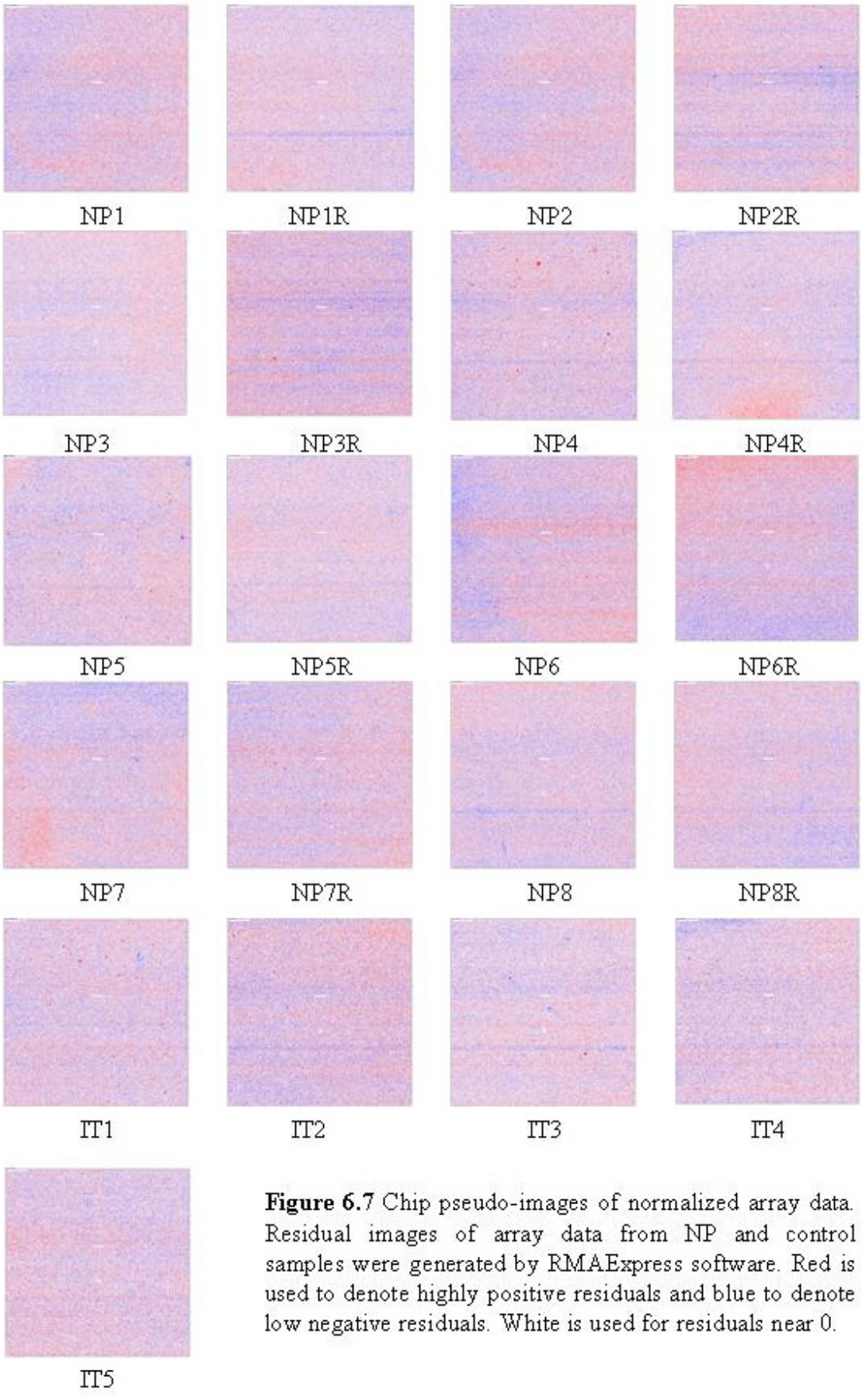


Figure 6.7 Chip pseudo-images of normalized array data. Residual images of array data from NP and control samples were generated by RMAExpress software. Red is used to denote highly positive residuals and blue to denote low negative residuals. White is used for residuals near 0.

(2) Probe level model (PLM) based quality assessment

Two main PLM based quality statistics are available in RMAExpress: Relative Log Expression (RLE) and Normalized Unscaled Standard Error (NUSE). To identify the lower quality arrays, RMAExpress also provides quality control cutoffs applied to the RLE/NUSE Multiplot. The arrays which fall well outside the control limits for both RLE and NUSE metrics are considered outliers. Within our dataset, the boxplots of all the arrays demonstrated similar shape and locate near the center position (data not shown here); moreover, no outlier array was found from the summarized RLE/NUSE Multiplot (**Figure 6.8**).

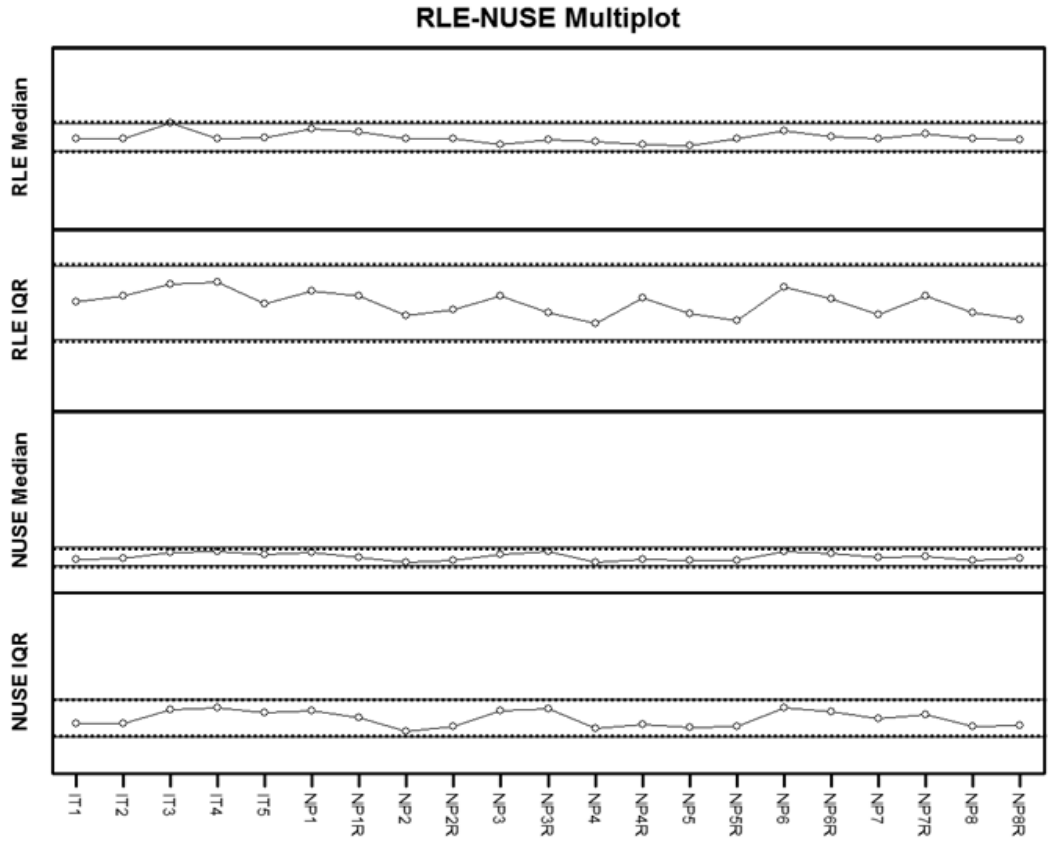


Figure 6.8 RLE and NUSE single summary plots of normalized array data. Normalized Unscaled Standard Error (NUSE) and Relative Log Expression (RLE) were used to access the quality of normalized data from NP and control samples. Dash lines are used to indicate the control limits.

In summary, the QC assessments of samples and array data are critical in successful

microarray analysis, since it facilitates filtering the outlier arrays and significantly reduces the obscuring variation which may arise during the experimental procedures. Our results indicate that (i) after step-by-step QC assessment, the final RMA-processed arrays are in good quality and comparable with each other; and (ii) these normalized array data are suitable for downstream statistical analysis.

6.1.4 Genome-wide transcriptional alterations

Following the normalization by RMA method and statistical analysis by SAM (*See* **Chapter 3.7.5,** *Page 51*), each transcript (with identified probeset ID) was assigned a score with a fold change and a proper false discovery rate (FDR) to represent its relative expression level. Therefore, we identified 3,833 differentially regulated transcripts in GC-naïve NP versus control. They differed in their relative intensities by at least 1.5-fold with FDR of 0.008. These transcripts represented 3,833/54,677 ≈ 0.07 (7%) of the total genes expressed in the nasal mucosal controls. Apart from the transcripts of GC-naïve NP versus control, comparisons between transcripts of GC-treated NP versus control and transcripts of GC-treated versus GC-naïve NP were also performed. In summary, 2,312 transcripts differed in relative expression (by at least 1.5-fold with FDR of 0.007) in GC-treated NP versus control, while 121 transcripts were different (by at least 1.5-fold with FDR of 0.06) in GC-treated versus GC-naïve NP.

After filtering the unmapped genes, genes with unknown gene ontology and genes with redundant probe identities, further search uncovered 1,989 and 1,330 genes which were differentially expressed in GC-naïve and GC-treated versus nasal mucosal controls, respectively (**Figure 6.9**). Expressions of 71 genes were different in

GC-treated versus GC-naïve NP tissues (**Figure 6.9, Table 6.4**). The Venn Diagram summarizes the relationship among the three comparisons (**Figure 6.9**): (i) among the 71 GC-responsive genes, most of them (n=51, 72%) were also found to be differentially expressed in GC-naïve NP versus control; (ii) these 51 genes represented 2.5% (51/1,989) of the NP disease associated genes, and underlie the molecular effects of GCs in NP; (iii) 88% (45/51) of these GC-responsive plus NP related genes were normalized in GC-treated NP; (iv) six out of the 51 genes were either under-expressed or over-expressed in GC-treated NP as compared to the controls; and (v) 48% (969/1,989) of the NP associated genes remained unchanged in GC-treated NP.

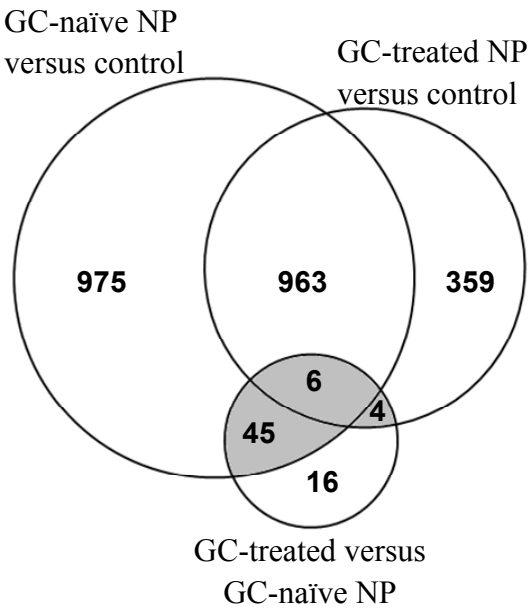


Figure 6.9 Overlapping genes in three datasets. Venn diagram shows the overlapping of differentially expressed genes in GC-naïve NP tissues versus nasal mucosal controls, GC-treated NP tissues versus nasal mucosal controls and GC-treated versus GC-naïve NP tissues.

Table 6.4 Significant GC-responsive genes in NP

Probe ID	Gene symbol	Gene title	Score (d)	Fold Change	FDR (%)
		Up-regulated genes in NP after GC treatment (n=55)			
222162_s_at	ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif 1.	3.319	2.464	0.000
233011_at	ANXA1	ANNEXIN A1.	5.260	5.905	0.000
201525_at	APOD	Apolipoprotein D.	3.218	2.412	0.000
221031_s_at	APOLD1	Apolipoprotein L domain containing 1.	3.139	4.004	0.000
205239_at	AREG	amphiregulin (schwannoma-derived growth factor)	3.024	6.474	0.000
203946_s_at	ARG2	Arginase type II.	2.624	3.299	3.560
202672_s_at	ATF3	Activating transcription factor 3.	2.472	7.548	3.904
238987_at	B4GALT1	BetaGlcNAc beta 1,4- galactosyltransferase, polypeptide 1.	2.459	2.254	3.904
205430_at	BMP5	Bone morphogenetic protein 5.	2.248	3.287	6.101
201236_s_at	BTG2	BTG family,member 2.	3.286	2.225	6.101
210735_s_at	CA12	Carbonic anhydrase XII.	3.044	2.145	1.309
1555827_at	CCNL1	Cyclin L1.	3.426	5.841	0.000
209795_at	CD69	CD69 molecule	3.357	2.999	0.000
206932_at	CH25H	Cholesterol 25-hydroxylase.Less info.	2.979	2.046	1.309
213992_at	COL4A6	Collagen,type IV,alpha 6. Less info.	3.071	2.262	1.309
209774_x_at	CXCL2	Chemokine (C-X-C motif) ligand 2, CXCL2	3.646	5.423	0.000
210764_s_at	CYR61	Cysteine-rich,angiogenic inducer,61.	2.503	4.127	3.904
201041_s_at	DUSP1	Dual specificity phosphatase 1.	5.316	4.977	0.000
204794_at	DUSP2	dual specificity phosphatase 2	2.391	3.197	4.413
208893_s_at	DUSP6	Dual specificity phosphatase 6.	3.549	2.077	0.000
227404_s_at	EGR1	Early growth response 1.	5.245	7.810	0.000
205249_at	EGR2	Early growth response 2.	5.097	4.242	0.000
206115_at	EGR3	Early growth response 3.	2.415	7.293	4.413
1564796_at	EMP1	Epithelial membrane protein 1.	3.049	4.734	1.309
209189_at	FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog, (known as c-Fos).	6.144	14.859	0.000
202768_at	FOSB	FBJ murine osteosarcoma viral oncogene homolog B.	4.002	16.671	0.000
209304_x_at	GADD45B	Growth arrest and DNA-damage-inducible,beta.	2.413	2.209	4.413
203821_at	HBEGF	Heparin-binding EGF-like growth factor.	3.382	5.454	0.000
208937_s_at	ID1	Inhibitor of DNA binding 1.	3.638	2.012	0.000
205207_at	IL-6	interleukin 6 (interferon, beta 2).	3.185	7.867	1.309
231183_s_at	JAG1	jagged 1 (Alagille syndrome).	3.711	2.007	0.000
201464_x_at	JUN	jun oncogene, (known as c-Jun).	4.119	3.390	0.000
201473_at	JUNB	Jun B proto-oncogene.	3.677	3.377	0.000
219371_s_at	KLF2	Kruppel like factor 2	2.933	2.084	2.046
221841_s_at	KLF4	Kruppel like factor 4	3.796	2.965	0.000
205266_at	LIF	Leukemia inhibitory factor.	3.337	2.402	0.000

Probe ID	Gene symbol	Gene title	Score (d)	Fold Change	FDR (%)
202431_s_at	MYC	v-myc myelocytomatosis viral oncogene homolog (avian)	2.561	2.610	4.413
223218_s_at	NFKBIZ	NF-kappaB inhibitor,zeta.	2.411	2.223	4.413
202340_x_at	NR4A1	Nuclear receptor subfamily4,group A,member1.	3.256	4.357	0.000
216248_s_at	NR4A2	Nuclear receptor subfamily4,group A,member2.	3.396	7.019	0.000
243296_at	PBEF1	Pre-B-cell colony enhancing factor 1.	4.973	3.707	0.000
204748_at	PTGS2	Prostaglandin-endoperoxide synthase 2, (known as COX-2)	3.565	3.469	0.000
216834_at	RGS1	Regulator of G-protein signalling 1.	3.350	3.195	0.000
212099_at	RHOB	ras homolog gene family, member B.	3.937	1.886	0.000
205725_at	SCGB1A1	secretoglobin, family 1A, member 1 (uteroglobin)	3.522	5.511	0.000
206211_at	SELE	Selectin E (endothelial adhesion molecule 1).	4.241	7.597	0.000
225660_at	SEMA6A	Sema,transmembrane domain, and cytoplasmic domain 6A.	2.331	1.710	6.101
204466_s_at	SNCA	Synuclein,alpha	3.680	2.008	0.000
227697_at	SOCS3	suppressor of cytokine signaling 3	3.537	4.202	0.000
212558_at	SPRY1	Sprouty homolog 1,antagonist of FGF signaling.	2.859	2.485	2.046
204011_at	SPRY2	Sprouty homolog 2.	2.734	2.114	2.840
221489_s_at	SPRY4	Sprouty homolog 4.	3.857	2.137	0.000
203888_at	THBD	Thrombomodulin.	3.676	2.631	0.000
1555938_x_at	VIM	Vimentin.	3.048	3.105	1.309
201531_at	ZFP36	Zinc finger protein 36.	3.467	3.777	0.000
		Down-regulated genes in NP after GC treatment (n=16)			
205692_s_at	CD38	CD38 molecule	-2.801	0.674	6.101
230609_at	CLINT1	Clathrin interactor 1.	-3.473	0.493	0.618
210163_at	CXCL11	Chemokine (C-X-C motif) ligend 11.	-3.752	0.404	0.000
203915_at	CXCL9	Chemokine (C-X-C motif) ligend 9.	-4.127	0.404	0.000
222858_s_at	DAPP1	Dual adaptor of phosphotyrosine and 3-phosphoinositides.	-3.444	0.651	2.282
227609_at	EPSTI1	Epithelial stromal interaction 1.	-4.264	0.467	0.000
205890_s_at	GABBR1	gamma-aminobutyric acid (GABA) B receptor, 1	-3.659	0.482	0.000
202270_at	GBP1	Guanylate binding protein 1 (interferon-inducible).	-4.711	0.475	0.000
211597_s_at	HOP	Homeodomain-only protein.	-5.424	0.420	0.000
204415_at	IFI6	Interferon,alpha-inducible protein 6.	-3.420	0.479	0.618
226757_at	IFIT2	Interferon-induced protein with tetratricopeptide repeats 2.	-2.703	0.624	6.101
204747_at	IFIT3	Interferon-induced protein with tetratricopeptide repeats 3.	-3.241	0.495	1.127
201744_s_at	LUM	Lumican.	-4.714	0.456	0.000
204259_at	MMP7	matrix metallopeptidase 7 (matrilysin, uterine)	-4.258	0.474	0.000
203936_s_at	MMP9	Matrix metallopeptidase 9.	-3.822	0.439	0.000
215342_s_at	RABGAP1L	RAB GTPase activating protein 1-like.	-3.118	0.632	6.101

6.1.5 Classification of samples based on gene expression patterns

Cluster analysis and principal component analysis (PCA) were first used to visualize the overall gene expression patterns throughout the three groups compared (GC-naïve NP, GC-treated NP and control). Cluster and PCA analyses were carried out by Genespring software (*See* **Chapter 3.7.7,** *Page 54*).

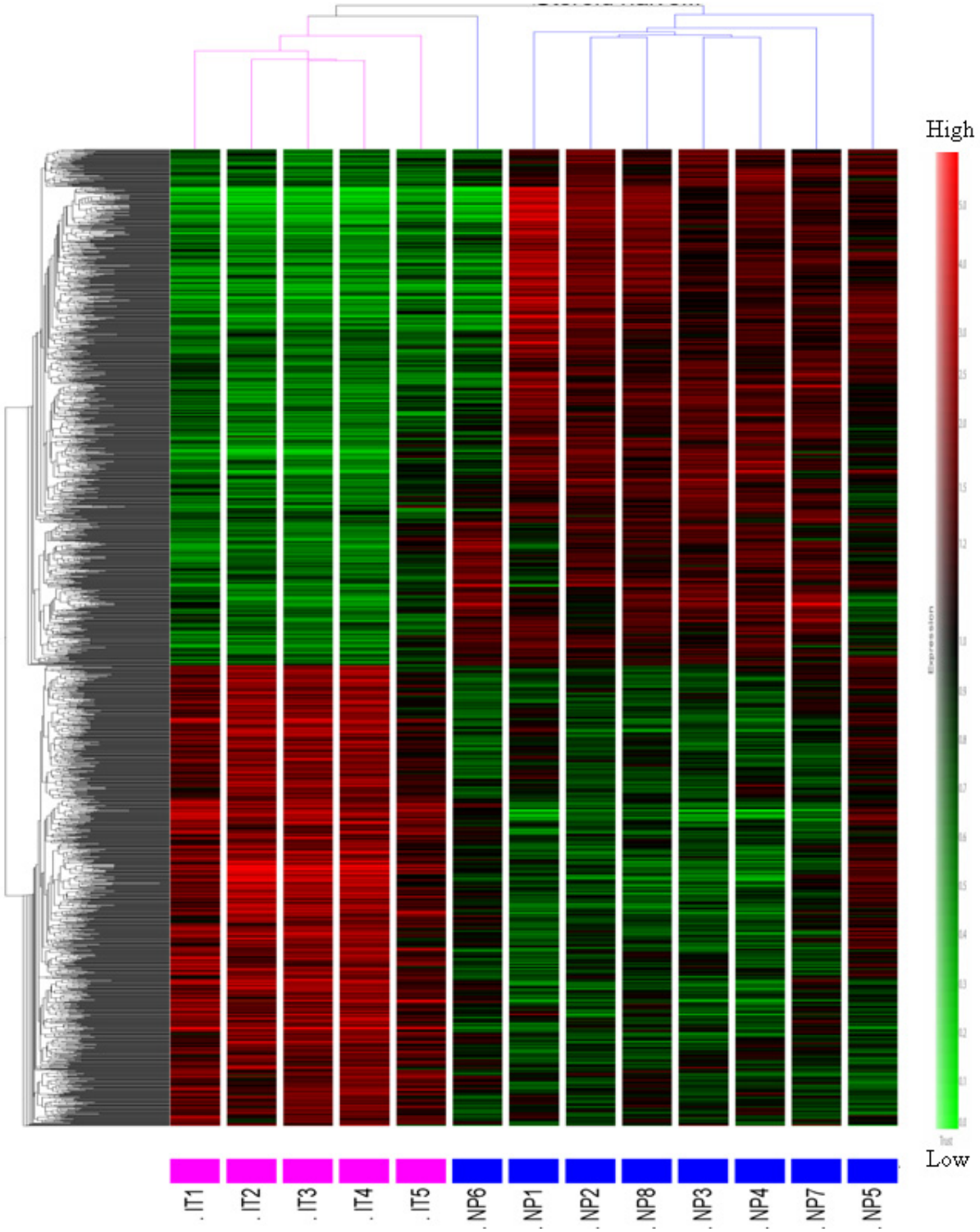
6.1.5.1 Tree view cluster

Cluster analysis for genome-wide expression data from the DNA microarray is described using standard statistical algorithms to arrange "conditions" (or say "samples") according to the similarity in pattern of gene expression, or genes according to similarity of expression profiles throughout the samples. Therefore, the relationships among objects (samples and genes) are represented by a tree whose branch lengths reflect the degree of similarity between the objects, i.e., genes/samples with similar expression patterns are placed closer to each other. The output is displayed graphically, conveying the cluster patterns and the underlying expression data simultaneously in a form intuitive for biologists.

In our study, two sets (GC-naïve NP vs. control and GC-treated vs. GC-naïve NP) of the significant genes were used to perform two-dimensional hierarchical clustering. The genes with similar expression profiles (i.e., up-regulation or down-regulation in NP or control group) were clearly separated (**Figure 6.10**) in each dataset. The more interesting dendrogram is to specify the arrangement of samples based on their gene expression patterns. **Figure 6.10** shows two distinct subclasses in each dataset: (i) for dataset of GC-naïve NP vs. control, one subclass consists entirely of NP tissues while

the other subclass consists primarily of control samples (except NP6); (ii) for the dataset of GC-treated vs. GC-naïve NP, one subclass consists primarily of GC-treated NP (except NP7) while the other consists primarily of GC-naïve NP (except NP4R). Moreover, NP4 and NP4R were grouped very closely.

(A)



(B)

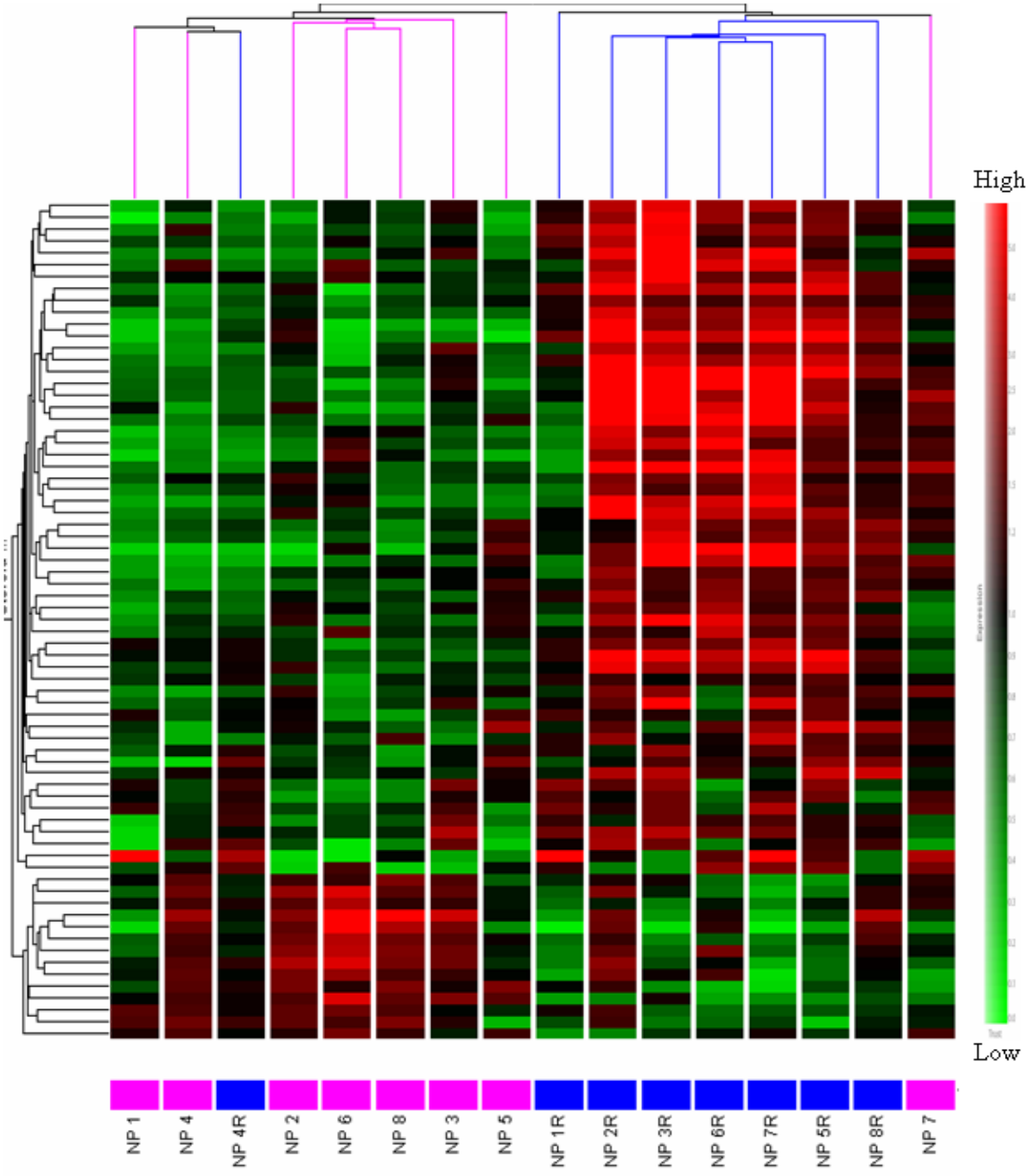


Figure 6.10 Cluster pictures generated from the results of significance analysis of microarrays (SAM) identified genes. Hierarchical cluster analysis was performed using the genes differentially expressed in (A) GC-naïve NP vs. control, and (B) GC-treated vs. GC-naïve NP. Each row represents an individual gene, and each column represents a tissue sample. Relative distance of each gene (vertical axis) and individual sample (horizontal axis) are also demonstrated. The color spectrum for the range of expression values is shown at the right: the red color indicates high expression and green color low expression. The tissue type color bar is shown at the bottom: in (A), purple color represents GC-naïve NP samples, while blue color represents control samples; in (B), purple color represents GC-naïve NP samples, while blue color represents GC-treated NP samples.

6.1.5.2 PCA classification

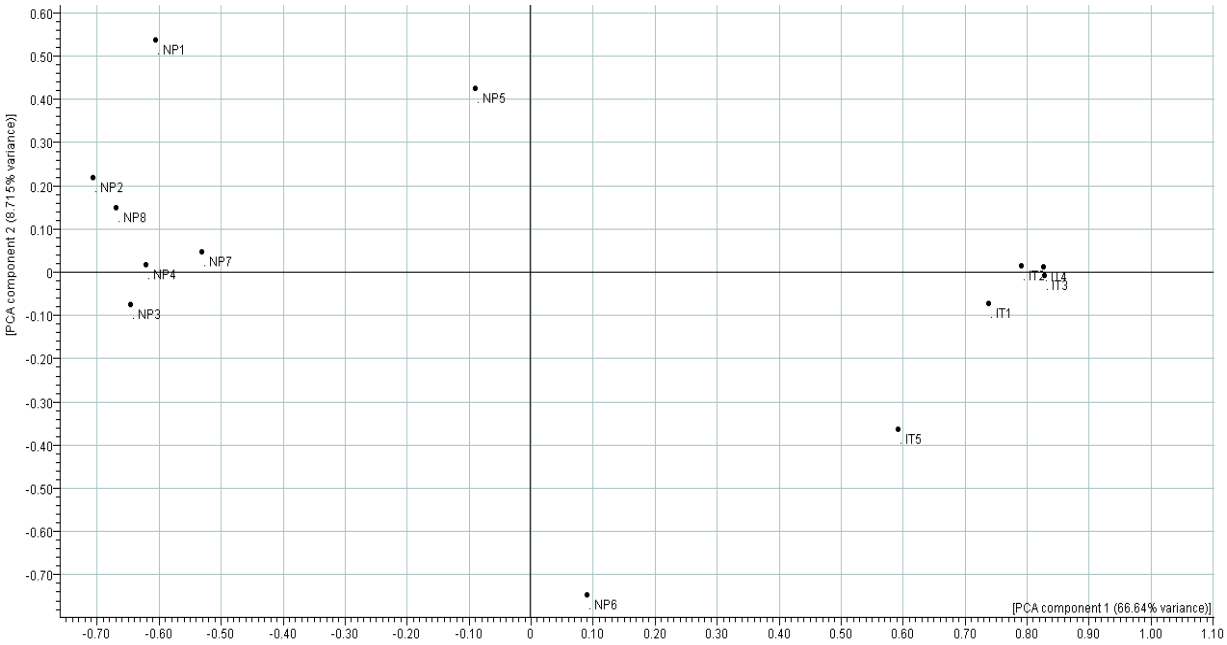
The expression of thousands of genes is measured across many conditions in a typical microarray experiment. Therefore, it becomes impossible to make a visual inspection of the relationship between genes or conditions in such a multi-dimensional matrix. PCA is a statistical method that can be used to reduce complex data sets with multiple variables into significantly smaller number of variables (known as components), which retain the relevant variance information used to distinguished the sample groups from one to another.

We applied PCA to the significant genes in each of the three comparisons to establish the inter-relationships among the tissue samples. **Figure 6.11** shows the two-dimensional (2D) plots for gene expression data of each comparison. The first two components, which accounted for more than 70% variance in our gene expression data, could be used to distinguish the overall gene expression profiles of GC-naïve NP, GC-treated NP and control. In order to interpret the variance of different samples intuitively, the 2D PCA plot can be separated like the four quadrants of a Cartesian coordinate system. Since the PCA component 1 represents the most variance of expression patterns (> 55%) among the objects, the samples in Quadrant II and III can be classified in one group, while the samples in Quadrant I and IV can be classified in another group (**Figure 6.11**).

The analysis demonstrates that most of the nasal tissues from the three sample groups can be separated easily in their pairwise comparisons, while the same cannot be said of one to two NP samples in these comparisons which were also described in cluster analyses. PCA figures showed that NP6 was located in the “control” Quadrant (I, IV)

in comparisons of GC-naïve NP versus control (**Figure 6.11**). Regarding the PCA for comparison of GC-treated versus GC-naïve NP (**Figure 6.11**), NP4R was located in the "GC-naïve NP" Quadrant (I, IV) and the distance between NP4 and NP4R was very close. The NP7 was located in the "GC-treated NP" Quadrant (II, III), but the distance between NP7 and NP7R was not close.

(A)



(B)

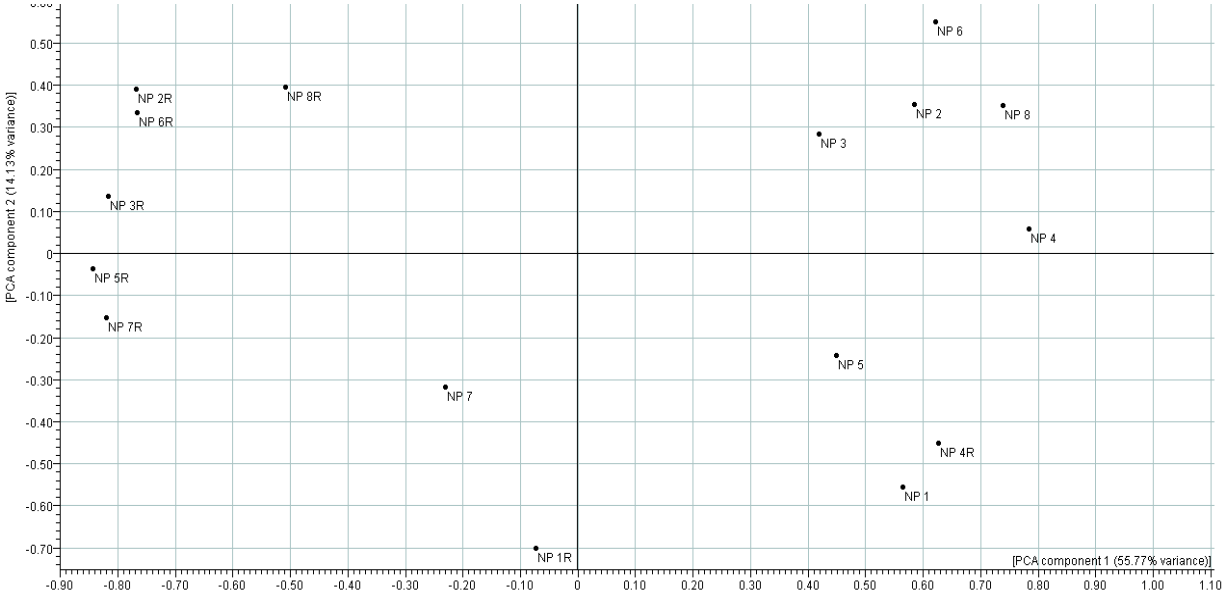


Figure 6.11 Principal component analysis (PCA) plots generated from the results of significance analysis of microarrays (SAM) identified genes. The gene expression profiles of (A) comparison of GC-naïve NP versus control and (B) comparison of GC-treated versus GC-naïve NP were analyzed by PCA. The two-dimensional plot view of gene expression data is shown, with respect to their correlation to the first two principal components, which account for more than 70% variance in the expression data. The PCA plot are separated into four quadrants (I~IV) like a Cartesian coordinate system for easy interpretation.

In summary, the results of cluster analysis and PCA were comparable to each other and both of them showed that different types of nasal tissues were almost classified into corresponding group.

6.1.6 Functions of the significant genes

Cluster and PCA analyses describe overall changes in apparent gene expression, but provide few insights into the biological process and signaling networks involved in the formation and development of NP, as well as its response to GC treatment. Therefore, it requires systematic analyses in the context of known biophysiological function and/or diseases, derived from the three datasets.

The three sets (GC-naïve NP vs. control, GC-treated NP vs. control and GC-treated vs. GC-naïve NP) of SAM identified significant genes accompanied by their effective significance as indicated by the *d* score, were uploaded to Ingenuity Pathway Analysis (IPA) for functional analysis. Biophysiological functions and/or diseases were assigned to each dataset by using the Ingenuity Pathway Knowledge Base (IPKB) as a reference set and a proprietary ontology representing over 500,000 classes of biological objects and consisting of millions of individually modeled relationships between proteins, genes, complexes, cells, tissues, small molecules and diseases. These encoded relationships are based on a continual formal extraction from the

public domain literature and cover more than 10,300 human genes. The biophysiological functions and/or diseases assigned to each dataset were ranked according to the significance (*p*-value) of that function to the dataset. Thus, the top significant and relevant functional annotations in each main category (Diseases & Disorders; Physiological System Development & Function; Molecular & Cellular Functions) of each dataset were listed in the respective tables (**Appendix I,** *Page 259*).

Briefly, the NP associated genes (from dataset of GC-naïve NP vs. control) and the GC-responsive genes (from dataset of GC-treated vs. GC-naïve NP) were most likely involved in: (i) the molecular functions such as cellular movement, growth, proliferation, development, and death; (ii) the physiological functions such as tissue morphology/development, organ development/function, and immune response; (iii) the diseases such as cancer, inflammatory and immunological diseases.

Once we get the top relevant functions in each primary category from each comparison, we can compare these relevant functions among the three datasets (i.e., GC-naïve NP vs. control, GC-treated NP vs. control, and GC-treated vs. GC-naïve NP) by IPA. This comparison analysis can be used to quickly gain an overview over the effect of GC treatment on a variety of high level functions (*See* **Chapter 3.7.8.1,** *Page 56*). **Figure 6.12** summarizes the comparison of the top relevant functional annotations in each primary category among these three datasets: (i) in the primary category of Diseases & Disorders, GC treatment mainly affects the genes involved in cancer, inflammatory disease, connective tissue disorders, and immunological disease; (ii) in the primary category of Physiological System Development & Function, GC

treatment mainly affects the genes involved in tissue development, organismal development, and cardiovascular system development & function; (iii) in the primary category of Molecular & Cellular Functions, GCs have potent effects on most of the selected functions, including cellular movement, cellular growth & proliferation, cell death, cellular development, cell morphology, cell cycle, and gene expression.

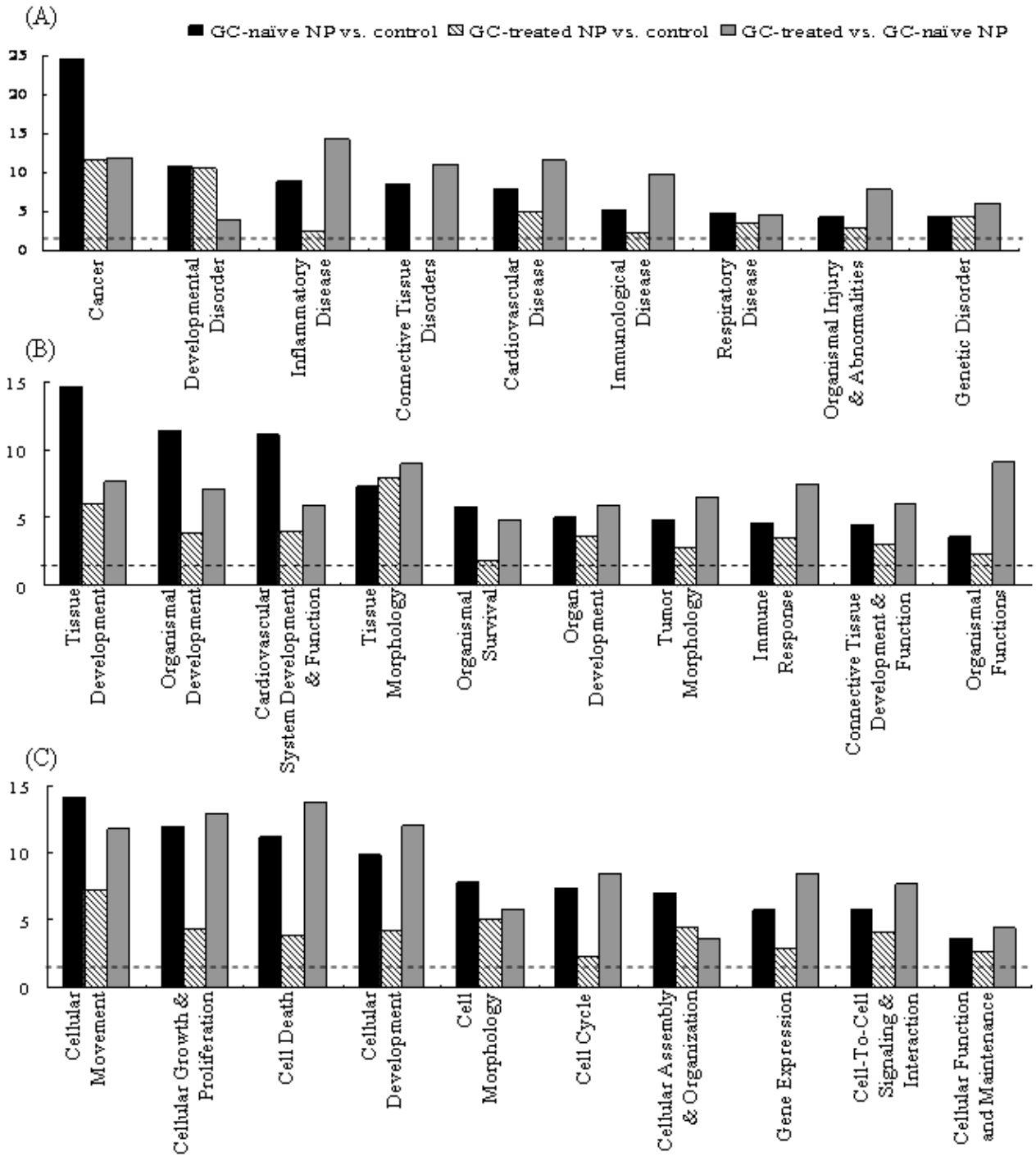


Figure 6.12 Functional comparison among the datasets. Top relevant functional annotations for gene sets differentially expressed in three comparisons: GC-naïve NP versus control, GC-treated NP versus control and GC-treated versus GC-naive NP. Three main categories of the functions are listed in (A) Diseases & Disorders, (B) Physiological System Development & Function, (C) Molecular & Cellular Functions. Statistic *p* value for a given function is calculated by considering 1) the number of functional analysis genes that participate in that function and 2) the total number of genes that are known to be associated with that function in the Ingenuity Pathway Knowledge Base (IPKB). On the y-axis of the diagram, the significance is expressed as the exponent of the *p* value calculated for each function. Dashed line represents the threshold ($p = 0.05$) of the significant level of the functions.

6.1.7 Identification of GC-responsive genes by network analysis

To further elucidate the global changes in NP tissue and its response to GC treatment, we sought to computationally decipher the principal networks involved in the given significant genes. Based on the computed scores, 68 and 5 significant networks were generated from the dataset of GC-naïve NP vs. control (data not shown here), and GC-treated vs. GC-naïve NP (**Table 6.5**), respectively. Since the numbers of significant networks and significant genes in the dataset of GC-naïve NP vs. control are huge, it is too complicated to select the representative networks and interpret the functional relationships among the interacting genes in the analysis of GC-naïve NP vs. control. In contrast, the dataset of GC-treated vs. GC-naïve NP contains fewer amounts of significant networks and genes, so that it is intuitive to explain the functional interaction among the genes of interest in the generated networks. For this reason, we intend to use network analysis to identify GC-responsive genes and their related network; but apply Canonical Pathway Analysis to identify the networks associated with NP pathogenesis. This part will be described in the latter section (**Chapter 6.1.8**).

Table 6.5 Functional network analysis for GC-responsive genes (Part I)

Network ID	Molecules in Network*	Score	Focus Molecules
1	**ANXA1**, **AREG**, **BTG2**, Calpain, Cbp/p300, Creb, **CXCL2**, Cyclin A, **DUSP6**, **EGR1**, ERK1/2, ETS, **ETS2**, Fgf, **FOS**, **GADD45B**, GC-GCR dimer, **HBEGF**, **ID1**, IFIT2, **IL6**, **JUN**, Mek1/2, MHC Class II, **PBEF1**, **PTGS2**, **SPRY1**, **SPRY2**, **SPRY4**, STAT5a/b, SWI-SNF, Tgf beta, **ZFP36**	43	20
2	Alkaline Phosphatase, **ATF3**, **B4GALT1**, **CCNL1**, CD38, CXCL9, CXCL11, Cyclin D, **CYR61**, DAPP1, **DUSP1**, **DUSP2**, **FOSB**, Gsk3, Hsp27, Ikb, IL1, JAK, **JUNB**, LDL, **LIF**, NFkB, **NFKBIZ**, Pdgf, PDGF BB, PDGF-AA, PI3K, Rb, **RHOB**, **SCGB1A1**, SOCS, **SOCS3**, STAT, **THBD**	35	18
3	14-3-3, Akt, Ap1, **CD69**, Cyclin E, **EGR2**, **EGR3**, **EMP1**, GABBR1, Hsp70, Hsp90, Ige, Insulin, Jnk, **KLF2**, **KLF4**, Mapk, Mek, MMP7, MMP9,**MYC**, Nfat, **NR4A1**, **NR4A2**, P38 MAPK, Pkc(s), Pld, Ras, Ras homolog, **RGS1**, Rxr, **SELE**, **SNCA**, TCR, Ubiquitin, Vegf	25	15
4	15-(S)-hydroperoxyeicosatetraenoic acid, **APOD**, ARF4, **CA12**, **CH25H**, CIDEC, Ck2, **COL4A6**, ELK3, **FZD5**, HAS1, HNRNPC, HOPX, HOXA11, IFI6, IFIT3, **JAG1**, JAG2, LRP6, LTBP2, MFAP2, MPZ, NOP5/NOP58, **PBEF1**, PDGFC, progesterone, RASA3, RNA polymerase II, SLC7A1, TERT, TGFB1, TGFBI, **THBD**, TNF	20	11
5	**ADAMTS1**, AP2A2, **APOLD1**, ARL6IP1, BCAS3, beta-estradiol, **BMP5**, CCNL2, CDKN1A, CLINT1, COMT, CTSH, DDR1, EGF, ELK3, ELL, FAT2, GBP1, **GPX3**, GSTA4, Hexokinase, Histone h3, HSPH1, KIAA0101, leukotriene D4, LUM, MCM5, MKI67, PELP1, TP53, UBE2S, **VIM**, **ZFP36**	18	9

*The underlied words in bold: Up-regulated genes in NP after GC treatment.

The words in grey color: Down-regulated genes in NP after GC treatment.

Table 6.5 Functional network analysis for GC-responsive genes (Part II)

	Top three functions/diseases of each network	*p*-value[#]
Network1	Cellular Development	1.61E-10
	Cellular Growth and Proliferation	2.50E-09
	Cell Death	6.09E-09
Network2	Immunological disease	2.89E-06
	Inflammatory disease	5.18E-06
	Cellular movement	3.56E-05
Network3	Tissue morphology	7.97E-06
	Cell cycle	1.13E-05
	Gene expression	2.72E-05
Network4	Cellular development	4.38E-07
	Cancer	4.34E-06
	Cell death	4.34E-06
Network5	Gene expression	5.07E-09
	Cancer	8.17E-08
	Cell death	8.17E-08

[#] Fisher's exact test was used to calculate a *p*-value determining the probability that each biological function and/or disease assigned to that network is due to chance alone.

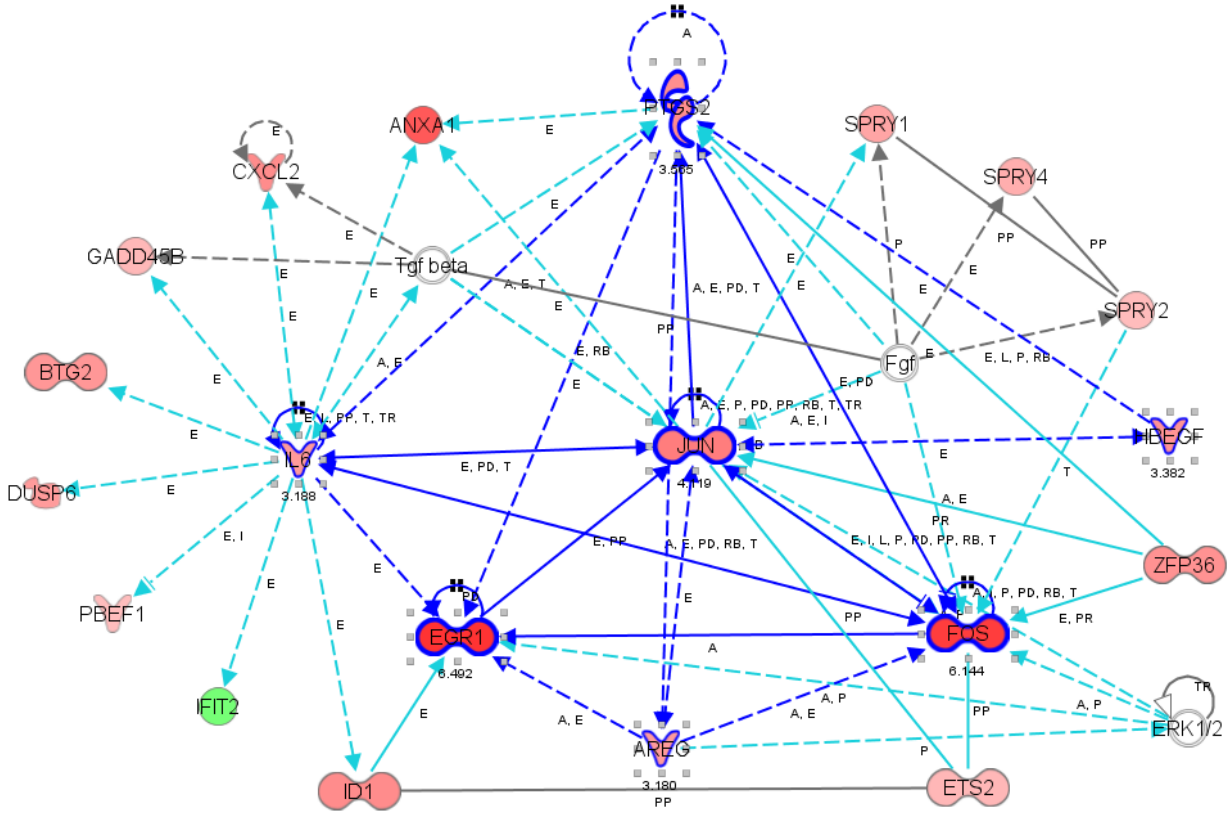
6.1.7.1 Identification of GC-responsive gene networks

In the dataset of GC-responsive genes, all five significant networks and their respective related functions are listed in **Table 6.5**. Among these networks, the top-scoring one (score = 43) contains two central molecules including JUN (also known as c-Jun) and FOS (also known as c-Fos), which are the most abundant components of activation protein 1 (AP-1) heterodimer (**Figure 6.13A**). Since these two important AP-1 genes are the prominent interaction partners at the level of interconnecting functional modules, this network is the so called AP-1 network. Notably, 20 of the 71 GC-responsive genes that were differentially expressed have at least one gene in the AP-1 pathway, underscoring its pivotal role in its corresponsive to GC therapy. In addition, evaluation of the AP-1 sub-network regions revealed that a group of AP-1 related genes (PTGS2, IL-6, AREG, HBEGF and EGR1) were modulated by GC administration in NP tissues (**Figure 6.13A**). Functional analysis

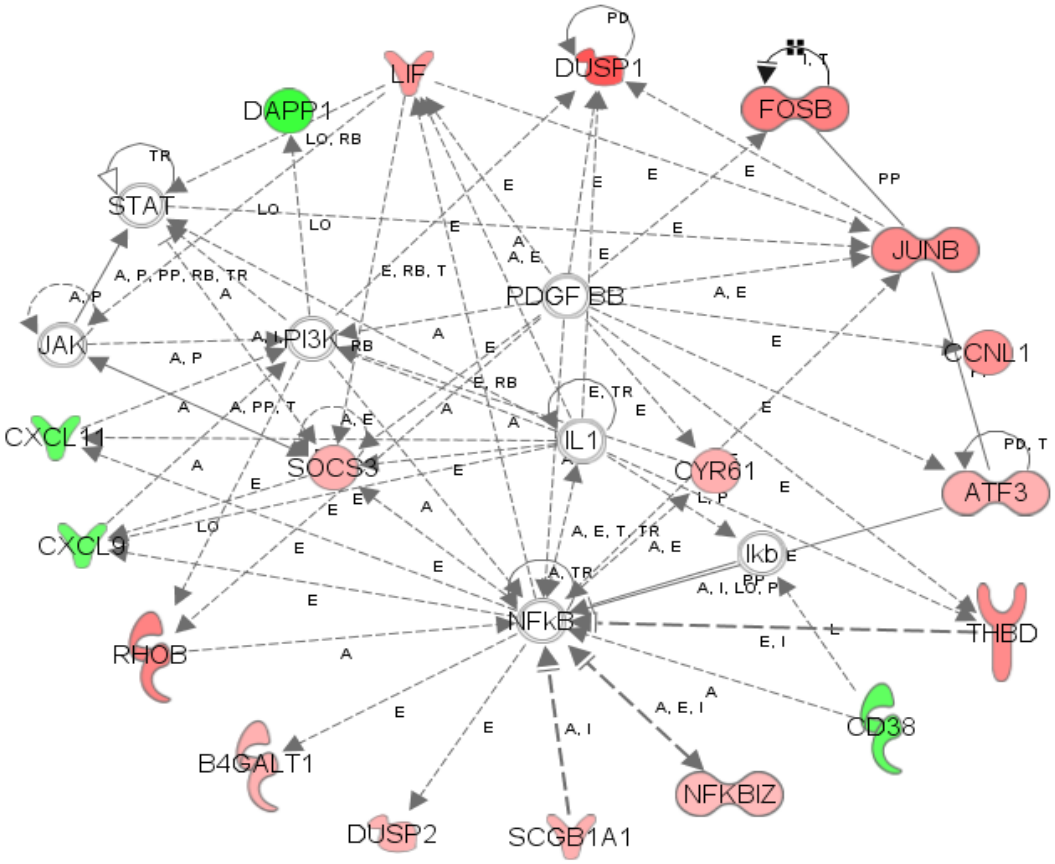
for this network indicates that GCs may have significant effects on NP by regulating cellular development, growth, proliferation and death (**Table 6.5**). Note that since AP-1/AP-1 related genes are altered in GC-naïve NP as compared to the control, they will also be identified in Canonical Pathways (*See* **Chapter 6.1.8,** *Page 126*).

The 2nd ranking network from the GC-responsive gene set is also of great interest, since the molecules in the network are likely associated with inflammatory and immunological diseases (**Figure 6.13B**). Although the core molecule (NF-kappa B) was not altered by GC treatment, most of its interacted genes were considered to have pro-/anti-inflammatory function, including CXCL9, CXCL11, DUSP1, DUSP2, NFKBIZ, SOCS3, SCGB1A1, and THBD. Some of the other anti-inflammatory genes (such as ANXA1, ZFP36, DUSP6, and SPRYs (1, 2, 4)) were also found in the top-scoring network, while two pro-inflammatory genes (MMP7 and MMP9) were found in the 3rd ranking network (**Table 6.5**). Moreover, two other AP-1 genes (FosB and JunB) were presented in this 2nd ranking network. Note that since some anti-inflammatory genes (such as DUSPs, SPRYs, SOCS3, and NFKBIZ) are also altered in GC-naïve NP as compared to the control, they will also be identified in the Canonical Pathways (*See* **Chapter 6.1.8,** *Page 126*).

(A)


ANXA1
CXCL2
SPRY1
SPRY4
SPRY2
GADD45B
Tgf beta
Fgf
BTG2
JUN
HBEGF
DUSP6
IL6
ZFP36
PBEF1
EGR1
FOS
IFIT2
AREG
ERK1/2
ID1
ETS2

(B)


DUSP1
LIF
DAPP1
FOSB
STAT
JUNB
PDGF BB
JAK
PI3K
CCNL1
CXCL11
IL1
SOCS3
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THBD
RHOB
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NFKBIZ
DUSP2
SCGB1A1

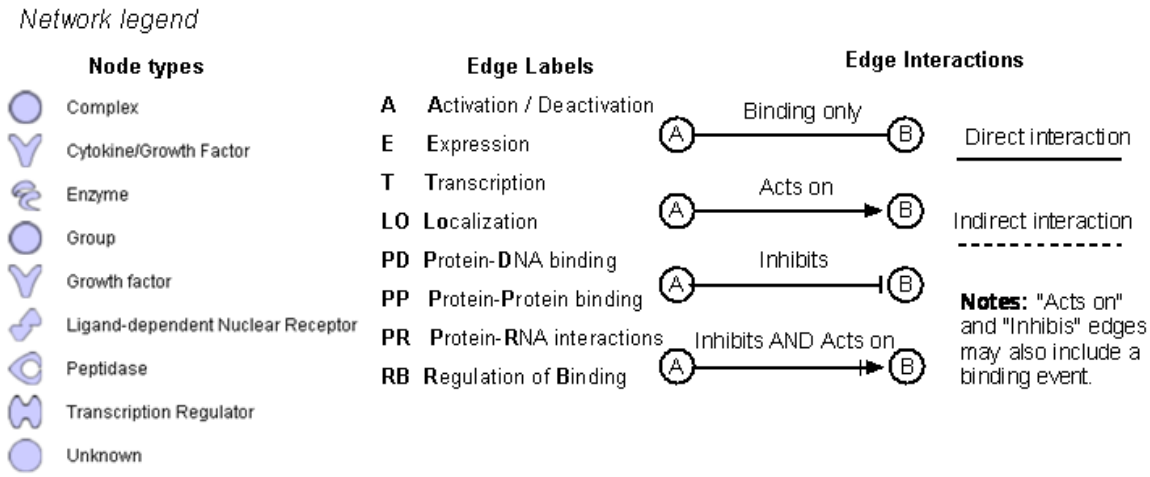


Figure 6.13 Network pathways of GC-responsive genes. Networks composed of GC-responsive genes were complemented by an unsupervised relevance network learning algorithm without any priori assumptions and post adjustment. (A) Top-scoring network with highlight (in dark blue color) of AP-1 and AP-1 related genes. (B) 2nd ranking network. Nodes represent genes, with their shape representing the functional class of the gene product, and edges indicate the biological relationship between the nodes (*see network legend*). Nodes are color coded according to their *d* score generated by SAM (red, overexpression in GC-treated vs. GC-naïve NP; green, underexpression in GC-treated vs. GC-naïve NP) and the color intensity increases with the magnitude of altered regulation.

6.1.7.2 Identification of core GC-responsive candidate genes

Another application of network analysis is to identify core genes among the total genes. This identification can be facilitated by merging the individual networks and then the graphic visualizations of all potential connections are generated. The genes with most prominent interaction partners within the merged networks are considered the central molecules. Since the total selected molecules for merging networks is limited to 500 nodes (i.e., focused genes), the total significant networks (containing more than 500 nodes) in dataset of GC-naïve NP vs. control cannot be merged. Therefore, only the networks from GC-responsive gene set were able to be merged. A high-resolution picture of the entire merged network and an enlarged portion with core genes of the merged network are shown in **Figure 6.14**.The combined networks revealed that c-Jun and c-Fos map the central nodes among all the GC-responsive

genes, which are the same as the core genes in the top-ranking GC-responsive network (AP-1 network). In addition, the merged networks showed that 3 of the AP-1 related genes (PTGS2, IL-6 and EGR1) in the AP-1 network also contain prominent interactions with other GC-responsive molecules (**Figure 6.14**).

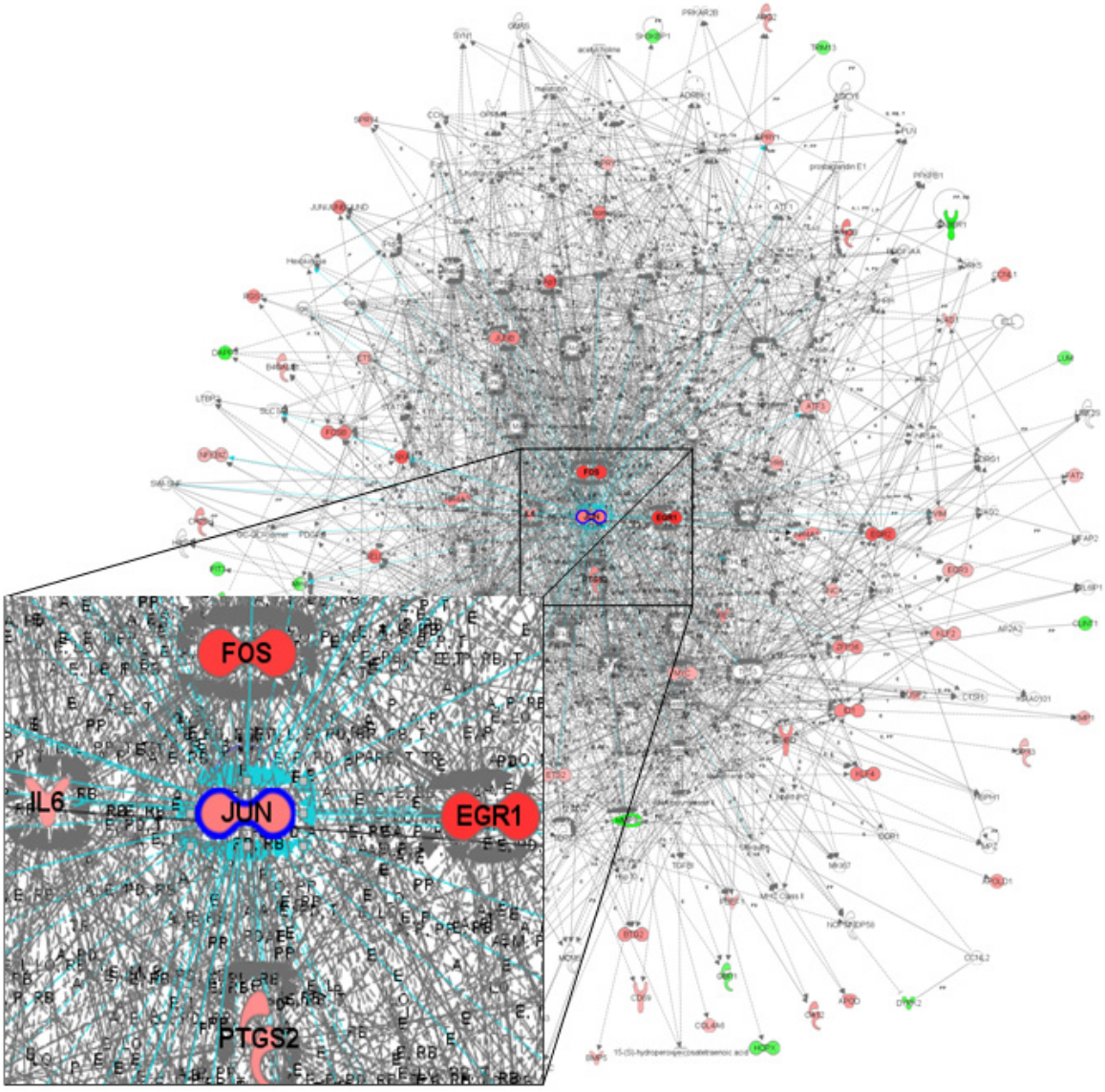


Figure 6.14 Merged network of GC-responsive genes. Networks composed by an unsupervised relevance network learning algorithm without any priori assumptions and post adjustment were generated by IPA. All five networks composed of GC-regulated genes were merged. The highlighted box shows that c-Jun and c-Fos are the core molecules. Nodes represent genes, with their shape representing the functional class of the gene product, and edges indicate the biological relationship between the nodes (*see network legend in* ***Figure 6.13***). Nodes are color coded according to their *d* score generated by SAM (red, overexpression; green, underexpression) and the color intensity increases with the magnitude of altered regulation.

In summary, the network analysis provides comprehensive molecular networks involved in GC mechanisms in NP, and also facilitates the identification of key genes underlying the GC effects on NP. The AP-1 network appears to be particularly important, because it is not only the top-ranking network but also includes the core genes (AP-1 genes) which interact with the most GC-responsive genes. In addition, those pro-/anti-inflammatory genes identified in the networks are also worth studying since GCs have been traditionally considered a potent anti-inflammatory agent.

6.1.7.2 Transcriptional levels of the GC-responsive genes

The network analysis identified that GCs mainly regulated AP-1/AP-1 related genes and inflammation related genes in NP. **Appendix II** (*Page 268*) lists the alteration (fold change) of these genes in GC-naïve NP, GC-treated NP, and control tissues. In addition, Appendix III (*Page 271*) describes the relative expression level of the genes validated by real-time RT PCR. c-Jun and c-Fos mRNA in GC-naïve NP tissues were only 29% and 8% of the nasal mucosal control, respectively. c-Jun and c-Fos mRNA were increased 3.39-fold and 14.85-fold in GC-treated versus GC-naïve NP, respectively. Both c-Jun and c-Fos mRNAs were normalized by GCs in NP. A similar trend was also observed in two other AP-1 members (FosB and JunB) and those 5 important AP-1 related genes. Transcripts of FosB, JunB, COX-2, IL-6, AREG, HBEGF, and EGR1 were markedly repressed in GC-naïve NP as compared to the control. Transcripts of these genes were increased in GC-treated NP tissues. However, expression of JunB, IL-6 and EGR1 were still lower in GC-treated NP tissues as compared to controls.

With regard to the inflammation related genes, expression of pro-inflammatory genes

(CXCL9, CXCL11, MMP7, and MMP9) was higher in GC-naïve NP than control; while anti-inflammatory genes (ANXA1, DUSP1, DUSP2, DUSP6, SPRY1, SPRY2, SPRY4, NFKBIZ, SOCS3, SCGB1A1, THBD, and ZFP36) were expressed at lower levels in GC-naïve NP than controls (**Appendix II,** *Page 268*). In response to GC treatment, down-regulation of these pro-inflammatory genes but up-regulation of the anti-inflammatory genes was evident in GC-treated NP tissues (**Appendix II,** *Page 268*). Since the expression levels of these pro-/anti-inflammatory genes were not significantly different between GC-treated NP and control, they were considered to be normalized by GCs. These results confirm the potent anti-inflammatory effect of GCs.

6.1.8 Identification of NP associated genes by Canonical Pathway analysis

The networks described above are generated *de novo*, based on the input data without any prior assumptions, while IPA also provides Canonical Pathways which are generated prior to data input and based on a pre-described well-known knowledge base. Since the amount of significant NP associated genes is large, and in order to narrow down the genes of interest which could represent NP pathogenesis, it is applicable to perform Canonical Pathway analysis. More importantly, it also provides a systemic view to reveal those well known signaling pathways underlying the NP pathogenesis.

As described in **Chapter 3.7.8.3** (*Page 59*), the top-7 relevant Canonical Pathways potentially associated with NP pathogenesis were selected: apoptosis signaling, complement system, EGF/EGFR signaling, eicosanoid signaling, ERK/MAPK signaling, IL-6 signaling, and NF-kappaB signaling. All these signaling pathways are regarded to be related to cellular proliferation, growth, and development and the

inflammatory process, which are consistent with the functions of NP associated genes (*See* **Chapter 6.1.6,** *Page 114*). The involved genes as well as their transcriptional levels in NP in these pathways are described in the following paragraphs. These Canonical Pathways are composed of genes differentially expressed in GC-naïve NP as compared to control. **Figure 6.15** demonstrates all of these 7 Canonical Pathways: the legend of **Figure 6.15A** describes the general components in these Canonical Pathways; and the legends of **Figure 6.15 B-H** describe the specific content of the signaling pathways. The fold changes of the genes which are indicated in the following paragraphs are listed in **Appendix II** (*Page 268*).

Figure 6.15 (A) General network legends of the Canonical Pathways

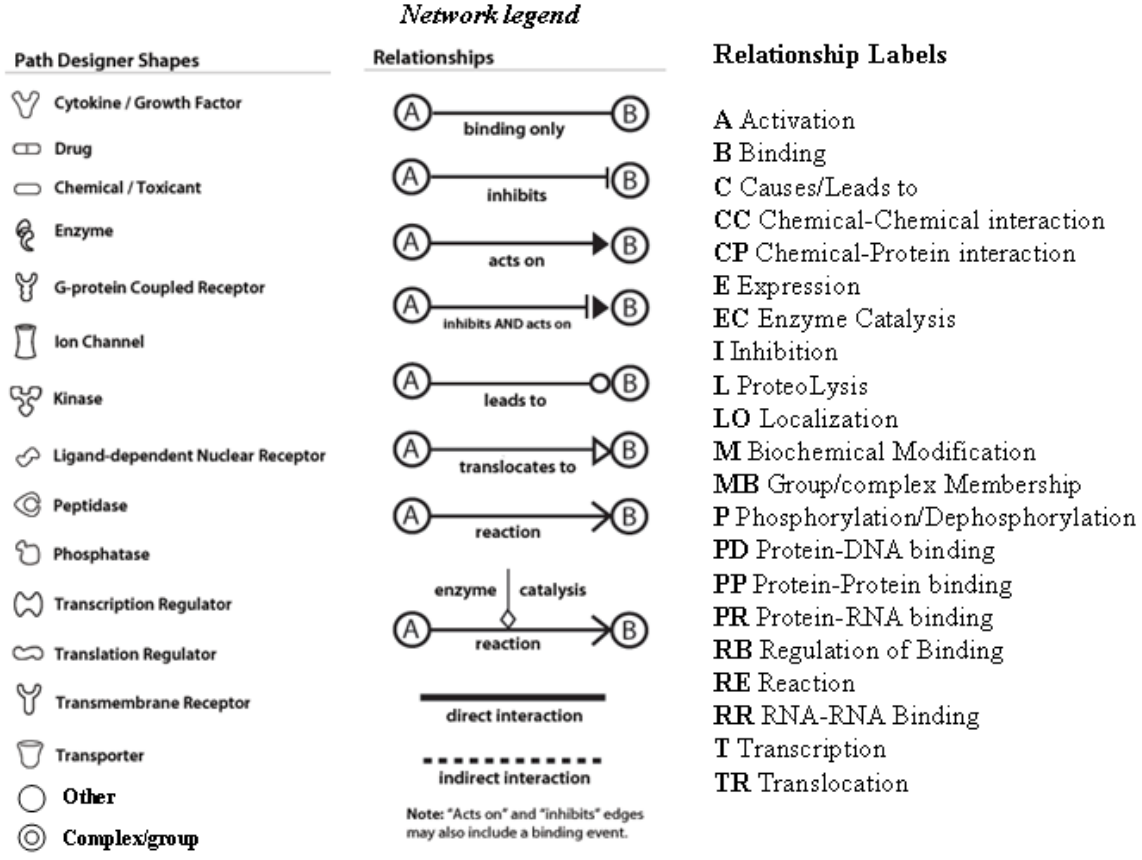


Figure 6.15 Canonical pathways in NP. (A) General network legends for **Figure 6.15 B-H**: nodes represent genes, with their shape representing the functional class of the gene product, and edges indicate the biological relationship between the nodes (*see network legend*). In **Figure 6.15 B-H**, nodes are color coded according to their *d* score generated by SAM (red, up-regulation in GC-naïve NP versus controls; green, down-regulation in GC-naïve NP versus controls) and the color intensity

increases with the magnitude of altered regulation. Up arrow in blue indicates up-regulation of the genes in NP after GC treatment.

6.1.8.1 Genes in apoptosis signaling

Apoptosis involves a series of biochemical events leading to a variety of morphological changes, including blebbing, changes to the cell membrane, cell shrinkage, nuclear fragmentation, chromatin condensation, and DNA fragmentation [Nagata, 1997]. **Figure 6.15B** highlights the key molecular events in triggering apoptosis. The central executioner of apoptosis, caspase 3 (CASP3) was up-regulated at 1.73-fold in GC-naïve NP as well as 1.85-fold up-regulation of caspase 7 (CASP7). BID, which act as a direct molecular link between the activated caspase 8 and mitochondrial death machinery, was increased at 1.65-fold in NP compared to the control; while AIF, which mediates caspase-independent DNA fragmentation, was up-regulated at 1.60-fold. However, none of these apoptosis related genes were changed in NP after GC treatment. These results indicate the increase of apoptosis signaling activity in GC-naïve NP.

Figure 6.15 (B) Apoptosis signaling

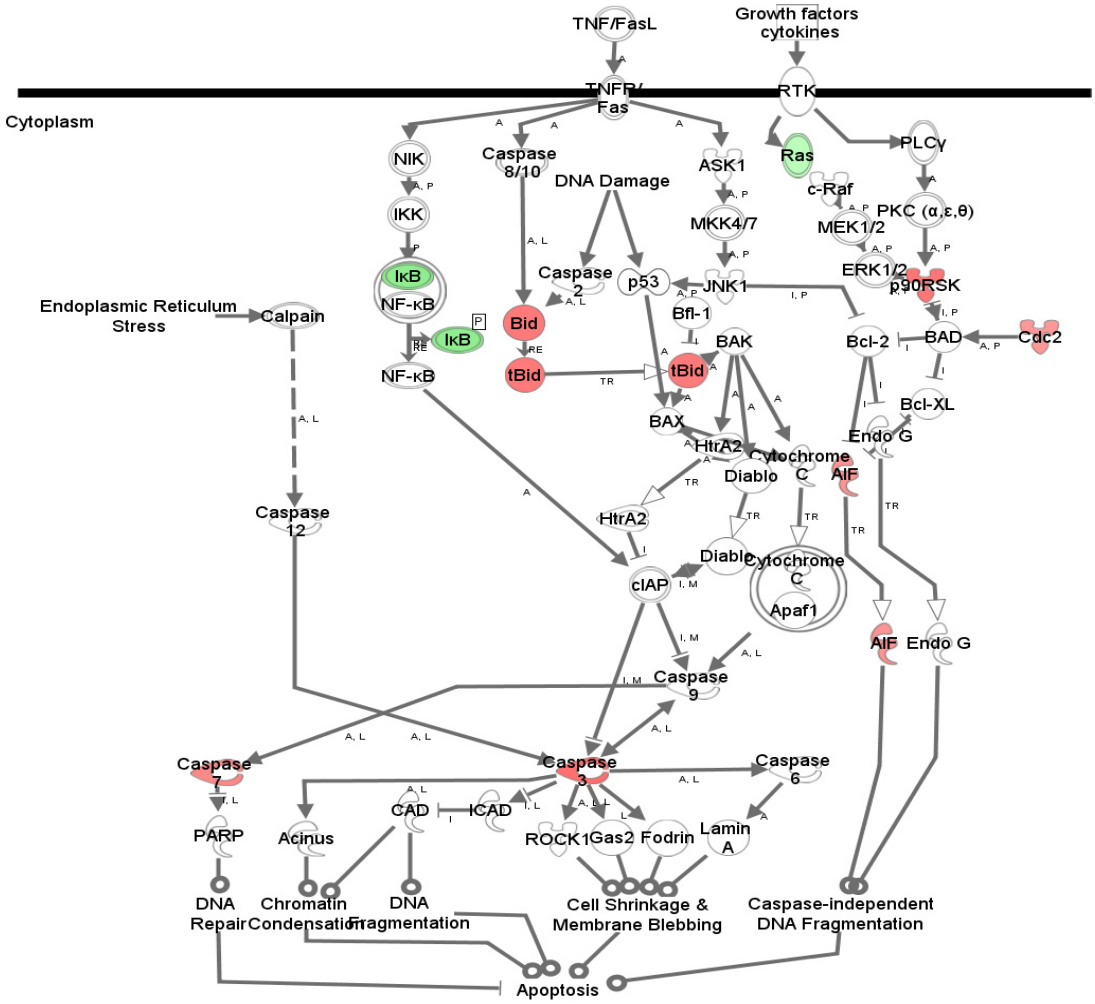


Figure 6.15 (B) Apoptosis signaling is a coordinated, energy-dependent process that involves the activation of a group of cysteine proteases called caspases and a cascade of events that link the initiating stimuli to programmed cell death. The two main pathways of apoptosis are the intrinsic and extrinsic pathways. The intrinsic signaling pathways are non-receptor-mediated and mitochondria-dependent. The end result is a change in mitochondrial transmembrane potential and release of two main groups of pro-apoptotic proteins from the intermembrane space into the cytosol. The first group consists of cytochrome c, DIABLO, and HTRA2, which activate the caspase-dependent mitochondrial pathway. The second group includes AIF and Endo G, which directly translocate to the nucleus and cause DNA fragmentation. The extrinsic signaling pathways that initiate apoptosis involve transmembrane receptor-mediated interactions (e.g., TNF receptors). The activation of these receptors triggers caspase 8 or 10 which can then activate the execution pathway. Alternatively, triggered caspase 8 or 10 could result in the activation of the pro apoptotic protein BID and then lead to a cross talk with the mitochondria-dependent intrinsic pathway of apoptosis. Each pathway requires specific triggers to initiate a cascade of molecular events that converge at the stage of caspase 3 activation. Up-regulation of caspase 3 &7, AIF, and BID in GC-naïve NP indicates the enhancement of apoptosis signaling in GC-naïve NP; while GCs have no effect on these apoptosis markers.

6.1.8.2 Genes in complement system

The complement system is not only important in innate immunity which contributes to host defense, but also mediates various inflammatory responses in airway disorders [Markiewski et al., 2007]. The Canonical Pathway of the complement cascade reveals the alteration of the complement components in NP (**Figure 6.15C**). Our results show that expression of the key complement components C3 and C4a was up-regulated at 2.64-fold and 5.54-fold in GC-naïve NP as compared to control, respectively. With regard to the other molecules related to complement pathways, C1QB was increased at 4.73-fold, while CFH (also known as HF1), which is the inhibitor of complement system, was decreased at 2.01-fold in GC-naïve NP as opposed to controls. However, expression of all the molecules in this complement pathway was not changed in NP after GC treatment. These results indicate the enhancement of the complement mediated inflammatory process in NP.

Figure 6.15 (C) Complement system

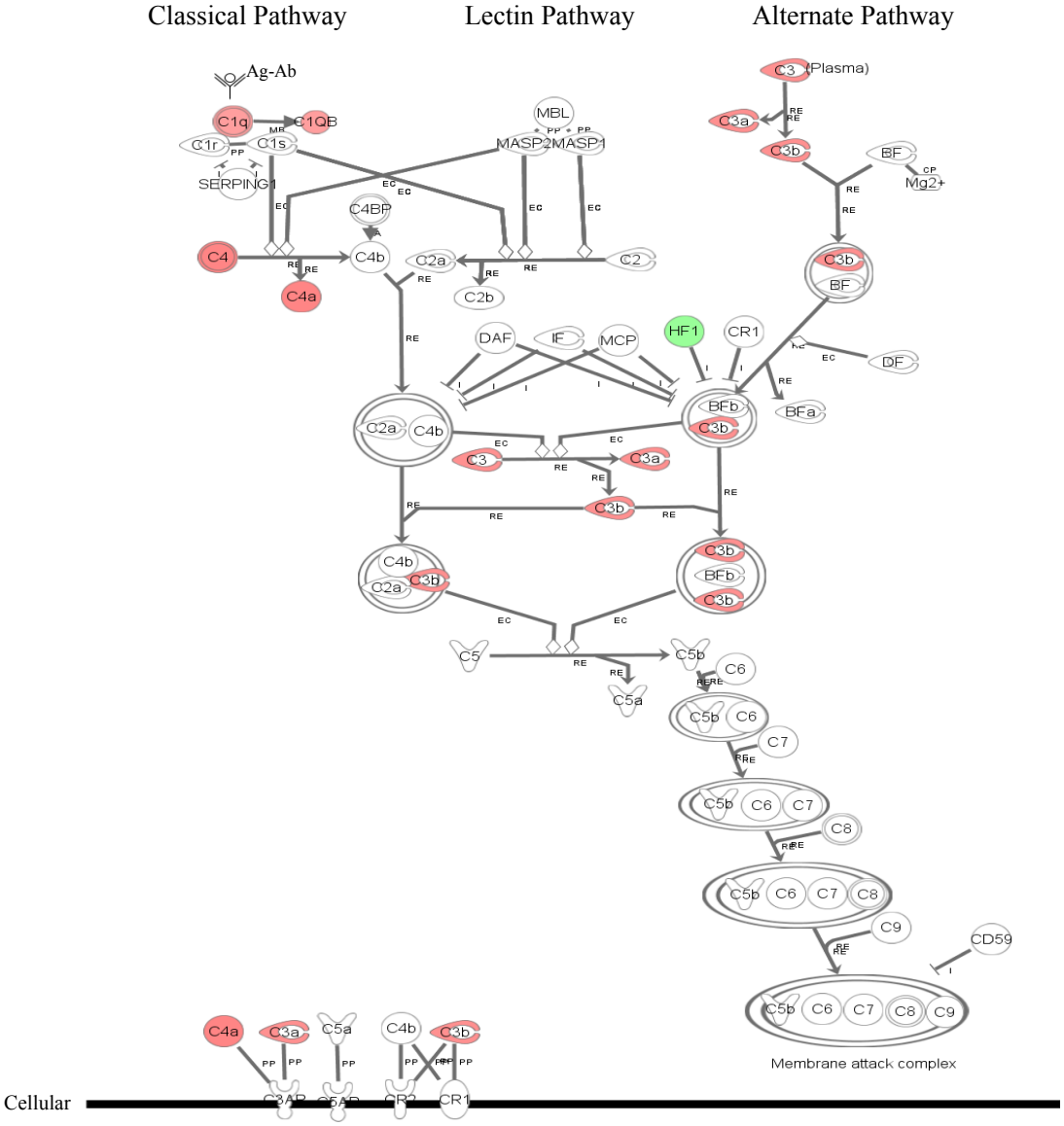


Figure 6.15 (C) The complement system has three pathways based on initiation of the complement cascade: classical pathway, alternate pathway and lectin pathway. The classical pathway is triggered by the binding of C1 to the antigen-antibody (Ag-Ab) complex. The lectin pathway is initiated by MBL (mannose binding lectin), a serum protein, binding to mannose groups on bacterial cell walls. The alternate pathway is activated by C3, a complement protein, binding to components of microbial cell surfaces. The three activation pathways converge into a final common pathway when C3 convertase cleaves C3 into C3a and C3b where C3b is directly linked to opsonization and also goes on to form part of the complex that cleaves C5 into C5a and C5b. C5b with C6, C7, C8, and C9 form (C5b6789) membrane attack complex, which is inserted into the cell membrane and initiates cells lysis. Moreover, fragments of C3, C4, and C5 can interact with the cell membrane receptor directly to trigger activation of inflammatory cells. The key promoters in complement system (C3, C4, and C1QB) are up-regulated while one complement inhibitor (HF1) is down-regulated in GC-naïve NP; however, none of them responds to GC treatment. These results indicate the increase of complement activity in NP.

6.1.8.3 Genes in EGF/EGFR signaling

The interaction between EGF receptors and their corresponding ligands have been considered an important mechanism in regulating cellular growth and proliferation [Citri et al., 2006]. In the Canonical Pathway of EGF/EGFR signaling, a uniform down-regulation of EGF receptor (ERBB4, 2.12-fold) and EGF ligands (AREG, 4.01-fold; HBEGF, 4.56-fold; EGF, 3.93-fold; NRG3, 4.44-fold) were found in GC-naïve NP as compared to controls (**Figure 6.15D**); while expression levels of AREG and HBEGF were significantly normalized after GC treatment with 6.48-fold and 5.45-fold up-regulation in GC-treated versus GC-naïve NP tissues, respectively. These results indicate the defect of EGF mediated proliferation signaling in NP, and GCs may correct the insufficient activity of EGF signaling.

Figure 6.15 (D) EGF/EGFR signaling

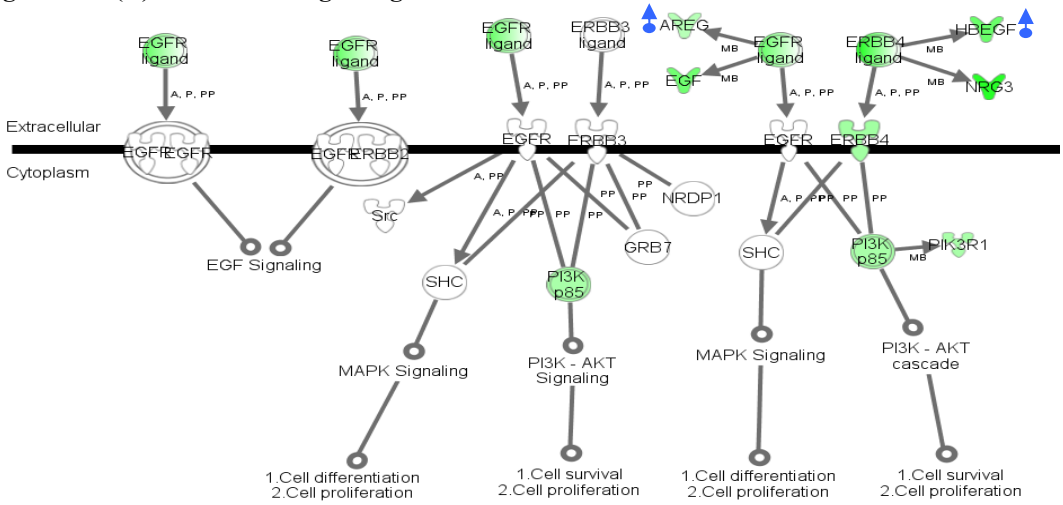


Figure 6.15 (D) The epiermal growth factor (EGF) receptors and their corresponding ligands play an important role in cell differentiation, proliferation, and survival. EGFR is the prototype of the EGF receptor family, and it can either homo-/or hetro-dimerize with other members including ERBB2, ERBB3, and ERBB4. The known EGFR ligands include EGF, AREG, HBEGF, NGR3, and TGF-alpha. Ligands bind to the EGF receptors results in series activation/phosphorylation events, which mainly trigger MAPK cascade and PI3K-AKT signaling. Down-regulation of the EGFR (e.g., ERBB4) and EGFR ligands (EGF, AREG, HBEGF, and NRG3) is significant in GC-naïve NP, and AREG and HBEGF are normalized by GCs. The results suggest the low activity of EGF signaling in NP, and GCs may promote EGF mediated proliferation signaling. Up arrow in blue indicates up-regulation of the genes in NP after GC treatment.

6.1.8.4 Genes in eicosanoid signaling

Eicosanoids transduce signals via their membrane receptors and mediate complex biological processes like inflammation, vascular permeability, allergic reactions, induction of labor and carcinogenesis. The Canonical Pathway picture of eicosanoid signaling (**Figure 6.15E**) highlights the alteration of some key receptors and enzymes in GC-naïve NP. In the leukotriene (LT) pathway, the LT receptors LTB4R and CYSLTR1 were up-regulated at 1.66-fold and 2.92-fold in GC-naïve NP, respectively; while the LT syntheis related enzymes ALOX5AP and LTA4H were increased at 2.61-fold and 1.53-fold, respectively. In the prostaglandin (PG) pathway, we found down-regulation of PTGS2 (or COX-2) (2.58-fold), PTGIS (3.88-fold) and PTGER3 (6.53-fold), but up-regulation of PTGER2 (1.99-fold) in GC-naïve NP compared to controls. Regarding the PLA_2 enzymes, expression levels of PLA2G4A (also known $cPLA_2$ alpha) and PLA2G10 were increased 2.61-fold and 2.51-fold in GC-naïve NP versus control, respectively. Among these molecules in the eicosanoid signal pathway, only COX-2 was normalized (3.47-fold up-regulation in GC-treated vs. GC-naïve NP) in response to GC treatment. The results suggest the increase of LT mediated inflammation and alteration of PGE_2 signaling activity in NP, and GCs mainly have an effect on the PG pathway.

Figure 6.15 (E) Eicosanoid signaling

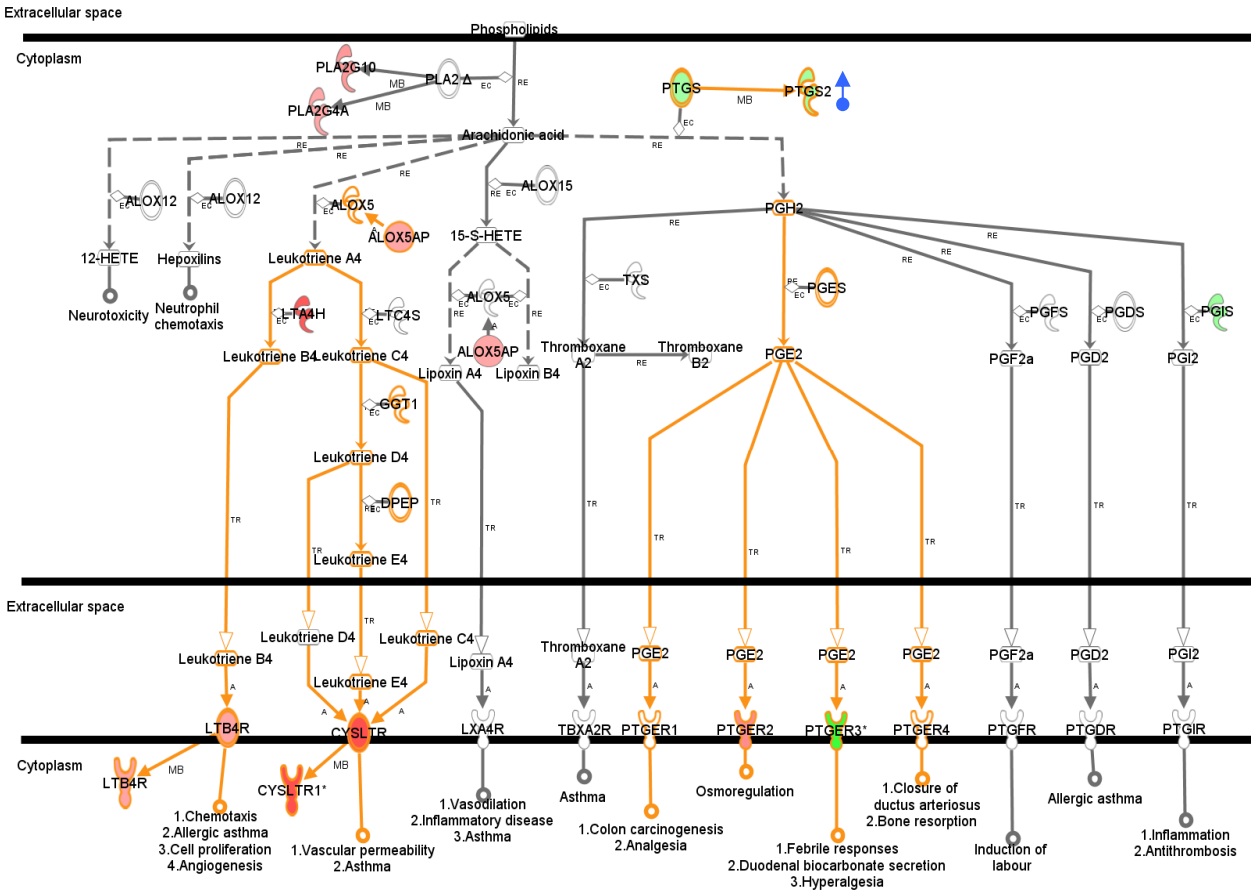


Figure 6.15 (E) Eicosanoid-mediated signaling plays a diverse role in many biological processes, such as inflammation, angiogenesis, and osmoregulation. There are four types of eicosanoids: prostaglandins (PGs), lipoxins, leukotrienes (LTs) and thromboxanes. Arachidonic acid (AA), the precursor for most eicosanoids, is produced by hydrolysis of membrane phospholipids by phospholipaseA2 (PLA2). AA is then converted to eicosanoids by one of two types of enzymes: (1) prostaglandin endoperoxide synthases (PTGS), commonly referred to as cyclooxygenases (COX-1 and COX-2), catalyze the key step in the synthesis of biologically active prostaglandins, the conversion of AA into prostaglandin H2 (PGH2). PGH2 serves as the precursor for thromboxanes and prostaglandins; (2) The lipoxygenases include ALOX5, ALOX12 and ALOX15; ALOX5 catalyzes the key step in the conversion of AA to leukotriene A4, B4 and C4; ALOX15 in concert with ALOX5 is involved in the formation of lipoxins A4 and B4; ALOX12 synthesizes 12(S)-HETE [12(S)-hydroxyeicosatetraenoic acid]. Eicosanoid receptors include LTB4Rs and CYSLTRs for leukotrienes, PTGERs for prostaglandin E2, PTGFR for prostaglandin F2, PTGDR for prostaglandin D2 and TBXA2R for thromboxane A2. The leukotriene and PGE2 pathways are highlighted in orange. Up-regulation of LT pathway related markers (such as LTB4R, CYSLTR1, ALOX5AP, and LTA4H) is evident in GC-naïve NP, and GCs have no effect on LT mediated signaling. In PG pathway, the key catalyzer COX-2 is down-regulated as well as down-regulation of PGER3; but PTGER2 is up-regulated. The response of COX-2 to GC treatment indicates that GCs may have effect on PG pathway. Up arrow in blue indicates up-regulation of the genes in NP after GC treatment.

6.1.8.5 Genes in ERK/MAPK signaling

Mitogen-activated protein kinase (MAPK) families mediate transcriptional and posttranscriptional changes in gene expression in response to proinflammatory stimuli and are considered to play an important role in inflammation [Chang et al., 2001]. Dual specificity protein phosphatases (DUSPs) (also known as MKPs) are the key negative regulators of the MAPK signaling cascade by dephosphorylating the theronine and tyrosine motifs on MAPK proteins [Farooq et al., 2004; Shepherd et al., 2007]. The canonical pathway of MAPK signaling reveals the down-regulation of these DUSPs in GC-naïve NP as compared to control (**Figure 6.15F**): DUSP1 (also known as MKP1) (9.61-fold), DUSP2 (also known as PAC1) (5.68-fold), DUSP4 (also known as MKP2) (4.78-fold), DUSP5 (4.50-fold), and DUSP6 (also known as MKP3) (2.91-fold). Furthermore, DUSP 1, DUSP2, and DUSP6 were normalized in NP in response to GC treatment with increase of 4.98-fold, 3.19-fold, and 1.73-fold, respectively.

Another gene family which also inhibits MAPK cascade is so called Sprouty (SPRY) homolog. SPRYs are essential in negative regulation of receptor tyrosine kinases (RTKs) signaling pathways. As shown in the canonical pathway figure, DUSPs specifically inhibit MAPKs at p38, ERK, and JNK levels, but SPRYs control the MEK or Raf level which is the up-stream kinase of MAPKs (**Figure 6.15F**). Similar to DUSPs, three SPRY homologies were observed to be down-regulated in GC naïve NPs compared to control nasal mucosa: SPRY1 (3.02-fold), SPRY2 (1.61-fold), and SPRY4 (1.62-fold); while all of them were normalized in NP in response to GC treatment: SPRY1 (2.49-fold), SPRY2 (2.11-fold), and SPRY4 (2.13-fold).

These results suggest that the negative regulation system of the inflammatory process

is inadequate in GC-naïve NP, leading to chronic inflammation; while GCs may perform the anti-inflammatory effect in NP via up-regulation of these negative regulators in the inflammatory pathway.

Figure 6.15 (F) ERK/MAPK signaling

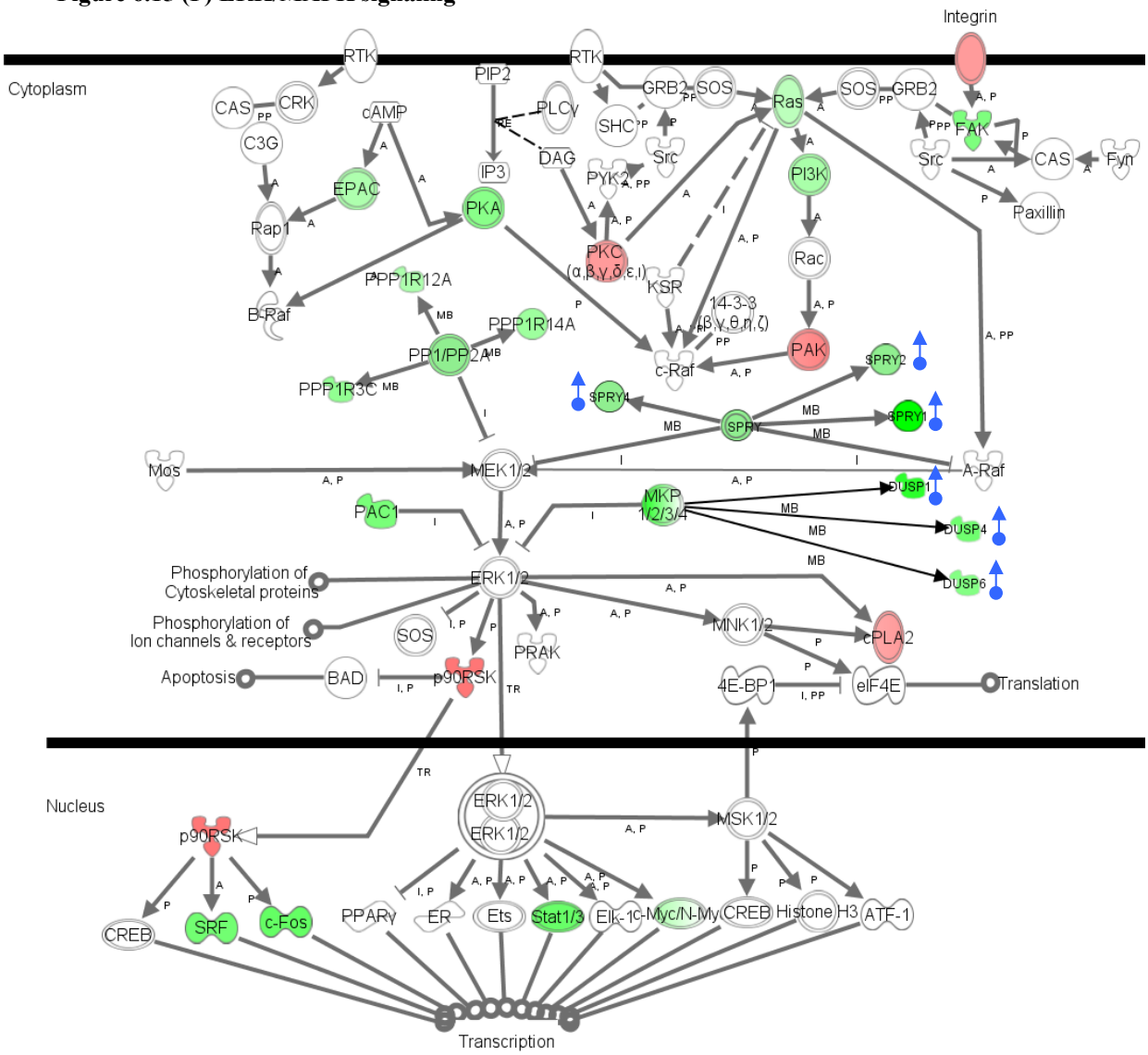


Figure 6.15 (F) The ERK (extracellular-regulated kinase)/MAPK (mitogen activated protein kinase) pathway is a key pathway that transduces cellular information on meiosis/mitosis, growth and differentiation during inflammatory process. Binding of ligand to RTK activates the intrinsic tyrosine kinase activity of RTK. Adaptor molecules like GRB2, SOS and Shc form a signaling complex on tyrosine phosphorylated RTK and activate Ras. Activated Ras initiates a kinase cascade, beginning with Raf (a MAPK kinase kinase) which activates and phosphorylates MEK (a MAPK kinase); MEK activates and phosphorylates ERK (a MAPK). ERK in the cytoplasm can phosphorylate a variety of targets which include cytoskeleton proteins, ion channels/receptors and translation regulators. The

ERK/MAPK signaling is controlled by its negative regulators at different cascade level. PP1/PP2A and Sprouty homolog (SPRYs) inhibit MEKs activity, while Dual specificity protein phosphatases (DUSPs) (known as MKPs) inhibit ERKs activity. DUSP1 known as MKP1, DUSP2 known as PAC1, DUSP4 known as MKP2 and DUSP6 known as MKP3. Down-regulation of the negative regulators of MAPK cascade was significant in GC-naïve NP, including DUSP1, DUSP2, DUSP4, DUSP6, SPRY1, SPRY2, and SPRY4; moreover, most of them (except DUSP4) are up-regulated in response to GC treatment. The results indicate that the negative regulation system of pro-inflammatory signaling is inadequate in NP, and the increase of these controllers represents the anti-inflammatory function of GCs. Up arrow in blue indicates up-regulation of the genes in NP after GC treatment.

6.1.8.6 Genes in IL-6 signaling

IL-6 mediated signaling pathway is important in the growth and differentiation of numerous cell types (including epithelial cells) [Gallucci et al., 2000]. As shown in the Canonical Pathway of IL-6 signaling, the key factors (including activation factors and inhibition factors) involved in the IL-6 signaling pathway were all down-regulated in GC-naïve NP as compared to controls (**Figure 6.15G**): IL-6 (6.10-fold), IL6ST (known as GP130) (1.69-fold), STAT3 (1.73-fold), and SOCS3 (3.21-fold). In response to GC treatment, IL-6 and SOCS3 were increased at 7.87-fold and 4.81-fold, respectively; but the expression level of IL-6 was still lower in GC-treated NP as compared to control. These results suggest that the IL-6 signaling is insufficient in GC-naïve NP, and the increase of both IL-6 and its inhibitor SOCS3 represents the regulatory role of GCs in IL-6 signaling mediated biological process.

Figure 6.15 (G) IL-6 signaling

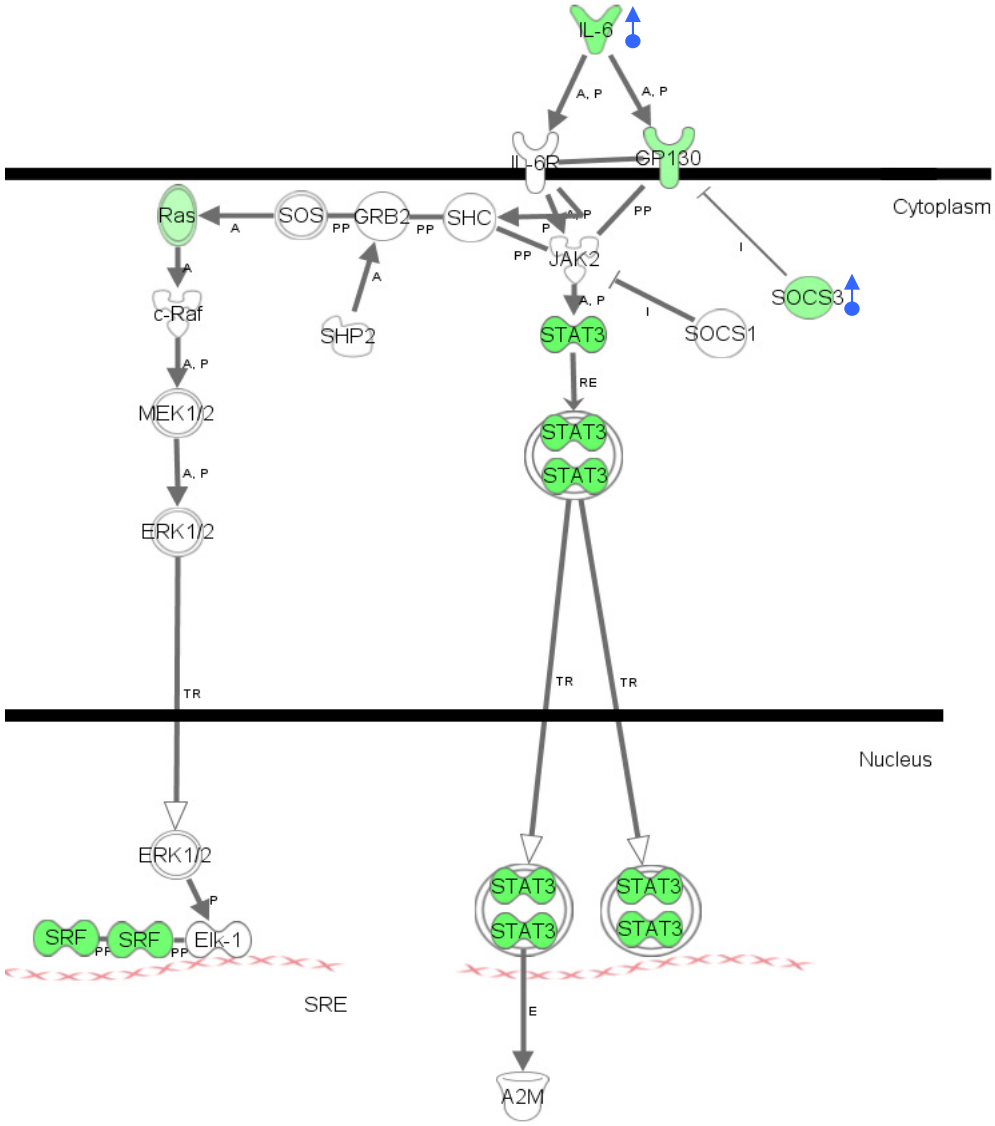


Figure 6.15 (G) IL-6 mediates both pro- and anti-inflammatory signaling. IL-6 responses are transmitted through Glycoprotein 130 (GP130), which serves as the universal signal-transducing receptor subunit for all IL-6-related cytokines. IL-6-type cytokines utilize tyrosine kinases of the Janus Kinase (JAK) family and signal transducers and activators of transcription (STAT) family as major mediators of signal transduction. Dimer of STATs translocate to the nucleus, where they regulate transcription of target genes. In addition, IL-6 also activates the extracellular signal-regulated kinases (ERK1/2) of the mitogen activated protein kinase (MAPK) pathway. The SHC protein is activated by JAK2 and thus serves as a link between the IL-6 activated JAK/STAT and RAS-MAPK pathways. Suppressor of cytokine signaling (SOCS) can negatively regulate IL-6-mediated JAK/STAT pathway. Down-regulation of the key genes in IL-6 signaling is obvious in GC-naïve NP, including IL-6, GP130, STAT3, and SOCS3; while up-regulation of IL-6 and SOCS3 after GC treatment. The results indicate the IL-6 pathway activity is low in GC-naïve NP, and GCs have an regulatory effect on IL-6 mediated proliferation signaling. Up arrow in blue indicates up-regulation of the genes in NP after GC treatment.

6.1.8.7 Genes in NF-kappaB signaling

NF-kappaB is a multi-subunit transcription factor that is involved in the regulation of a large number of genes that control various aspects of the immune and inflammatory response [Baldwin et al., 1996]. The activity of NF-kappaB is largely controlled by inhibitors of NF-kappaB (IkappaB), which bind to NF-kappaB, preventing its association with DNA and causing its localization to the cytoplasm [Baldwin et al., 1996]. The Canonical pathway of NF-kappaB signaling showed a decrease of IkappaB in GC-naïve NP (**Figure 6.15H**): IkappaB-α (also known as NFKBIA) and IkappaB-ζ (also known as NFKBIZ) were down-regulated at 1.53-fold and 2.19-fold respectively, while only NFKBIZ was increased at 2.22-fold in NP in response to GC treatment. These results suggest that the negative regulation of NF-kappaB signaling in GC-naïve NP is insufficient, and GCs may have an inhibitory effect on NF-kappaB mediated inflammatory process via regulating NF-kappaB inhibitors.

Figure 6.15 (H) NF-kappaB signaling

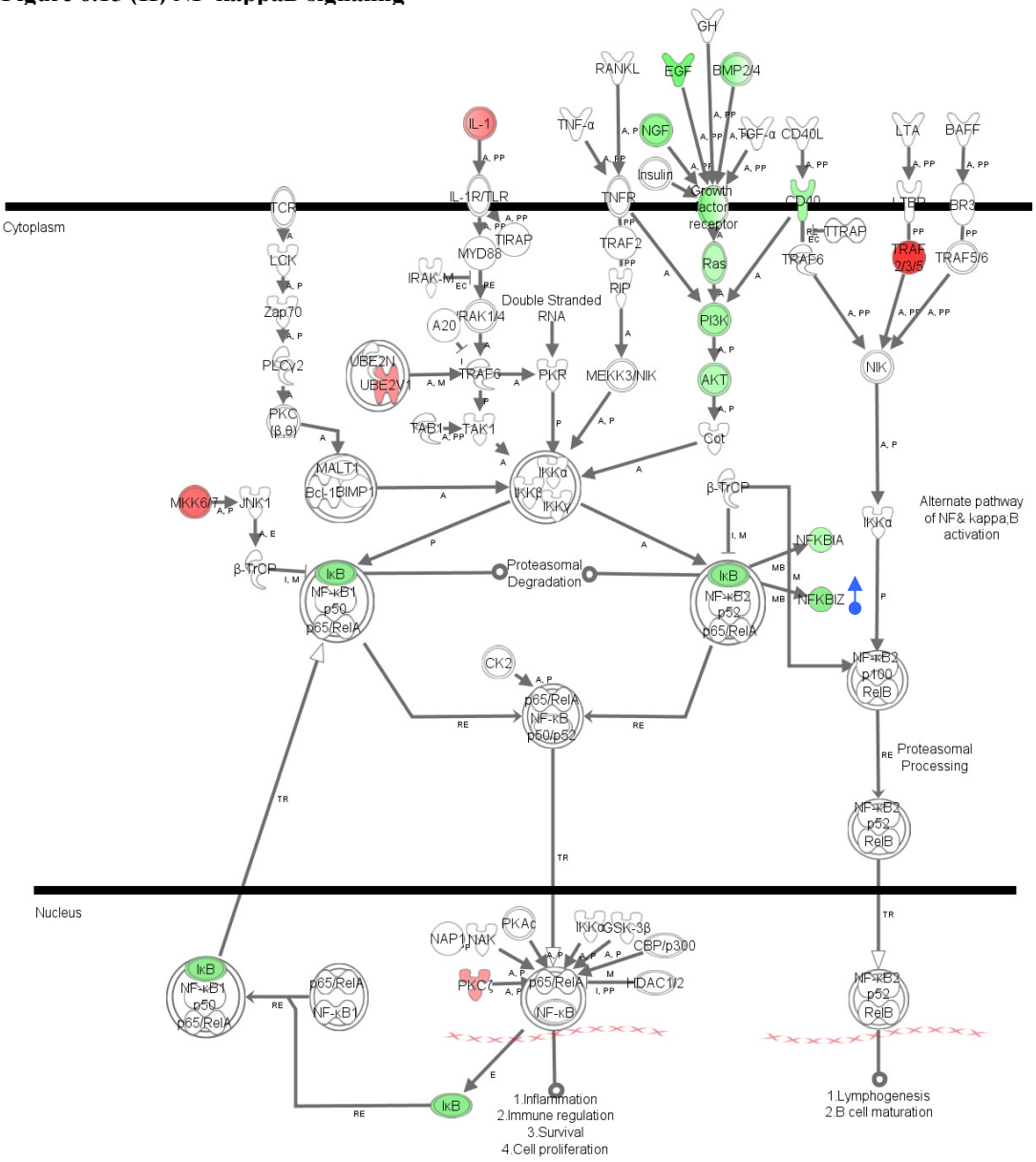


Figure 6.15 (H) The Nuclear factor kappa B (NF-kappaB) transcription factors are key regulators of gene expression culminating in response to stress and the development of innate and acquired immunity, leading to activation of inflammatory process. RELA/p65 and p50 are the two major subunits of NF-kappaB hetero-/homodimers. In quiescent situation, cytoplasmic NF-kappaB are bound to inhibitor of NF-kappaB (IkappaB), thereby sequestering them in the cytoplasm. Activators of NF-kappaB mediate the site-specific phosphorylation of seriene on IkappaB, making IkappaB for ubiquitination and destruction. NF-kappaB is then free to translocate to the nucleus and bind DNA leading to the activation of a host of inflammatory response target genes. IkappaB not only inhibits NF-kappaB mediated transcription, but also transports NF-kappaB back to the cytoplasm. Two members of NF-kappaB inhibitor, NFKBIA and NFKBIZ are down-regulated in GC-naïve NP, leading to increase of NF-kappaB signaling. However, only NFKBIZ is responded to GC treatment, indicating the inhibitory effect of GCs on NF-kappaB mediated inflammatory process. Up arrow in blue indicates up-regulation of the genes in NP after GC treatment.

6.1.9 Identification of NP associated genes by literature reviews

To broaden the identification of NP related genes in the microarray results, we intend to review more genes associated with NP histopatholgical patterns by literature reports, such as infiltration of eosinophils and neutrophils, edema, tissue damage, and mucus hypersecretion. The following paragraphs describe these disease related genes as well as their expression profiles in response to GC treatment. The fold changes of the genes which are indicated in the following paragraphs are listed in **Appendix II** (*Page 268*).

6.1.9.1 Eosinophil related genes

NP has been considered an eosinophil predominant inflammatory disease and GC can potently suppress eosinophil infiltration in NP (**Table 6.1,** *Page 90*). To identify the eosinophil associated genes, we mainly relied on the authoritative reviews which discussed eosinophil biological features [Rothenberg, 1998; Rothenberg & Hogan, 2006] and then 60 important genes were selected from the published sources. **Table 6.6** summarizes and categorizes these eosinophil related genes based on the biological process of eosinophils. Among the 60 eosinophil-associated genes, microarray data suggested that 31 genes (21 up-regulated and 10 down-regulated genes) differed significantly in GC-naïve NP compared to controls. Hierarchical cluster results indicate that the differential expression of these 31 genes may reflect the phenotypic differences in eosinophil infiltration between NP and control samples (**Figure 6.16**). Results showed a clear separation of NP and control subjects, i.e., the tissue sample dendrogram (condition tree) divided NP samples as one group and control samples as another group; while the gene dendrogram (gene tree) also divided up-regulated and down-regulated genes in two distinct groups. Furthermore, two GC-naïve NP subjects

(NP6 and NP7) with a low grade (grade=1) of eosinophil count have shown a difference in expression pattern with other NP subjects with higher grades (grade=2 or 3) of eosinophil infiltration.

Effects of oral GCs on 5 important eosinophil-associated genes (MMP9, CD69, DUSP1, NR4A1, and NR4A2) were evident. Expression of MMP9 in GC-treated NP tissues was significantly reduced as compared to GC-naïve tissues. Moreover, expression of CD69, DUSP1, NR4A1 and NR4A2 in GC-treated NP tissues was completely normalized as compared to controls.

Table 6.6 Microarray expression profiles of eosinophil-associated genes in NP (before GC treatment) as compared to control

Biological process of Eosinophil	**Genes without significant change***	**Genes with significant change***	
		Up-regulation	***Down-regulation***
Development & Maturation	CSF2 (GM-CSF); IL-3; IL-5; GATA1.	None	None
Adhesion & rolling	ITGA4 (VLA-4); ITGB7; SELEP; ICAM1; VCAM1; ITGB1(CD29).	ITGB2 (CD18); SELPLG; ADAM8.	None
Chemo-attraction	C5; CCL5 (RANTES); CCL7 (MCP-3); CCL13 (MCP-4); CCL24; CCL2; CCR3; CYSLTR2; HRH4; GPR44 (CRTH2); IL-4; IL-5; IL-8; IL-13.	**MMP9**; CCL11 (eotaxin)#; CCL15; CCL28; CYSLTR1#; LTB4R#; LTA4H#; ALOX5AP#; PLA2G4A#; LGALS9; IL-18; C3; SCG2.	CXCL12; IL13RA2.
Survival, signaling and others	CD95L; BCL2L1 (Bcl-xl); TGFβ; CD95; CYSLTR2; CCR3; Siglec-8; Bax; CSF2 (GM-CSF); IL-4; IL-5; IL-13	CCL11 (eotaxin)#; IL5RA; NOS2A; CD86; MIF; LYN; CYSLTR1#; LTB4R#; LTA4H#; ALOX5AP#; PLA2G4A#.	**DUSP1**; **NR4A1**; **NR4A2**; **CD69**; NR4A3; CD9; CD40; NFKBIA.

Genes in bold and underlines style were found significant changes after GC treatment.

*: Significant change in microarray analysis.

#: Genes have dual functions and been listed twice.

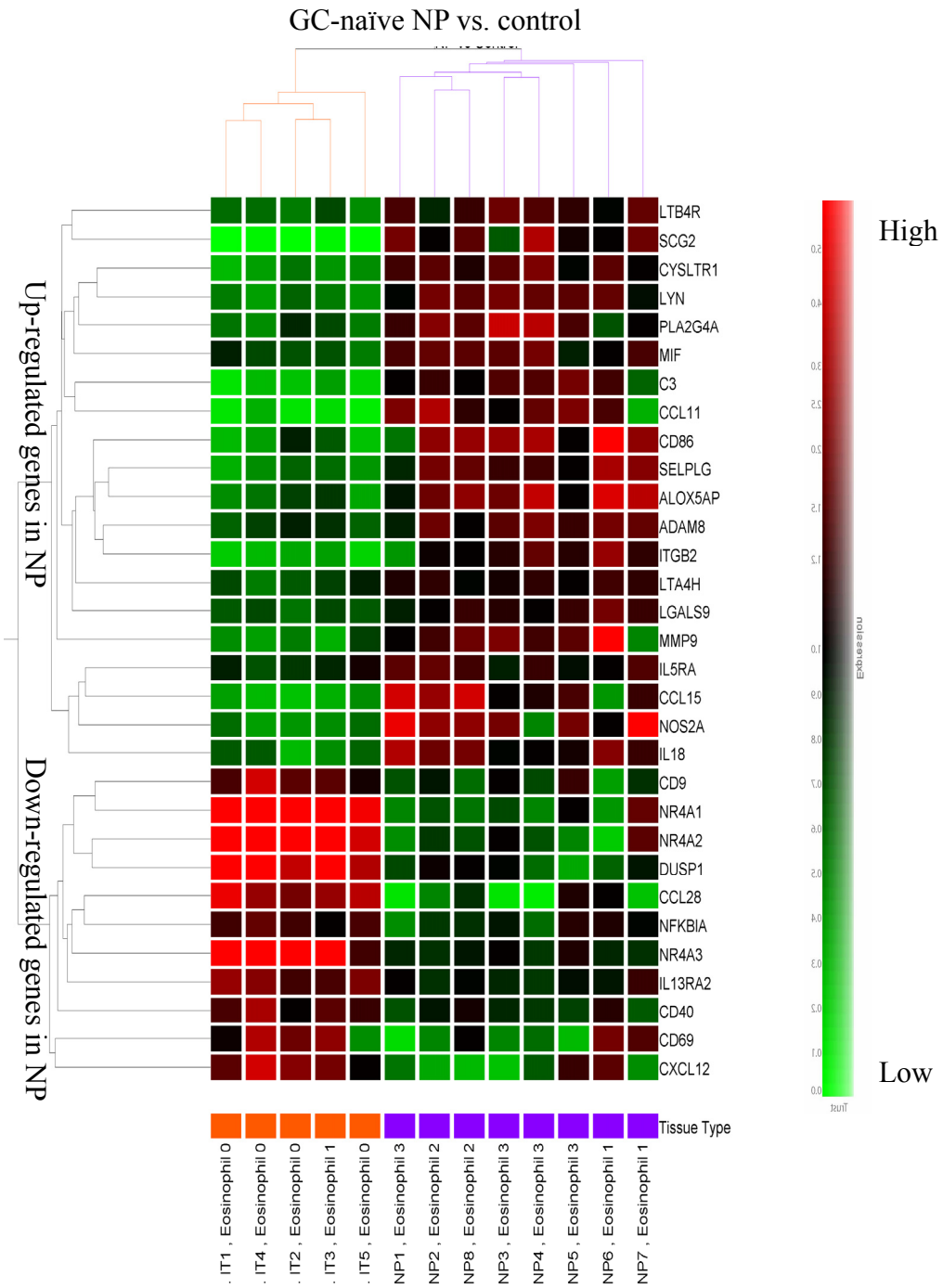


Figure 6.16 Cluster views of eosinophil associated genes in nasal tissues. Supervised hierarchical cluster analysis based on analysis of significance of microarrays (SAM) selected genes which are associated to eosinophil bio-physiological function. Each row represents an individual gene, and each column represents a tissue sample (GC-naïve NP or control) with grades (0 to 3) of eosinophil infiltration. Relative distance of each gene (vertical axis) and individuals (horizontal axis) are also demonstrated. The color spectrum for the range of expression values is shown at the left: the red color indicates high expression and green color low expression. The tissue type color bar is shown in the bottom: the purple color represents NP group, and orange color represents control group.

6.1.9.2 Neutrophil associated genes

Besides well-recognized eosinophil-dominated inflammation in Caucasian studies, predominant infiltration of other types of cells, especially neutrophils, could be a key component underlying the pathogenesis of NP in Asian populations [Jareoncharsri et al., 2002; Zhang et al., 2006]. The results from our research group also reported a high infiltration of neutrophils in Chinese NP [Hao et al., 2006]. Therefore, it is worthwhile to review and identify the genes associated with the neutrophil biological process in NP, particularly in the Asian polyps.

Following a strategy similar to the selection of eosinophil associated genes, 77 important neutrophil associated genes were chosen based on several authoritative reviews [Borregaard et al., 2007; Kobayashi, 2008; Theilgaard-Mönch et al., 2005]. Analysis of the gene expression profiles demonstrated that 14 genes were differentially expressed in GC-naïve NP versus controls. Ten genes were up-regulated (CXCL6, CXCL12, IFNAR1, LGALS8, ITGB2, CEACAM6, MMP7, MMP9, SERPINA1, and DEFB1) while 4 genes were down-regulated (CXCL2, CRISP3, CEACAM1, and PTX3) in GC-naïve samples (**Table 6.7**). Among those 14 genes, only MMP7, MMP9 and CXCL2 mRNA levels were modified by the short course of GC treatment (**Table 6.7**).

Table 6.7 Microarray expression profiles of neutrophil associated genes in GC-naïve NP as compared to control

	Genes without significant change*	**Genes with significant change***	
		Up-regulation	***Down-regulation***
Neutrophil migration & activation	CXCR4; CCR1; CCR2; CCR3; CCR6; IL1β; IL8; CXCL1; CXCL3; CXCL5; CXCL10; CCL2; CCL3; CCL4; CCL19; CCL20; IFNGR1; IFNGR2; IL8RA; IL8RB; IL4R; IL6R; IL10RB; IL13RA1; IL17RA; TNFRSF1A; TNFRSF1B; TGFBR2; LILRB1; LILRA2; LILRA5; TNF; TGFB1.	CXCL6; IFNAR1; LGALS8; ITGB2.	**CXCL2**; CXCL12.
Neutrophil granule proteins	BPI; CST3; CTSG; CYBB; SNAP23; STOM; MPO; ELA2; DEFA1; DEFA4; PRTN3; AZU1; LCN2; MMP8; CAMP; CEACAM1; CEACAM8; LTF; ITGAM; PGLYRP1; CFP; VAMP2; VTI1B; PLAUR; HP; SLPI; ORM1; HPSE; B2M; SLC11A1.	**MMP7; MMP9**; SERPINA1; DEFB1; CEACAM6.	CEACAM1; CRISP3; PTX3.

Genes in bold and underlines style were found significant changes after GC treated.

*: Significant change in microarray analysis.

6.1.9.3 Edema associated genes

(1) Bioelectric genes

Normal airway epithelium maintains the homeostasis of water transport across the epithelial tissues via regulating anion secretion and cation absorption. In NP and other airway disorders, dysregulation of the ion channels is thought to result in airway tissue edema [Boucher, 1994].

In our study, we looked for the ion channel genes based on the literature reports [Bernstein 1997; Boucher, 1994; Yasuda et al., 2007a; Yasuda et al., 2007b]. One family of sodium channels, sodium channel nonvoltage-gated (SCNN) (also known as amiloride-sensitive epithelial sodium channel, ENaC), was uniformly up-regulated in

GC-naïve NP as compared to controls: SCNN1A (1.96-fold), SCNN1B (4.93-fold), and SCNN1G (5.65-fold). Another family of chloride channels, chloride intracellular channels (CLICs), was up-regulated in GC-naïve NP: CLIC3 (2.03-fold), CLIC5 (1.85-fold), and CLIC6 (3.57-fold). With regard to the molecules which maintain the homeostasis of intracellular Na^+ levels, ATP1A2 (ATPase, Na^+/K^+ transporting, alpha 2) was down-regulated in GC-naïve NP at 2.96-fold. Although these bioelectric markers were differentially expressed in NP versus controls, none of them was altered after GC treatment.

(2) Angiogenesis related genes

In NP tissues, small vessels have been observed in the stroma, suggesting angiogenesis activity in NP. Angiogenesis in NP has been considered to be associated with vascular permeability, leading to tissue edema [Coste et al., 2000]. Several angiogenesis related genes have been reviewed. One important angiogenesis gene family, so called angiopoientins (ANGPTs), was identified in the NP disease gene list. Angiopoientins as well as its functional receptor (TEK) were down-regulated in GC-naïve NP as compared to controls: angiopoietin 1 (ANGPT1), 3.66-fold; angiopoietin 2 (ANGPT2), 2.94-fold; TEK, 3.19-fold.

6.1.9.4 Genes related to tissue damage

Airway tissues are susceptible to injury by various stimuli and environmental factors. Atmospheric oxygen species and free radicals have been thought to result in tissue damage of both upper and lower airways, such as asthma, chronic obstructive pulmonary disease and NP [Rahman et al., 2006]. Based on searching for gene ontology information related to oxygen species metabolism by online database

(http://www.geneontology.org/), we found that the expression of a number of oxidant/antioxidant related genes was significantly changed in GC-naïve NP compared to the control. Those enzymes which promote oxidative stress were uniformly up-regulated in GC-naïve NP: NOS2A, 3.96-fold; NOX4, 3.03-fold; DUOX1, 6.67-fold. However, some common antioxidant related enzymes were differentially expressed in GC-naïve NP: down-regulation of SOD3 (2.56-fold), GPX3 (2.59-fold), OXR1 (2.37-fold), and LPO (35.7-fold); while up-regulation of GCLM (3.65-fold), TXN (1.64-fold), PRDX1 (1.62-fold), and PRDX5 (1.79-fold). Among these oxidant/antioxidant markers, only GPX3 was up-regulated at 1.59-fold in NP after GC treatment.

6.1.9.5 Genes with mucus hypersecretion

Mucus hyper-secretion is a feature of several airways diseases such as chronic rhinosinusitis, asthma, and cystic fibrosis. Our histological results showed that submucosal glands in NP were dilated in pathological condition, leading to an increase of mucus production (**Figure 6.1**). Mucins are the major components of mucus. Our results showed that several mucin genes were altered in GC-naïve NP compared to control: up-regulation of MUC4 (5.47-fold), MUC16 (11.12-fold), and MUC20 (4.51-fold), but down-regulation of MUC7 (41.67-fold). However, none of these mucins responded to GC treatment in NP.

6.1.10 Target genes validation by quantitative PCR

To verify the findings from the microarray analysis, the same starting materials for the microarray study were used for subsequent quantitative PCR measurements. Several target genes deemed biologically interesting because of their differential expression in

GC-naïve NP versus control and/or in GC-treated versus GC-naïve NP were selected for validation. Selection of verified genes was based on the following criteria: (i) the key genes in the top ranking GC-responsive gene network; (ii) the important pro-/anti-inflammatory genes among GC-responsive genes; (iii) those NP associated genes which respond to GC treatment; (iv) the key genes in NP related Canonical Pathways; and (v) some important eosinophil related genes in NP. Appropriate TaqMan™ assays for genes of interest and GAPDH were purchased (**Table 3.3)**. PCR data of validated genes were comparable to the results generated from microarray experiments (**Figure 6.17**). The rank order and magnitude of gene expression profile derived from quantitative PCR were in accordance with the microarray data.

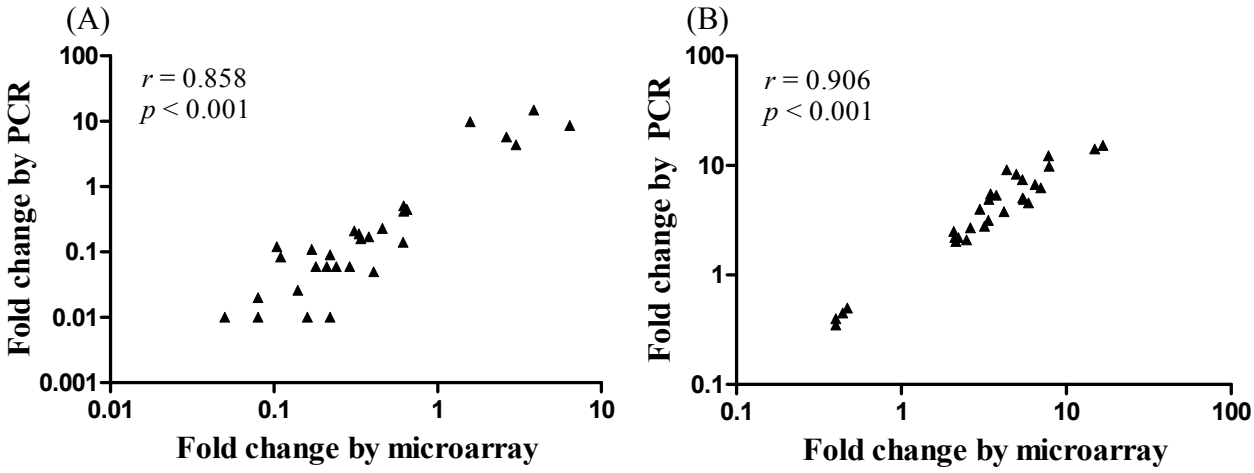


Figure 6.17 Correlation of gene expression levels between Real-time RT PCR and microarray assays. Fold changes of each gene between GC-naïve NP and control (A), and between GC-treated and GC-naïve NP (B), were determined by means of real-time PCR and microarray. Twenty-nine validated genes in (A) include AREG, C3, CCL11, CD69, c-Fos, c-Jun, COX-2, CXCL2, DUSP1, DUSP2, DUSP6, EGR1, FosB, HBEGF, IL-18, IL5Ra, IL-6, JunB, MMP7, NFKBIA, NFKBIZ, NR4A1, NR4A2, SOCS3, SPRY1, SPRY2, SPRY4, THBD, and ZFP36. Twenty-nine validated genes in (B) include ANXA1, AREG, CD69, c-Fos, c-Jun, COX-2, CXCL2, CXCL9, CXCL11, DUSP1, DUSP2, DUSP6, EGR1, FosB, HBEGF, IL-6, JunB, MMP7, MMP9, NFKBIZ, NR4A1, NR4A2, SCGB1A, SOCS3, SPRY1, SPRY2, SPRY4, THBD, and ZFP36. Scale of X and Y axis is logarithmic (base 10) transformed.

Due to the importance of the AP-1 network in GC-responsive genes, the correlation

between gene expression in AP-1 and AP-1 related genes was further evaluated based on fold change by PCR measurement. c-Jun mRNA is positively correlated with c-Fos and JunB mRNA (Spearman, $r = 0.924$, $p < 0.001$ and $r = 0.827$, $p = 0.003$, respectively; **Figure 6.18**). Similarly, c-Jun gene expression is positively correlated with AP-1 related genes (COX-2, IL-6 and EGR1) in GC-treated NP tissues (COX-2, $r = 0.827$, $p = 0.003$; IL-6, $r = 0.662$, $p = 0.03$ and EGR1, $r = 0.893$, $p = 0.001$; **Figure 6.18**). Although the expression levels of the other two AP-1 related genes (AREG and HBEGF) were not significantly correlated, their correlation coefficients range from medium to large (AREG, $r = 0.558$; HBEGF, $r = 0.426$).

Two glucocorticoid receptor (GR) isoforms were also measured by quantitative PCR (**Appendix II,** *Page 268*). GRα mRNA was significantly decreased in patients with NP, either with or without GC treatment, when compared to control tissues. Compared with GRα mRNA expression, the level of GRβ mRNA expression was undetectable in all groups.

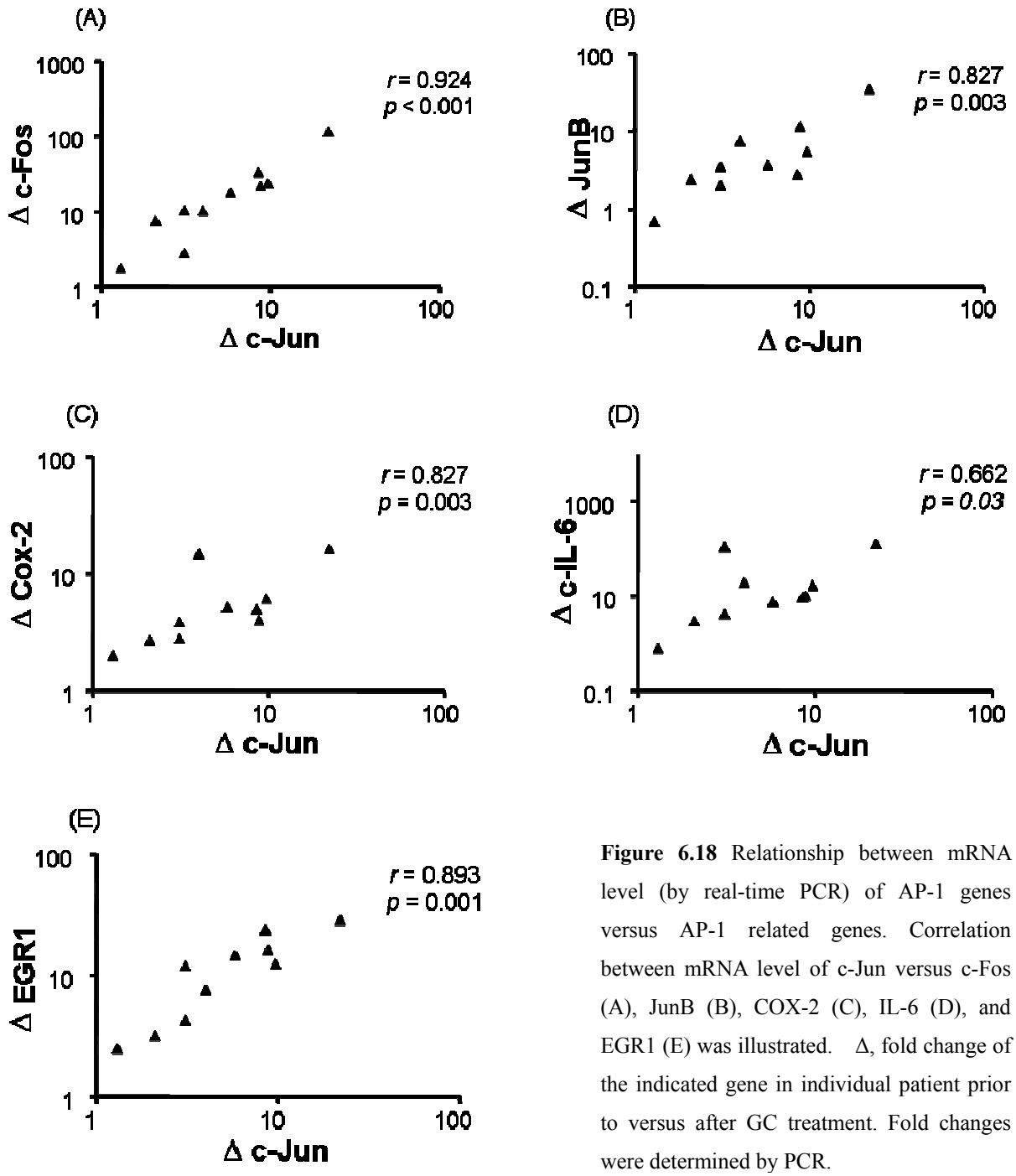


Figure 6.18 Relationship between mRNA level (by real-time PCR) of AP-1 genes versus AP-1 related genes. Correlation between mRNA level of c-Jun versus c-Fos (A), JunB (B), COX-2 (C), IL-6 (D), and EGR1 (E) was illustrated. Δ, fold change of the indicated gene in individual patient prior to versus after GC treatment. Fold changes were determined by PCR.

6.1.11 Protein expression evaluated by immunohistochemistry

Since two AP-1 components (c-Jun and c-Fos) are the core genes among those GC-responsive genes, protein expression levels of these AP-1 genes were further evaluated by immunohistochemistry. c-Jun was present predominantly in epithelium of both NP tissues and nasal mucosal controls (**Figure 6.19**). c-Jun protein was

markedly lower in 6 of 10 GC-naïve NP tissues, but significantly elevated in GC-treated NP, suggesting usage of GC contributed to the increase of c-Jun expression (**Table 6.8**). In turbinate control tissues, the intensity of c-Jun staining in epithelial cells was similar to that in GC-treated NP tissues (**Figure 6.19**).

c-Fos protein was expressed among various cell types such as epithelial cells, glandular cells, vascular endothelial cells, lymphocytes, granulocytes and monocytes. Interestingly, the expression of c-Fos was similar among GC-naïve, GC-treated and control tissues (**Figure 6.19**), suggesting that GC treatment does not alter the protein expression of c-Fos in NP.

Because the current study showed that one of the major effects of GCs in NP was to improve epithelial structure (**Table 6.1,** *Page 90*) and the functions of the AP-1 gene network are involved in cellular development, growth, and proliferation, we intended to evaluate the relationship between c-Jun protein expression and epithelial improvement by GCs. Interestingly, the results showed that c-Jun expression was positively related to the epithelial integrity in both GC-naïve and GC-treated NP tissues (**Table 6.8**), suggesting its role in growth and development of epithelial cells.

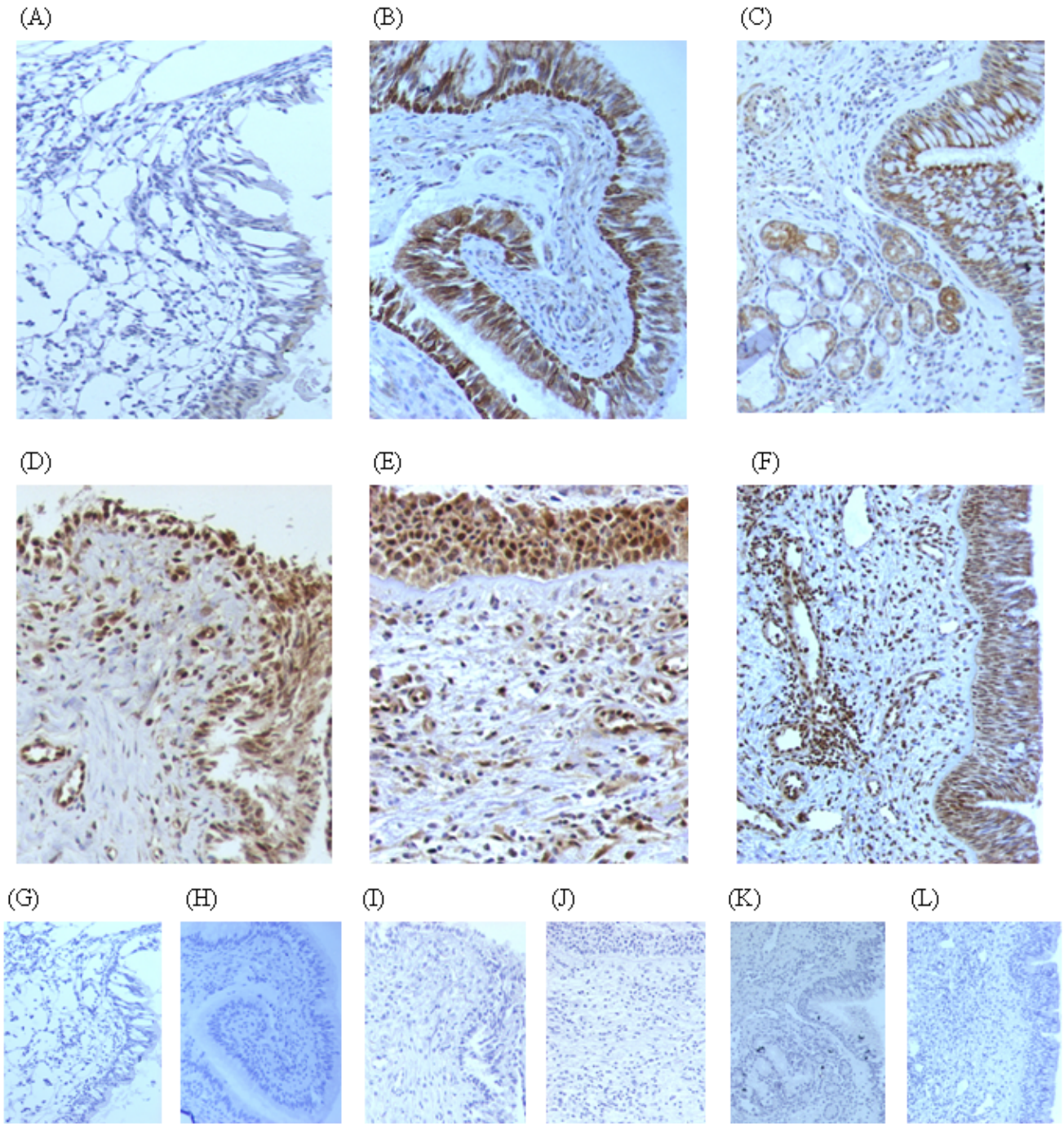


Figure 6.19 Expression of c-Jun and c-Fos protein in nasal tissues. Protein levels of c-Jun and c-Fos were determined by means of immunohistochemistry in the nasal mucosa of 2 representative NP patients and 2 controls: plates A, B, G and H are from patient NP9 and plates D, E, I and J are from patient NP7 while plates C and K are from control IT5, and plates F and L are from control IT3. Plates A and B represent c-Jun staining in patient NP4 prior to versus after GC treatment and plates G and H are stains with isotype control antibody prior to versus after GC treatment. Similarly, plates D and E represent c-Fos staining in patient NP5 prior to versus GC treatment while plates I and J are stains with isotype control antibody prior to versus GC treatment. Plate C represents c-Jun staining in control IT5, while plate K is the corresponding isotype control. Plate F represents c-Fos staining in control IT3, while plate L is the corresponding isotype control. Original magnification: 200x.

Table 6.8 Comparisons of c-Jun immunohistochemistry in NP epithelium from GC naïve and GC treated subjects

Group	Evaluation of c-Jun staining *		
	Weak	Strong	*p* value #
GC naïve NP	6	4	0.01
GC treated NP	0	10	
Damaged Epithelium †	6	4	0.01
Intact Epithelium †	0	10	

* Referring to c-Jun staining in Table 6.1: overall score of ≥ 6 defined as "strong" expression, and < 6 as "weak" expression.

† Referring to epithelium damage in Table 6.1: Grade 0 defined as intact epithelium; Grade 1 and 2 defined as damaged epithelium.

p value obtained by fisher's exact test.

Part II Discussion

Gene profiling technologies have demonstrated considerable power in the generation of cell and tissue molecular signatures, identification of disease-associated gene, determination of candidate genes in response to drug effects and exploration of molecular signaling pathways. DNA microarray technology consists of a matrix with attached sequences that allow simultaneous analysis of expression of panels of human genes. Comparison of gene expression profiles in disease versus healthy tissues or in drug effects often highlights the involvement of both expected and unsuspected pathologic pathways.

Inflammatory processes within the mucosa of the upper respiratory tract are believed to play an important role in the development of NP. Infiltration of eosinophils and epithelial damage followed by abnormal remodeling are the most characteristic features of NP. Some of the mediators that participate in the NP formation have been already

identified in previous studies (*See* **Chapter 1.4.6,** *Page 15*). However, considerable doubt remains concerning the role of each molecule in this complex and dynamic process. In addition, the molecular mechanisms underlying the ameliorative response of NP to GCs are poorly understood. Therefore, based on increasing recognition that a systems approach is necessary to view the overall molecular events responsible for NP and GC effects on NP, we have combined large-scale analysis of gene expression profile with knowledge-based and relevance network analyses.

In our study, DNA microarrays with 38,500 human genes were used to screen for those in which expression was altered in GC-naïve NP versus control and GC-treated versus GC-naïve NP. This strategy identified both up-regulated and down-regulated genes. Most importantly, complex networks involved in inflammation, cellular infiltration and tissue development were mapped and subsequently explored in the context of genes deemed important in NP development as well as its response to GC treatment by inferential statistics for a refined molecular pathway picture. The candidate genes for GC effects on NP have been subsequently suggested.

In the following sections, the important findings of the current microarray study will be highlighted and discussed: (i) indication of the general microarray analysis (including PCA/cluster analysis and functional network analysis); (ii) the major molecular events underlying the GC effects on NP; (iii) the important molecular mechanisms underlying the pathological patterns of NP.

6.2.1 Indication of microarray analysis

Microarray analyses describe the general expression profiles (by PCA/cluster

analysis), and the functions as well as molecular interaction networks among the gene sets. These analyses themselves reveal some interesting indications.

6.2.1.1 Indication of PCA/cluster analysis

Based on these significantly different genes, advanced computational methods (PCA/cluster analysis) were applied to study the relationships among the nasal samples according to similarity in pattern of gene expression. The PCA and cluster analysis were consistent with each other, and they demonstrated a near-perfect separation of three sample groups (GC-naïve NP, GC-treated NP, and control), indicating there were prominent underlying differences in gene expression associated with the development of NP, as well as the GC effects on NP (**Figure 6.10,** *Page 111***; Figure 6.11,** *Page 113*). However, one to two NP samples were not contained within their corresponding clusters. They did not cluster tightly with the majority of the other NP samples in cluster and PCA analyses likely because they have distinct gene expression patterns at least with respect to the dataset of the significant genes used in these analyses.

In the comparisons between GC-naïve NP and controls, NP6 was clustered opposite to the NP samples. This observation may be attributed to the distinct histological pattern of NP6 compared to the other GC-naïve NP samples. NP6 was the only polyp sample highly infiltrated with lymphocytes but without eosinophil infiltration. Considering the comparison of GC-treated vs. GC-naïve NP, NP7 (GC-naïve polyp) and NP4R (GC-treated polyp) were not clustered in the corresponding groups. This observation could also be explained by the different histological patterns of these NP samples from the others. In the studied NP samples, most GC-naïve NP represent high

infiltration of eosinophils and severe edema, while most GC-treated NP represent low infiltration of eosinophils and absence of edema (**Table 6.1,** *Page 90*). NP7 was the only NP sample which simultaneously showed little eosinophil infiltration and edema among the GC-naïve NP group, so that the histological characters of NP7 were close to those of GC-treated NP tissues. As far as the similarity between NP4 and NP4R are concerned, the histological changes among this pair of NP were not obvious: eosinophil infiltration was not significantly reduced in NP4R compared to NP4, and both NP4 and NP4R were not edematous polyps. Therefore, the close cluster of NP4 and NP4R may indicate the limited effects of GCs on change of histopathologic profiles (eosinophils and edema) in NP4 patient.

In summary, these analyses suggest that varied histological features of NP may have a basis in distinct gene expression patterns and this observation is consistent with the great biological variability observed *in vivo* as manifested in patient clinical behavior and the heterogeneous features of NP.

6.2.1.2 Indication of functional analysis

In **Chapter 6.1.6** (*Page 114*), we described and compared the most significant functional groups in three datasets (GC-naïve NP versus control, GC-treated versus GC-naïve NP, and GC-treated NP versus control). The significant functions were classified into three categories (i.e., at disease, tissue, and molecular/cellular levels). The most significant functions related to NP and the response of NP to GCs were in agreement with each other, including inflammatory/immunological diseases, cancer, tissue development, cellular development, growth, proliferation, and death. Indeed, these functions may contribute to the pathogenesis of NP at different levels (i.e. from

tissue to cell to molecule). These results could explain that NP is a chronic inflammatory disease with abnormal tissue development process in epithelium and stroma area, which are attributed to dysregulation of cellular activation and infiltration. Furthermore, these functions are also considered to be the underlying mechanism for the potent anti-inflammatory and tissue repair effects of GCs.

One interesting finding of the functional analysis is that the top significant disease related to NP associated genes is cancer. Although NP has not been considered a cancer-prone lesion, the hyperplastic features and high recurrence rate of NP raise attention to whether NP could share some pathological mechanisms with those malignant (e.g. nasopharyngeal carcinoma) and benign (e.g. inverted papilloma) neoplasms in upper respiratory tissues, or whether NP could serve a link between neoplasia and inflammation. This suggests that some cancer related genes/mechanisms could be considered alternative approaches to study NP pathogenesis.

6.2.1.3 Indication of functional network analysis

The effect of GC treatment on NP is attributed to the complex interaction among those functional related genes, so that we have identified distinct molecular networks implicated in the mechanism of GCs. These networks include both directed and undirected interactions. Directed interactions are characterized by a well-defined information flow (e.g., from a transcription factor to the gene it regulates). Undirected interactions do not have an assigned direction (e.g., mutual binding relationships). The ability to rank the networks based on their relevance to the GC-responsive gene set allowed for rapid prioritization of networks with highest importance. The

top-scoring network, AP-1 network is related to cellular development, growth, and proliferation; while the 2nd ranking network is associated with inflammatory disease. These results are consistent with those in functional analysis and could represent the molecular evidence of GCs, at least in our studied NP samples.

Another important application of network analysis in the current study is to identify the core/candidate genes among the GC-responsive genes. This is based on the searching of most prominent interaction partners within the merged networks. Other than those previous microarray studies in NP which focused on the top over-expressed/under-expressed genes [Fritz et al., 2003; Benson et al., 2004; Liu et al., 2004; Lee et al., 2006; Bolger et al., 2007; Figueiredo et al., 2007], network analysis identifies the most functional interacting genes which may represent the molecular candidates in response to GC treatment. The identified candidates, AP-1 genes (c-Jun and c-Fos) are critical factors which govern the transcription status of numerous growth related genes, mapping the central position among the GC-responsive gene network. Again, these results are also in agreement with those significant functions related to the gene sets.

6.2.2 Summary of the functional network pathways

Based on the network analysis (**Chapter 6.1.7,** *Page 118*) and Canonical Pathway analysis (**Chapter 6.1.8,** *Page 126*), the present microarray study reveals several important molecular signaling pathways which contribute to the pathogenesis of NP and its response to GC treatment. Network analysis suggests that AP-1 network and the network composed of pro-/anti-inflammatory genes would underlie the GC effects on NP. The Canonical Pathways suggests that apoptosis signaling, complement

system, EGF/EGFR signaling, eicosanoid signaling, ERK/MAPK signaling, IL-6 signaling, and NF-kappaB signaling would be associated with NP pathogenesis. **Table 6.9** summarized the relevant roles of these signaling pathways in NP before and after GC treatment based on the evaluation of the expression levels of the key genes. Note that some genes identified in Canonical Pathways are also determined in GC-regulated network pathways. The Canonical Pathways are discussed in this section; while those GC-responsive pathways will be discussed in a latter section (*See* **Chapter 6.2.3,** *Page 169*; **Chapter 6.2.4,** *Page 179*).

Table 6.9 Summary of the functional network pathways in NP

Pathway	**Relevant biological/immunological functions**[†]	**Response to GC therapy**[‡]	
		Before	**After**
AP-1 network[*]	Epithelial restitution	Down	Up
Anti-inflammatory gene network[*]	Anti-inflammatory process	Down	Up
Caspase-mediated apoptosis signaling[#]	Epithelial damage	Up	*NS*
Complement system[#]	Eosinophil infiltration	Up	*NS*
EGF/EGFR signaling[#]	Epithelial restitution	Down	Up
Leukotriene signaling[#]	Eosinophil infiltration	Up	*NS*
Prostaglandin E_2 signaling[#]	Epithelial restitution	Down	Up
ERK/MAPK signaling[#]	Pro-inflammatory process	Up	Down
IL-6 signaling[#]	Epithelial restitution	Down	Up
NF-kappaB signaling[#]	Pro-inflammatory process	Up	Down

[*] Networks generated from GC-regulated genes.

[#] Networks generated from NP disease associated genes.

[†] Only list the most relevant function of the indicated pathway based on the results of current study.

[‡] Response of indicated network is evaluated by the expression levels of the involved key genes.

NS, not significant change.

6.2.2.1 Apoptosis signaling in NP

Apoptosis, a form of cell death characterized by cell shrinkage, membrane blebbing, nuclear breakdown, and DNA fragmentation, is essential for development, maintenance of tissue homeostasis and elimination of harmful cells in metazoan organisms [Nagata, 1997]. Caspases, a group of cysteine proteases that cleave protein substrates after aspartic acids, play a central role in the regulation and execution of apoptosis [Cryns et al. 1998]. Canonical Pathway of apoptosis described the caspase-mediated apoptosis cascade in GC-naïve NP (**Figure 6.15B,** *Page 129*).

There is less information about the apoptotic factors in NP, and only one recent report showed that down-regulation of cIAP1 (apoptosis inhibitor 1) may contribute to the increase of cellular infiltrates in NP [Cho et al., 2008]. Our results showed that, although GCs did not change expression level of the apoptotic factors in NP, two critical downstream caspase genes (CASP3 and CASP7) as well as Bid and AIF were constituently expressed higher in GC-naïve NP. The previous study also reported that CASP3 was only expressed in the epithelial region in NP, but not in subepithelial constituents [Cho et al., 2008]. Since epithelium damage was detected in GC-naïve NP in our study, it suggests that the caspase pathway may not be an important pro-apoptotic mechanism in the stromal cellular infiltration, but may be essential in epithelial damage/remodeling in NP. In addition, up-regulation of pro-apoptotic genes may also indicate the heterogeneity of apoptotic environment in NP, i.e., the anti-apoptotic milieu in eosinophil/neutrophil infiltration, but pro-apoptotic milieu in epithelial cells.

6.2.2.2 Complement components in NP

The complement system is perceived as a central constituent of innate immunity, defending the host against pathogens, coordinating various events during inflammation, and bridging innate and adaptive immune responses [Markiewski & Lambris, 2007]. However, disturbance in this defense system leads to inflammatory disorders. The Canonical Pathway of complement system showed the activation cascades of complement components in GC-naïve NP (**Figure 6.15C,** *Page 131*).

The roles of complement in asthma are considered to promote migration of inflammatory cells, resulting in the rapid degranulation of mast cells [Guo et al., 2005; DiScipio et al., 2007]. However, the effects of complement signaling in NP inflammation have been rarely reported. Only two studies have reported the findings of complement levels in NP, but the results were controversial since both up- and down-regulated production of complement was found [Zídková et al., 1993; Baruah et al., 2007].

Our results showed that the key complement component C3 was associated with eosinophil infiltration (**Table 6.6,** *Page 142*) and other components such as C4a and C1QB were also up-regulated in GC-naïve NP. Moreover, C3 has also been recognized as a potent mediator for the induction of Th2-induced allergic asthma [Drouin et al., 2001]. Therefore, the increase of complement components in NP may reflect the ongoing inflammatory response (e.g. recruitment of leukocytes and up-regulation of inflammatory cytokines) in exposure to microbes in nasal mucosa, leading to an increase of eosinophils.

6.2.2.3 EGF/EGFR signaling in NP

The EGF/EGFR signaling is important to regulate cell proliferation, migration and differentiation on target cells (**Figure 6.15D,** *Page 132*). The EGF/EGFR signaling has been suggested to play a critical role in tissue repair/remodeling in both healthy airway tissues and airway inflammatory disorders (e.g., asthma) [Davies et al., 1999; Holgate, 2000; Watelet et al., 2006]. Most importantly, the localization of EGF genes and EGFR in nasal epithelium from healthy inferior turbinate indicates that they have an essential role in epithelial development and repair [Polosa et al., 2000]. However, its function in NP has been less documented. Our results showed that both EGFR ligands (e.g., AREG, EGF, HBEGF and NRG3) and EGF receptors (e.g., ERBB4) were down-regulated in GC-naïve NP as compared to controls, suggesting the dysregulation of EGF/EGFR mediated proliferation signaling in NP, especially in NP the epithelial region. Furthermore, an increase of AREG and HBEGF in response to GC treatment suggests the epithelial repair function of GCs (more detail in **Chapter 6.2.3,** *Page 169*).

6.2.2.4 Eicosanoid signaling in NP

Abnormal regulation of both leukotriene (LT) and prostaglandin (PG) pathways appear to be involved in the chronic inflammation of NP [Picado et al., 1999; Mullol et al., 2002; Pérez-Novo et al., 2005; Pérez-Novo et al., 2006; Adamjee et al., 2006; Hyo et al., 2007]. The Canonical Pathway picture showed the interactions of the molecules involved in the eicosanoid signaling network (**Figure 6.15E,** *Page 134*).

The enzymes (PLA_2, ALOX5AP, and LTA4H) in the LT synthesis pathway as well as the LT receptors (LTB4R and CYSTLR1) were up-regulated in GC-naïve NP and

up-regulation of these LT related markers were significantly correlated with an increased infiltration of eosinophils in nasal samples (**Table 6.6,** *Page 142***; Figure 6.16,** *Page 143*). These data are in line with the previous studies showing that eosinophils are one of the most important sources of LT receptors in inflamed upper airways [Sousa et al., 2002]; and that an LT signaling pathway may be involved in eosinophil migration and survival [Holgate et al., 2003; Saito et al., 2004].

As far as in the PG pathway, we found that the key enzyme (COX-2) for PGE_2 production and the PGE_2 receptors (PTGER2 and PTGER3) were changed in GC-naïve NP as compared to controls and only COX-2 expression responded to GC treatment in NP. The anti-inflammatory roles of COX-2/ PGE_2 in NP have been documented. Previous studies reported that the imbalance of AA/PGE_2 metabolism in aspirin-intolerant/aspirin-tolerant NP patients could be due to down-regulation of COX-2 [Picado et al., 1999; Mullol et al., 2002; Pujols et al., 2004]. Another important role of COX-2/PGE_2 is considered to be associated with epithelial repair and it will be discussed in **Chapter 6.2.3** (*Page 169*). The complexities of PGE_2-mediated effects may be attributed to the diversified properties of PGE_2 receptors [Funk, 2001; Tilley et al., 2001]. PGE_2 promotes vasodilatation by activation of cAMP-coupled PTGER2 on vascular smooth muscle and increase of vascular permeability by enhancing the release of histamine and other mediators from leukocytes. Regarding PTGER3, a previous study showed that the prevalence of a certain haplotype in *PTGER3* was significantly higher in mild asthmatics than in moderate and severe asthmatics, suggesting that genetic variations in PTGER3 may play an important role in asthma severity [Park et al., 2007]. PTGER3 action was mediated by an increase of intracellular cAMP signaling, which is associated with an inhibition of leukocyte activation in

inflamed tissues [Gerlo et al., 2004]. Therefore, up-regulation of PTGER2, but down-regulation of PTGER3 may promote the chronic inflammation of NP.

In summary, mRNA patterns of the genes involved in LT and PG pathways are different between NP and control. Up-regulation of the markers related to LT synthesis/activation would contribute to eosinophil infiltration in NP. In contrast to the effects of LT signaling, PGE_2 pathway (COX-2 – PTGERs) may have diverse modulatory roles in the development of NP and up-regulation of COX-2 may represent the beneficial effects of GCs in NP inflammation. In addition, our results also indicate that to determine the role of PGE_2 in a given inflammatory response, one should keep in mind not only knowledge of the lipid mediators (PGs) presented in the tissues, but also the PG receptor profile and its corresponding biochemical signaling on immune cells.

6.2.2.5 ERK/MAPK signaling in NP

The ERK/MAPK signaling pathway has an important role in cellular processes, such as proliferation, stress responses, apoptosis, and inflammation. MAPK pathways are activated through a cascade of sequential phosphorylation events. Activated MAPKs can phosphorylate a wide array of downstream targets, including protein kinases and transcription factors (e.g. ATF/CREB and AP-1 family) that facilitate the transcription of pro-inflammatory genes [Johnson et al., 2007]. Meanwhile, activation of the MAPK signaling cascade also triggers negative-feedback mechanisms, which can restrain and terminate the inflammatory response [Lang et al., 2006]. The ERK/MAPK Canonical Pathway (**Figure 6.15F,** *Page 136*) revealed altered expression of two major negative regulators of MAPK signaling pathway in NP, including dual specificity phosphatases

(DUSPs) and sprouty homologys (SPRYs), which control the signaling cascade at different activation/phosphorylation levels. Moreover, most of the DUSPs and SPRYs responded to GC treatment in NP, which were also identified in the GC-responsive gene networks.

In many cases, the expression of DUSPs is regulated by MAPKs and the participation of DUSPs in feedback regulation of MAPK activity is thought to be critical to the dynamic regulation of MAPK responses. GCs have been shown to inhibit the activity of different MAPK members [González et al., 1999; González et al., 2000; Lasa et al., 2002]. However, the response of most DUSP members to GCs is poorly understood; only induction of DUSP1 was found in several cell types by GC treatment and it has been regarded as one potential anti-inflammatory mechanism of GCs [González et al., 1999]. Furthermore, DUSP1 is the founding member of the MAPK phosphatase family and is expressed as an immediate-early gene in response to serum, growth factors, or cellular stresses [Sun et al., 1993; Zheng et al., 1993].

Sprouty homology family represents a major class of ligand-inducible inhibitors of receptor tyrosine kinase (RTK)-dependent signaling pathways, particularly the RTK-RAS-RAF-ERK/MAPK signaling cascade [Mason et al., 2006]. Expression of SPRY proteins is induced by various growth factor signals, including fibroblast growth factors (FGFs) [Hanafusa et al., 2002] and epidermal growth factor (EGF) [Reich et al., 1999]. The inhibitory activity of SPRY is exerted upstream of ERK/MAPK and downstream of the RTK, but the precise point at which SPRY intercepts RTK-ERK/MAPK signaling varies depending on the biological context. For example, in mouse fibroblasts, SPRY1 and SPRY2 interfere with ERK/MAPK signaling at the

level of Ras activation [Gross et al., 2001], while in human epithelial cells, SPRY2 and SPRY4 functions at the level of RAF activation [Yusoff et al., 2002].

Although the knowledge of MAPK signaling pathway in inflammation is well established, the mechanism of MAPKs in NP has been rarely reported. Only the induction of RANTES [Yamada et al., 20001], MUC5AC [Young et al., 2004], MUC8 [Cho et al., 2005], and CCL2 [Lin et al., 2007] was involved in MAPK mediated inflammatory pathways. In addition, no report has described the expression and function of the MAPK negative regulators (DUSPs and SPRYs) in NP. Our study found that five main DUSPs (DUSP1, DUSP2, DUSP4, DUSP5, and DUSP6) and three major SPRYs (SPRY1, SPRY2, and SPRY4) were expressed lower in GC-naïve NP, indicating the negative regulation process on MAPK was inadequate in NP. Therefore, the response of DUSP1, DUSP2, DUSP6, SPRY1, SPRY2, and SPRY4 to GC treatment in NP suggests the anti-inflammatory effects of GCs on ERK/MAPK signaling pathways.

6.2.2.6 IL-6 signaling in NP

As shown in **Figure 6.15G** (*Page 138*), IL-6 signaling is mediated by IL-6 signal transducer (IL6ST, or known as gp130), which leads to Jak activation, receptor phosphorylation, and consequently activation of the transcription factor STAT3 [Bravo et al., 2000; Schmitz et al., 2000]. Upon phosphorylation, STAT3 forms homodimer and translocates into the nucleus, where it activates transcription of target genes, such as Bcl-2, cyclin D1, cyclin E1, CDKN1A, MYC, c-Jun, and c-Fos [Alvarez et al., 2004]. To control the IL-6 mediated proliferation signal, gp130 can recruit the key feedback inhibitor, suppressor of cytokine signaling 3 (SOCS3) by interacting with the Y759

motif of gp130 [Starr et al., 1997; Nicholson et al., 2000]. SOCS3 is induced by IL-6 and has an important role in the regulation of IL-6 signaling pathway [Croker et al., 2003].

The expression of IL-6 in NP has been reported controversially, and both up- and down-regulated alteration has been found [Bachert et al., 1997; Scavuzzo et al., 2005; Danielsen et al., 2006], indicating IL-6 may participate in both pro- and anti-inflammatory actions. Since all the key markers (including IL-6, gp130, STAT3, and SOCS3) were down-regulated in NP, IL-6 signaling may be defected in GC-naïve NP samples (**Figure 6.15G**, *Page 138*). Reduction of IL-6 signaling may reflect the damage of epithelium and low growth potential of epithelial cells in NP. So that the increase of IL-6 expression in NP after GC treatment suggests the promotion of epithelial restitution by GCs; while the simultaneous up-regulation of SOCS3 by GCs may indicate the homeostatic effect of GCs on IL-6 mediated proliferation signaling.

6.2.2.7 NF-kappaB signaling in NP

NF-kappaB acts on genes for proinflammatory cytokines, chemokines, enzymes that generate mediators of inflammation, immune receptors, and adhesion molecules that play a key part in the initial recruitment of leukocytes to sites of inflammation [Hayden et al., 2004]. The activated form of NF-kappaB is a heterodimer, which usually consists of two proteins, a p65 subunit and a p50 subunit. In unstimulated cells, NF-kappa B is found in cytoplasm and is bound to inhibitors of NF-kappaB (IkappaB), which prevent it from entering the nuclei [Baldwin, 1996]. NF-kappaB Canonical Pathway revealed that two important inhibitors (NFKBIA and NFKBIZ) of NF-kappaB signaling were decreased in GC-naïve NP (**Figure 6.15H**, *Page 140*).

There are six IkappaB family members, including IkappaB-α (NFKBIA), IkappaB-β (NFKBIB), IkappaB-γ, IkappaB-ε (NFKBIE), BCL3, and IkappaB-ζ (NFKBIZ), which are characterized by the presence of five to seven ankyrin repeats that assemble into elongated cylinders that bind the dimerization domain of NF-kappaB dimmers. Various members of the IkappaB family target different NF-kappaB complexes, for example, NFKBIA and NFKBIB interact preferentially with p65/p50 heterodimers [Thompson et al., 1995], while NFKBIZ preferentially associates with the NF-kappaB subunit p50 rather than p65 [Yamazaki et al., 2001].

Previous studies showed that an increase of NF-kappaB activity and expression has been found in NP, which was accompanied with up-regulation of pro-inflammatory genes (such as GM-CSF, IL-5, IL-8, and eotaxin) [Takeno et al., 2002; Valera et al., 2008]. Direct interactions between the glucocorticoid receptor and NF-kappaB account for the inhibitory effects of GCs on NF-kappaB signaling [McKay et al., 1999]. However, glucocorticoid response element (GRE) was found in IkappaB genes, and GCs are able to induce expression of IkappaB, indicating another way by which GCs inhibit NF-kappaB activity [Auphan et al., 1995]. Therefore, the lower expression of NFKBIA and NFKBIZ suggest that the control mechanism of NF-kappaB is insufficient, contributing to the chronic inflammatory milieu in NP. Although we did not find the change of expression in NF-kappaB factors in NP, the results of up-regulation of NFKBIZ are in line with the concept that GCs can inhibit activation of NF-kappaB by promoting IkappaB expression [Scheinman et al., 1995].

6.2.2.8 Conclusion of Canonical Pathways in NP

Systemic pathway analysis reveals the well-established signaling pathways which

contribute to the NP pathogenesis. Referring to the histopathological findings of the current study, we suggest that the dysfunction of NP epithelium would be associated with the alteration of caspase-mediated apoptosis signaling, EGF/EGFR signaling, PGE_2 signaling, and IL-6 signaling, while the increase of eosinophil infiltration and inflammatory process in NP would be related to the enhanced activity of complement system, LT signaling, ERK/MAPK signaling, and NF-kappaB signaling (**Table 6.9,** *Page 159*). Most of the Canonical Pathways responded to GC treatment (except apoptosis signaling, complement system, and LT signaling), indicating the potential effects of GCs on NP could be epithelial repair and anti-inflammation (**Table 6.9,** *Page 159*). These two major beneficial effects will be discussed in detail in the following two sections (**Chapter 6.2.3 & 6.2.4**).

6.2.3 Epithelial repair effect of GCs in NP

In the current study, the advanced functional and network analyses were used to identify the key networks and candidates in response to GC treatment. This is the first *in vivo* study deciphering the role of the AP-1 gene network and epithelial remodeling in NP. The major findings are: (i) epithelial damage is an important feature of NP and a remarkable epithelial restitution is observed after GC treatment; (ii) AP-1 and its related genes appear to be a key molecular network underlying the wound healing in NP; (iii) epithelial c-Jun protein expression is up-regulated by oral GC therapy and is positively correlated with epithelium restitution in NP tissues.

6.2.3.1 Background information of epithelial repair in airway tissues

The epithelial repair process is a highly organized and well coordinated process, which is generally categorized into three steps: the inflammatory, proliferative, and

remodeling phases. During inflammation, various inflammatory cells (including neutrophils, lymphocytes and macrophages) infiltrated the wounded epithelium, and secrete pro-inflammatory mediators (e.g. cytokines and growth factors) which are vital for pushing the wound healing process into the proliferative phase. After the inflammatory stage, fibroblasts begin to enter the wound site, marking the onset of the proliferative phase. During this period, epithelial cells migrate across the new tissue to form a barrier between the wound and the environment. Finally, tissue remodeling occurs, including re-epithelialization and matrix deposition.

The human nasal epithelium represents the first line of defense against a number of toxic substances, allergens and infectious agents. In order to maintain its integrity, the nasal epithelium has the capacity to repair itself in response to the chronic exposure of damaging agents. However, the feature of mucosal inflammation in NP is epithelial damage followed by abnormal tissue repair and remodeling [Holgate, 2000; Watelet et al., 2006]. The NP epithelium is "passively" under attack by the infiltrating eosinophils and inflammatory mediators, leading to damage of epithelium [Uneri et al., 2005; Cheng et al., 2006]. Consequently, the epithelial damage of NP causes the release of pro-inflammatory signals which pass down into the submucosa and also removes some anti-inflammatory mediators such as PGE_2, leading to uncontrolled remodeling. Hence, under chronic inflammatory conditions, the epithelial repair process may become inadequate in NP, resulting in abnormal tissue repair and remodeling. In this context, rapid repair of damaged epithelium without causing uncontrolled re-epithelialization process is critical to the resolution of NP inflammation.

GC treatment is considered the most effective pharmacological therapy for chronic

upper airway inflammation including NP and asthma [Fokkens et al., 2007; Bateman et al., 2008]. The predominant effects of GCs are to switch off multiple inflammatory genes that have been activated during the inflammatory process and to promote tissue repair, such as immunoregulatory and remodeling effects by induction of TGF-β in NP [Mastruzzo et al., 2003], and by activation of EGF/EGFR signaling in the asthma model [Wadsworth et al., 2006]. However, the previous studies only specify selected known markers, while a systematic approach is necessary to view the overall molecular events responsible for GC therapy and their effects on epithelial repair in NP.

6.2.3.2 Histolgoical findings regarding epithelial repair by GCs in the current study

On the basis of a grading system that we used to quantify epithelial integrity, the epithelium of most GC naïve NP samples was substantially damaged, while remarkable epithelial restitution with no abnormal remodeling was observed after oral GC treatment (**Table 6.1,** *Page 90*). Moreover, improvement was evident in GC-naïve NP tissues with areas of squamous epithelium (NP3 and NP10), and GC-treated NP3 and NP10 presented pseudostratiried columnar epithelium without aberrant hyperplasia, indicating the regulatory effects of GCs. Our findings are consistent with those reports which demonstrate improvement in the structure of airway epithelium after GC treatment [Mastruzzo et al., 2003; Wadsworth et al., 2006]. Note that the previous studies focused on the effect of topical GC treatment, while our study observed that short course (3-5 days) of oral GC treatment indeed had a tissue repair effect on NP *in vivo*.

6.2.3.3 Molecular events underlying the wound healing effects of GCs in NP

Histological evaluation has confirmed the epithelial change at the tissue level, while microarray analyses explored the molecular evidence underlying this healing process of GCs. In this context, the top-scoring network (AP-1 network) and the involved GC candidate genes (AP-1 molecules) as well as their major interacting genes are of particularly interest. The functions of AP-1 network and AP-1 genes/AP-1 related genes are associated with cellular development, growth, and proliferation, which are considered to contribute to the histological change of NP epithelium. The discussion of AP-1 and AP-1 related genes is described in the following paragraphs.

(1) Background information of AP-1

AP-1 regulates a wide range of cellular processes by induction of various pro-/anti-inflammatory molecules, including inflammation, cell migration, proliferation, death, survival and differentiation [Shaulian et al., 2002]. AP-1 is formed by dimerization of members of Jun (c-Jun, JUNB and JUND) and Fos (c-Fos, FOSB, FOSL1 and FOSL2) proto-oncogene families, while the most abundant AP-1 heterodimer is c-Fos:c-Jun [Karin et al., 1997]. c-Jun is the central component of all AP-1 proteins and the key factor for AP-1 activity, due to its wide range of dimerization with other AP-1 members and regulation (e.g. phosphorylation) by various mediators [Karin et al., 1997]. The Fos proteins, which cannot homodimerize, form stable heterdomiers with Jun proteins and thereby enhance their DNA binding activity.

AP-1 activity is regulated by a broad range of extracellular stimuli including physiological agents (mitogens, growth factors, hormones, extracellular matrix and inflammatory cytokines), bacterial and viral infections, pharmacological compounds

(anisomycin, phorbol esters, and okadaic acid) and cellular stress (ultraviolet or ionizing radiation) [Karin et al., 1997]. Many of these stimuli activate the mitogen-activated protein kinase (MAPK) cascades [mostly p38, JUN N-terminal kinases (JNKs) and extracellular signal-related kinases (ERKs)], leading to the phosphorylation of c-Jun [Karin, 1995]. In turn, phosphorylated JUN heterodimers bind DNA sequences (AP-1 response elements) and induce the transcription of downstream genes which are most involved in cellular development, growth and proliferation. AP-1 induces a variety of growth related genes (e.g. EGF protein family) [Grose, 2003], cytokines (e.g. IL-6) [Gallucci et al., 2000], and enzymes (e.g. COX-2) [Yamaguchi et al., 2005]. Moreover, AP-1 can also interact with other transcription factors (e.g., EGR1), which enhance AP-1 activity [Levkovitz & Baraban, 2001].

Regulation of AP-1 gene expression in response to GCs is highly complex and may be cell-type-specific. Subramaniam *et al.* demonstrated that dexamethasone (Dex) up-regulated the mRNA levels of c-Jun and c-Fos in human osteoblasts [Subramaniam et al., 1992]. Zhou *et al.* showed that both c-Jun mRNA and protein, but not other members of the AP-1 family (c-Fos, JUNB, JUND, FOSB), were induced by Dex in human leukemic lymphoblasts [Zhou et al., 1996]. Boudreau *et al.* reported that both the mRNA and protein of c-Jun, c-Fos, and JUNB were increased in intestinal epithelial cells by Dex treatment [Boudreau et al., 1999]. However, Lee *et al.* reported that the expression of c-Jun was repressed by Dex in fibroblasts [Lee et al., 1991]. In addition, c-Jun transcription was directly stimulated by its own gene product [Angel et al., 1988].

(2) AP-1 gene expression in nasal tissues

We determined the expression of two main components of AP-1 (c-Jun & c-Fos) in

NP as well as in nasal mucosal control at mRNA and protein level. A deficiency in both c-Jun and c-Fos mRNA expression in NP tissues was evident. A restoration of both c-Jun mRNA and protein was found after GC therapy in NP tissues. With regard to c-Fos, its mRNA was up-regulated in GC-treated NP, but immunohistochemical staining suggests that GC treatment did not improve c-fos protein level in extracted NP tissues.

Our results are in disagreement with a previous report in which elevated c-Fos protein was detected in NP tissue and GC decreased c-Fos protein, but did not affect c-Fos mRNA level in NP tissues [Baraniuk et al., 1998]. In that study, mRNA expression of c-Fos was measured by RT-PCR that is considered as qualitative but not quantitative measurement. Furthermore, determination of c-Fos mRNA level was normalized by β-actin intensity band, which is not a reliable internal control for GC-treated nasal samples [Bolger et al., 2007]. c-Fos mRNA and protein expression may also be affected by broad alterations in post-transcriptional processes: (i) increased turnover of c-Fos protein may not be detectable by immunohistochemistry [Kruijer et al., 1984]; (ii) c-Fos or AP-1 may negatively auto-regulate c-Fos protein translation [Sassone-Corsi et al., 1988]; (iii) binding of activated GR and c-Fos may lead to the mutual inactivation and proteolytic degradation and then hamper the c-Fos production [Yang-Yen et al., 1990]. Other plausible explanations for such a conflicting result are due to variable factors such as choice of different subjects (same patient versus different patient before and after treatment), dosage and type of GC treatment (oral versus topical GC), and different experimental protocols (e.g. different RNA detection method, different immunohistochemistry methods). Nevertheless, the role of c-Fos protein in NP and its response to GC treatment needs to be further clarified, and trying to minimize

confounding factors in detecting c-Fos is recommended.

(3) Tissue repair role of c-Jun/AP-1 and its related genes in nasal polyp epithelium

Circumstantial evidence suggests a critical role of AP-1 (especially c-Jun) and its related genes in the maintenance and repair of epithelial tissues and reduction of AP-1 expression may interrupt a critical autocrine/paracrine pathway of signaling to the epithelial restitution [Grose, 2003; Li et al., 2003; Shaulian et al., 2002]. In our results, elevated expression of c-Jun in GC-treated NP tissues was associated with the significant improvement in NP epithelial structure (**Figure 6.19,** *Page 152***; Table 6.8,** *Page 153*), indicating that GCs may perform the tissue repair effect via regulating c-Jun expression.

c-Jun/AP-1 is a transcription factor, which induces many mRNAs for cytokines and growth factors (AP-1 downstream genes), while c-Jun/AP-1 itself is also regulated by various growth related factors (AP-1 upstream genes). Therefore, these c-Jun/AP-1 related genes (both downstream and upstream) indeed participate in the epithelial repair function of c-Jun/AP-1. The integrated analysis of multi-dimensional microarray data has proved informative. Our network analyses have identified a number of AP-1 related genes (COX-2, IL-6, AREG, HBEGF, and EGR1) which appear to modulate airway epithelial remodeling and are GC dependent.

AP-1 induces transcription of COX-2 which is the key enzyme required for the conversion of arachidonic acid into prostaglandins (PGs) [Yamaguchi et al., 2005]. COX-2/PGE_2 plays an important role in the homeostasis of epithelial remodeling process in airway tissues [Savla et al., 2001]. Decreased expression of COX-2 and

diminished PGE_2 production seem to be an important feature of untreated NP tissues, especially those with aspirin intolerance [Picado et al., 1999; Mullol et al., 2002]. We found an up-regulation of COX-2 mRNA in NP tissues after oral GC treatment, which is in line with the report by Dworski *et al.*, who found that treatment with prednisone resulted in a significant increase in COX-2 mRNA and protein in atopic subjects *in vivo* [Dworski et al., 1997].

In our study, up-regulation of c-Jun/AP-1 is associated with IL-6. c-Jun/AP-1 was shown to induce IL-6 expression through the CRE binding region [Franchimont et al., 1999], while IL-6 mediated signal pathway could increase expression of c-Jun [Solis-Herruzo et al., 1999]. IL-6 is a pleiotropic cytokine that exerts both pro- and anti-inflammatory activities, as well as cellular proliferation. GCs were found to enhance the induction of IL-6 mRNA in human bronchial epithelial cells, indicating the host defense activity of GCs [Homma et al., 2004]. In addition, IL-6 together with growth related factors (e.g. EGF and COX-2) are important to protect epithelium from injury in a variety of tissues, including skin [Gallucci et al., 2000], gastrointestinal tract [Tebbutt et al., 2002], and kidney [Nechemia-Arbely et al., 2008].

This study shows evidence of an up-regulation of two EGFR ligands (AREG and HBEGF) in response to GC treatment. The EGF protein family is thought to be the primary cytokines in the process of epithelium healing [Holgate, 2000; Watelet et al., 2006]. The GC mediated wound healing process in airway epithelium requires the activation of the EGF/EGFR signaling pathway [Wadsworth et al. 2006]. The response of EGF genes is clearly linked to AP-1 transcription factor activity, since both the EGFR and its known ligands are direct AP-1 target genes [Fu et al., 1999; Johnson et al., 2000]; and

activation of EGF/EGFR signaling also leads to the induction of AP-1 [Grose 2003; Li et al., 2003].

In this study, we found an increase of EGR1 that has been regarded to induce c-Jun expression and trigger c-Jun/AP-1 activation [Levkovitz et al., 2001]. EGR1 functions as a transcriptional regulator and is an example of an "immediate early response protein" because it is rapidly induced by numerous cytokines, growth factors, injurious stimuli, stress, and GC hormone [Liu et al., 2000; Revest et al., 2005]. EGR1 is also involved in the tissue repair process mediated by AP-1/EGF signaling [Warburton et al., 2005] and activated GC receptors could increase the levels of both EGR1 mRNA and protein [Revest et al., 2005].

6.2.3.4 Conclusion of the epithelial repair effect of GCs

Our results indicate the epithelial repair effect of GCs on NP from histological to molecular levels. AP-1 (especially c-Jun) and its related genes are all involved in the top function network composed of GC-regulated genes in NP (**Figure 6.13,** *Page 122*). The network analysis has revealed an extended pathway map potentially underlying the epithelial healing process in NP in response to GC treatment. c-Jun/AP-1 has been considered the core gene in this network, while the interactive positive feedback loop between c-Jun/AP-1 and its related genes (including AREG, HBEGF, COX-2, IL-6, and EGR1) will contribute to the epithelial repair effects of GCs on NP. The interaction among c-Jun/AP-1 and its related genes are summarized in **Figure 6.20** (*Page 179*): (i) expression level of c-Jun/AP-1, EGF molecules (AREG & HBEGF), COX-2, IL-6 and EGR1 in GC-naïve NP may be too low to perform their normal physiological process (e.g. wound healing), resulting in epithelial damage; (ii)

inadequate expression of AREG, HBEGF, COX-2, and IL-6 in NP may contribute to the lower expression of c-Jun/AP-1; (iii) since these growth related genes are c-Jun/AP-1 target genes, it may further potentiate the reduction of AREG, HBEGF, COX-2, and IL-6; (iv) after GC treatment, GR may directly/indirectly induce the expression of c-Jun/AP-1, AREG, HBEGF, COX-2, IL-6, and EGR1 in NP, and the positive feedback loop among these factors is activated, resulting in the promotion of epithelial repair in NP.

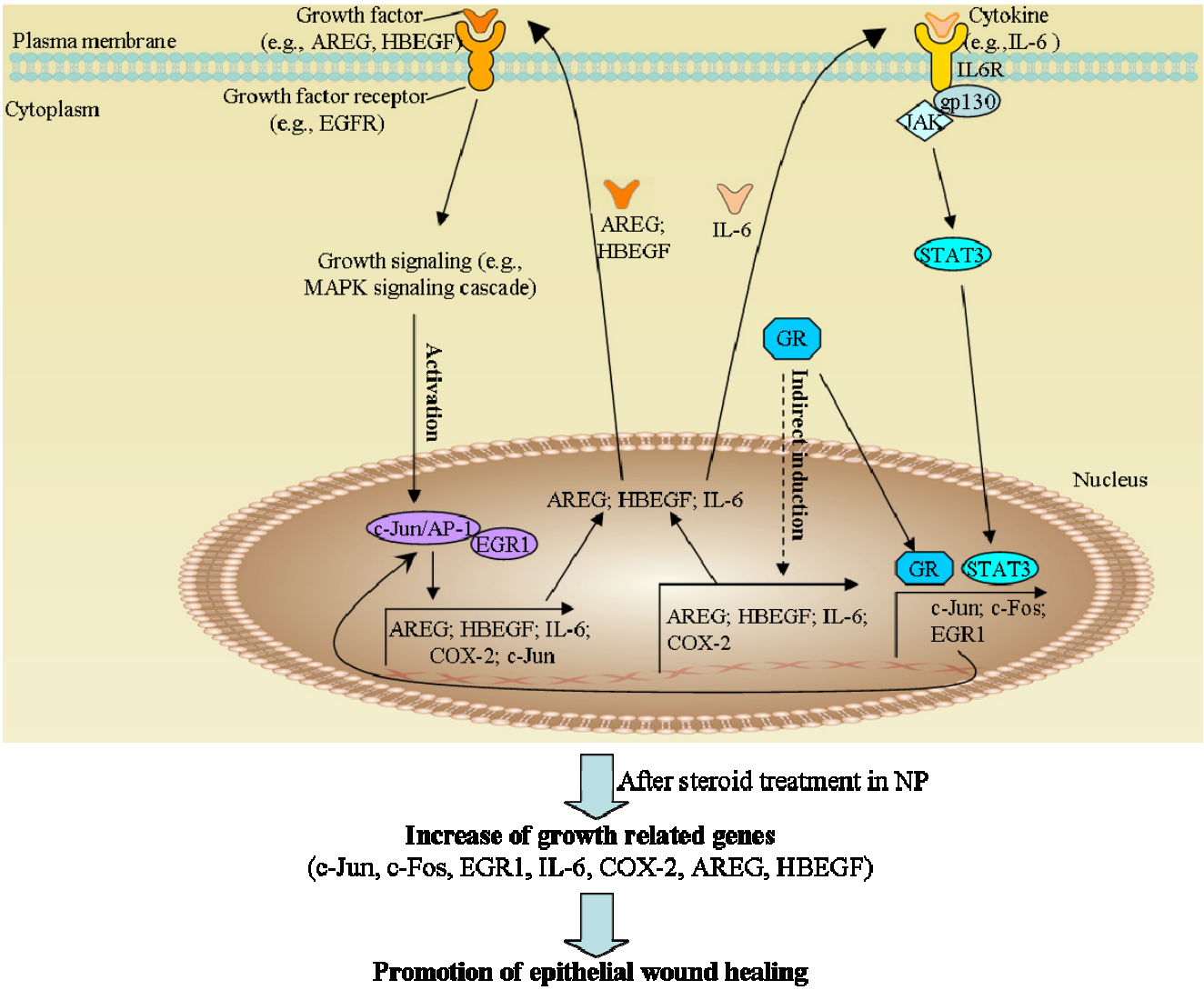


Figure 6.20 Schematic diagram of AP-1 and AP-1 related genes in epithelial repair in NP after GC treatment. AREG, HBEGF, COX-2, and IL-6 are both AP-1 upstream activators and AP-1 downstream target genes; and EGR1 is the co-activator of AP-1. Before GC treatment, AP-1 and AP-1 related genes are expressed at a low level in NP; while after GC treatment, all these genes are up-regulated and then the positive loop of epithelial cell proliferation is activated, leading to enhance of epithelial restitution. GR, glucocorticoid receptor.

6.2.4 Anti-inflammatory effect of GCs in NP

In previous paragraphs, we suggested that GCs may promote re-epithelization during the tissue repair process. Indeed, the anti-inflammatory action of GCs is widely accepted, and this effect is mediated by glucocorticoid receptor (GR), which migrates from the cytoplasm to the nucleus upon binding of ligand and exerts both positive and

negative effects upon transcription [Newton, 2000]. In our study, the network analysis identified several important pro- and anti-inflammatory genes involved in the GC gene networks (**Chapter 6.1.7,** *Page 118*). We suggest that GR modulates the expression of anti-inflammatory genes (e.g., inhibitors of NF-kappaB, inhibitors of MAPKs, SOCS3, ANXA1, SCGB1A1, ZFP36 and THBD) in a positive way, either directly or indirectly [Castro-Caldas et al., 2003; Usmani et al., 2005; Ishaq et al., 2007]; while it modulates the expression of some pro-inflammatory genes (e.g., MMP7, MMP9, and chemokines) in a negative regulation way.

6.2.4.1 Glucocorticoid receptor

Two human isoforms of glucocorticoid receptor (GR), GRα and GRβ, have been identified, which originate from the same gene by alternative splicing of the GR primary transcript [Encío et al., 1991]. GRα is the predominant isoform of the receptor and has GC-binding activity [Pujols et al., 2004]. In contrast, GRβ does not show ligand-binding activity, but may act as a dominant negative inhibitor of GRα activity [Pujols et al., 2004]. Moreover, recent evidence suggests that increased expression of GRβ may be associated with GC resistant asthma and NP [Hamid et al., 1999].

Our results showed a decrease of GRα mRNA in GC-naïve NP compared to controls, which is in line with previous reports [Pujols et al., 2008]. It has been reported that most of the eosinophils in NP were stained slightly with GR [Pujols et al., 2008], and GR expression was prominent in airway epithelial cells [Adcock et al., 1996]. These findings indicate that the local inflammatory milieu (high infiltration of eosinophils) of NP may be related to the down-regulation of GRα. In contrast to our results, Choi et al.

reported the higher level of GRα mRNA in NP than in control nasal mucosa [Choi et al., 2006]. These controversial results may be attributed to the cellular complexity of the NP tissues, heterogeneity of patient background and different detection methods. In agreement with previous data, expression of GRβ mRNA was too low to be detected in both NP and control tissues by real-time RT PCR.

GC resistance in asthma may result from the elevated level of GRβ expression and deficiency of GRα [Leung et al., 1997]. Although GRβ expression is considered a marker of GC resistance in NP [Hamilos et al., 2001], GRα expression level appears to have no effect on those NP patients who is resistant to the GC treatment [Choi et al., 2006]. Our results confirmed no detection of GRβ in GC-naïve NP, indicating that no GC resistance occurs in the studied samples. In addition, down-regulation of GRα in GC-naïve NP also appears not to be associated with the GC insensitivity in NP patients, since GCs could alter the expression of many GC responsive genes in the current NP samples.

There was no significant difference in GRα expression in NP before and after GC treatment. These results contrast with the data showing down-regulation of the GR after *in vitro* treatment of different airway cell types with GCs [Pujols et al., 2001; Pujols et al., 2004]. In keeping with our results, Henriksson et al. reported that NP did not respond with down-regulation of GR mRNA following GC treatment *in vivo* [Henriksson et al., 2001]. The conflicting findings may be attributed to the different experimental designs (*in vitro* vs. *in vivo*).

6.2.4.2 Anti-inflammatory molecules regulated by GCs

The inhibitors of pro-inflammatory signaling pathways including NFKBIZ, DUSPs, SPRYs, and SOCS3 were discussed in **Chapter 6.2.2** (*Page 158*). Moreover, some other important anti-inflammatory genes were also identified by network analysis, such as ANXA1, SCGB1A1, ZFP36, and THBD.

(1) Annexin A1

Annexin A1 (ANXA1) belongs to a family of Ca^{2+}-dependent phospholipid binding proteins and has phospholipase A2 (PLA2) inhibitory activity. Since PLA2 is required for the biosynthesis of the potent mediators of inflammation, such as prostaglandins, and leukotrienes. ANXA1 may have potential anti-inflammatory activity in inflammatory response [Wallner et al., 1986]. Besides the inhibition of arachidonic acid metabolism, ANXA1 also contributes to a variety of anti-inflammatory pathways, including inhibition of iNOS expression [Wu et al., 1995], stimulation of IL-10 release [Ferlazzo et al., 2003], inhibition of leukocyte migration [Perretti et al., 1996], and induction of apoptosis of inflammatory cells [Solito et al., 2001]. Regulation of ANXA1 by GCs has been well established and ANXA1 is able to mimic the anti-inflammatory effects of GCs in several experimental models of inflammation both *in vivo* and *in vitro* [Flower et al., 1988]. Our study showed that expression of ANXA1 was not different between NP and controls, but augmented ANXA1 expression was found in NP after GC treatment. These results are in agreement with the previous study [Sena et al., 2006], indicating that up-regulation of ANXA1 in NP may be attributed to the feedback response of the immune system to chronic inflammation, while GCs may enhance such anti-inflammatory action.

(2) Secretoglobin, family 1A, member 1

Secretoglobin, family 1A, member 1 (SCGB1A1; also know as uteroglobin), is an important GC-induced anti-inflammatory gene, which was originally found to inhibit PLA2 activity [Mukherjee et al., 2007]. It is the founding member of a newly designated Secretoglobin superfamily of proteins [Klug et al., 2000], constitutively expressed by the epithelial lining of all organs that communicate with the external environment. It has a broad range of anti-inflammatory effects [Mukherjee et al., 2007], including the inhibition of PLA2, leukocyte chemotaxis, and pro-inflammatory cytokines. Furthermore, peptides derived from SCGB1A1 are among the most potent anti-inflammatory agents identified to date [Miele, 2003]. Our study found that SCGB1A1 was expressed at a similar level between GC-naïve NP and control, but it was up-regulated at 5.19-fold in NP in response to GC treatment. The result is consistent with the previous NP microarray study, which revealed that SCGB1A1 was increased most in NP after GC treatment [Benson et al., 2004].

(3) Zinc finger protein 36

Zinc finger protein 36 (ZFP36; also known as tristertraprolin, TTP) is an immediate-early gene induced by various stimuli (e.g. LPS, TNF-α, p38 MAPK, IFN-γ) [Sauer et al., 2006], and destabilizes several pro-inflammatory cytokine mRNAs by binding to AU-rich elements with their 3' untranslated regions, targeting degradation [Carballo et al., 1998]. Moreover, dexamethasone treatment was found to inhibit TNF-α expression by sustained induction of the ZFP36 mRNA level in lung epithelial cells [Smoak et al., 2006]. This evidence suggests that ZFP36 performs its anti-inflammation activity by a negative feedback loop which is induced by

inflammatory signalings. Although GCs can induce ZFP36 and control the post transcription of pro-inflammatory genes, the role of ZFP36 in airway inflammation (e.g. NP and asthma) as well as its response to GC treatment has not been investigated as well. Our results demonstrated that ZFP36 was expressed lower in GC-naïve NP, but up-regulated in NP after GC treatment, suggesting the degradation effect of GCs on pro-inflammatory gene mRNA.

(4) Thrombomodulin

Ample evidence has suggested the cross-talk between inflammation and coagulation, i.e. inflammation leads to the activation of coagulation, meanwhile coagulation also significantly affects inflammatory activity, moreover, several anti-coagulant molecules are supposed to modulate inflammation [Levi et al., 2004]. Thrombomodulin (THBD) is an important anticoagulant factor. It plays an indirect role in modulating inflammation by inhibiting thrombin mediated inflammatory and remodeling processes [Van de Wouwer et al., 2004]. For example, the C-type lectin-like N-terminal domain of THBD could suppress activation of the MAPK cascade and prevent expression of leukocyte adhesion molecules, consequently interfering with the leukocyte migration to inflammatory tissues [Conway et al., 2002]. However, the roles of THBD in NP and its response to GC treatment have not yet been studied. Our study found down-regulation of THBD in GC-naïve NP, while it was increased in NP after GC treatment, indicating the effect of GCs on the coagulation system mediated inflammation and remodeling process in NP.

6.2.4.3 Pro-inflammatory molecules regulated by GCs

In addition to the positive effect on anti-inflammatory genes, GCs also suppress the

expression of those pro-inflammatory genes, such as Matrix metalloproteinase and CXC-chemokines.

(1) Matrix metalloproteinase (MMP)

Among the GC-responsive genes, two pro-inflammatory genes, MMP7 and MMP9 were down-regulated in GC-treated NP. MMP7 and MMP9 belong to the matrix metalloproteinase (MMP) family, which is capable to cleave a diverse array of extracellular matrix (ECM), thereby modulating cellular infiltration and tissue structure during both health and disease stages. Substantial evidence suggests that MMP is an important modulator of the inflammatory and remodeling processes. Increased or mis-regulated levels of MMPs have been observed in many inflammation associated diseases [Malemud, 2006]. The natural inhibitors of MMPs, so called tissue inhibitors of metalloproteinases (TIMPs), were used as anti-inflammatory drugs in inflammatory conditions such as arthritis [Burrage et al., 2007]. Steorids have been found to reduce expression of MMPs in airway tissues [Hoshino et al., 1999; Profita et al., 2004].

MMPs was found to be involved in tissue remodeling and eosinophil migration in NP due to the property of ECM degradation [Lechapt-Zalcman et al., 2001; Watelet et al., 2004; Kostamo et al., 2007; Can et al., 2008]. There is evidence that eosinophils are a source of MMP9 in NP and asthmatic tissues [Ohno et al., 1997]. In agreement with the previous studies, we found the up-regulation of MMP7 and MMP9 in GC-naïve NP, and increase of MMP9 was associated with eosinophil/neutrophil infiltration in NP, while MMP7 was only associated with a neutrophil influx. Increase of MMP9 and MMP7 production could degrade components of endothelial basement membrane and then enhance microvascular permeability, leading to eosinophil or neutrophil

transmigration [Lechapt-Zalcman et al., 2001; Yoon et al., 2007; Can et al., 2008; Swee et al., 2008]. Hence, after GC treatment, decrease of MMP7 and MMP9 may not only contribute to the suppression of leukocyte migration, but also maintain the homeostasis of tissue remodeling in NP.

(2) CXC-chemokines

Two CXC-chemokines, CXCL9 and CXCL11 were down-regulated in NP in response to GC treatment. CXCL9 and CXCL11 genes are closely located to each other on human chromosome 4. Both of them are considered T-cell chemoattractants, which interact with the T-cell surface receptor CXCR3. Since gene expression of CXCL9 and CXCL11 is strongly induced by IFN-gamma and CXCR3 is primarily expressed on activated Th1 cells, CXCL9 and CXCL11 are associated with Th1 response [Cole et al., 1998]. In addition, CXCL9 and CXCL11 are commonly produced by local cells in inflammatory lesions, suggesting that CXCR3 and its chemokines ligands participate in the recruitment of inflammatory cells. With regard to the response of GCs, CXCL9 and CXCL11 which were induced by IFN-gamma were found to be decreased after GC treatment [Egesten et al., 2007; Widney et al., 2000].

Although CXCL9 and CXCL11 have not been reported in any NP studies, their role in allergic rhinitis is regarded to suppress Th2 response [Sun et al., 2007]. Our results showed that before GC treatment, expression of CXCL9 and CXCL11 was not significantly different between NP and control. However, they were expressed at a high level in one NP sample (NP6) which was prominently infiltrated by lymphocytes (data not shown). These results are consistent with the histological patterns of these studied NP samples, most of which are eosinophil dominant. The decrease of CXCL9

and CXCL11 by GCs suggests that GC attenuation of CXCL9 and CXCL11 expression may contribute to the anti-inflammatory effects of GCs in Th1-dependent processes [Goleva et al., 2004].

6.2.4.4 Conclusion of the anti-inflammatory effects of NP

Our observation suggests that the anti-inflammatory effects of GCs in NP may not only be due to transrepression of pro-inflammatory genes by the GR, as indicated in previous studies [De Bosscher et al., 2003], but also involves positive regulation of anti-inflammatory genes. This finding is in agreement with the new concept of "non-transrepression" anti-inflammatory actions of GCs, i.e., activated GR initiates a signaling cascade with an induction of anti-inflammatory genes [Limbourg & Liao, 2003; Hayashi et al., 2004]. Most importantly, the identified anti-inflammatory genes are all involved in the negative feedback loops of the inflammatory signaling pathways. For example, NFKBIZ inhibit NF-kappaB activation, DUSPs, SPRYs, ZFP36, THBD inhibit MAPK cascade activation, SOCS3 inhibit IL-6 activity, ANXA1 and SCGB1A1 antagonize arachidonic acid mediated inflammatory response. Nevertheless, those pro-inflammatory markers such as MMP7, MMP9, CXCL9, and CXCL11 were also attenuated by GCs. These findings emphasize the diverse anti-inflammatory functions of GCs in modulating cellular signaling events in NP.

6.2.5 Hypothesis of the GC beneficial effects on NP

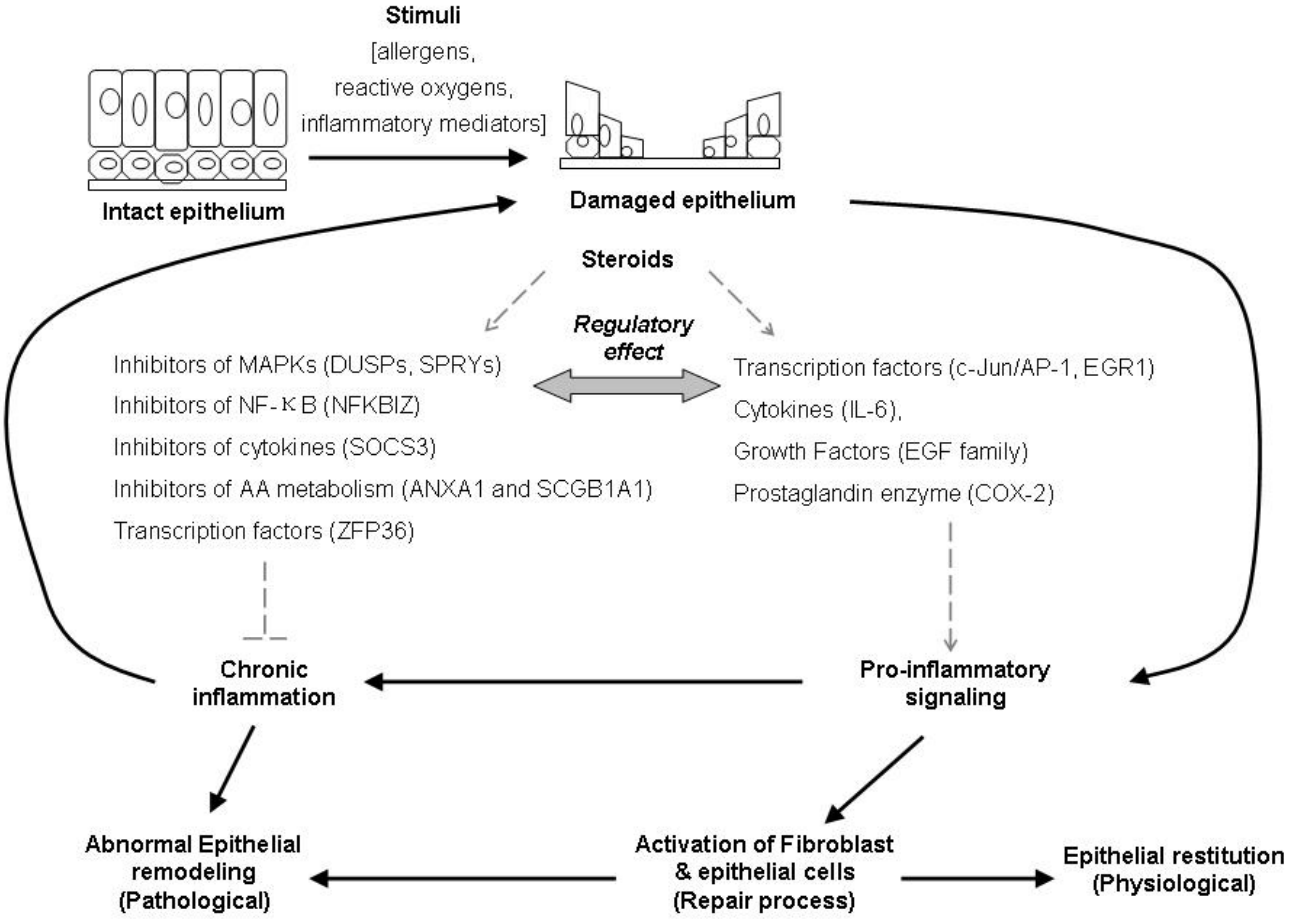
With regard to the GC effects on NP, we discussed two major findings: epithelial repair and anti-inflammatory function. AP-1 and AP-1 related genes respond to the growth effect on damaged NP epithelium and they are mainly involved in MAPK, IL-6, and arachidonic acid signaling pathways; however, most identified

anti-inflammatory genes negatively regulate this growth related signaling. Although these two effects seem opposite, they may represent a regulatory effect of GCs on the epithelial repair process. This is because wound healing itself is a process that involves both inflammation and the resolution of the inflammatory response, which culminates in remodeling.

Figure 6.21 summarizes the epithelial repair process under physiological and pathological conditions: the increase of pro-inflammatory signaling markers (like AP-1 and AP-1 related genes) in response to epithelial damage results in epithelial restitution, while if these growth signals are not controlled, it will cause chronic inflammation and then potentiate the epithelial injury, leading to abnormal epithelial remodeling. In this scenario, the increase of the inhibitors (like DUSPs, SPRYs, SOCS3, etc.) of these inflammatory signaling pathways would be considered to control the aberrant remodeling. Indeed, the histological evaluation of NP epithelium was concordant to the effects of these genes, and showed improvement of epithelial structure but without severe remodeling (e.g., squamous metaplasia).

Based on these observations, we hypothesize that the beneficial effects of GCs on epithelial healing may be at least in part, mediated by the induction of c-Jun/AP-1 and its related genes; and the promotion of anti-inflammatory effects of GCs could control the remodeling process of epithelium in a proper situation. Therefore, regulation of these gene markers would be useful in the resolution of NP inflammation, suggesting their potential therapeutic benefit.

Figure 6.21 Schematic representation of the epithelial repair process as well as its response to GC treatment in NP



6.2.6 Combination of eosinophil- and neutrophil-infiltration in NP

The general histopathological classification of NP is eosinophil-dominated inflammation (65-90%), which appears to be a hallmark of Caucasian NP [Fokkens et al., 2007]. However, in the Caucasian population, neutrophilic NP may only account for 15% to 20% of the studied cases [Bachert et al., 2003]. In contrast to the studies in Caucasians, recent studies reported a lower incidence of eosinophil-dominated inflammation in Asian population with tissue infiltration of neutrophils was as high as 40% [Jareoncharsri et al., 2002]. We have investigated the type of cellular inflammation in

Singaporean Chinese patients with NP. Our results indicated a combined cell infiltration with eosinophil, neutrophils and CD4+/CD8+ T lymphocytes [Hao et al., 2006]. Our recent findings lead to the following intriguing theory. Besides well-recognized eosinophil-dominated inflammation in Caucasian studies, predominant infiltration of other types of cells, especially neutrophils, could be also a key component underlying the pathogenesis of NP in Asian populations.

The current study confirmed that infiltration of eosinophils and neutrophils was evident in Chinese patients with NP. The concept that NP in Chinese population may be a separate entity from those NP in Caucasian population is an interesting postulation. However, it is also possible that NP in Chinese population may represent a whole constellation of disease states with differing etiologies that converge upon a common final pathway of clinical manifestation as those NP in Caucasian populations. The current knowledge about the pathogenesis of NP is insufficient to test this hypothesis as some Chinese patients may undergo medical management for their disease after it has been present for a long period of time, making it difficult to determine what the inciting factors may have been. We postulated that a specific cell type-associated genetic analysis of differential gene expression could be a relevant indicator of difference or similarities among various disease states in NP. The questions to be addressed in this research, therefore, were whether eosinophil- or neutrophil-associated gene expression differs in NP patients and, if so, how it differs.

6.2.6.1 Eosinophil-related genes in NP

In rhinosinusitis, with or without NP, eosinophils have accumulated in the nasal or paranasal sinuses and caused profound clinical effects. Important immunoregulatory

genes influence the function of eosinophils. By using the microarray approach, we were able to assess the gene expression levels of most candidate genes (n=60) which are associated with eosinophil biology from published reviews [Lampinen et al., 2004; Rothenberg, 1998; Rothenberg, 2006], as well as their response to GC treatment in the same study. Among those 60 important eosinophil-associated genes, expression levels of 31 genes were different in NP patients versus controls. Aberrant expression of those 31 genes has been associated with the migration and survival of eosinophils. Ten out of these 31 eosinophil-associated genes were down-regulated while 21 genes were up-regulated in NP when compared to controls (**Table 6.6,** *Page 142*). In addition, it is not surprising that we did not find any genes related to eosinophil development due to the tissue specificity of our study samples.

Hierarchical clustering indicates the relationships among samples (NP and control) with different histological patterns dependent on the expression of those 31 eosinophil-associated genes. The expression profile of eosinophil-associated markers was distinct between NP and control nasal mucosa due to their different patterns of eosinophil infiltration, (**Figure 6.16,** *Page 143*). In addition, two NP samples (NP6 and NP7) with weak eosinophil infiltration were not clustered tightly with the majority of NP, suggesting the expression profile may also be different between eosinophilic and non-eosinophilic NP. Note that the histological feature of NP6 represented edema with highly infiltrated lymphocytes, while the feature of NP7 represented increased number of glands without edema. These results suggest the correlation between the expression levels of these 31 genes and the extent of eosinophil infiltration.

Among those 31 eosinophil-associated genes, differential expression of 12 genes (MMP9, IL5RA, ITGB2, SELPLG, CCL11, LGALS9, LTA4H, ALOX5AP, PLA2G4A, CYSLTR1, LTB4R and NOS2A) in NP subjects versus controls have been reported previously [Chen et al., 2007; Gevaert et al., 2003; Hebestreit et al., 1998; Matsumoto et al., 2002; McNulty et al., 1999; Olze et al., 2006; Parnes, 2002; Ponath et al., 1996; Sousa et al., 2002; Woltmann et al., 2000]. As far as we are concerned, this is the first study suggesting that aberrant expressions of 19 eosinophil-associated genes are important aspects conditioning the severity and prognosis of NP. Perturbations of these 19 genes (ADAM8, CCL15, IL-18, C3, SCG2, CXCL12, IL13RA2, NR4A1, NR4A2, NR4A3, CD69, CD86, LYN, MIF, NFKBIA, DUSP1, CCL28, CD9 and CD40) have been implicated in many diseases associated with eosinophila, such as asthma [Adachi et al., 1999; DiScipio et al., 2007; Foerster et al., 2002; Hashida et al., 2007; Kobayashi et al., 2006; Matsuno et al., 2008; Yasunaga et al., 2003], allergic rhinitis [Korsgren et al., 2003; Sebelova et al., 2007], and atopic dermatitis [Kagaya et al., 2005]. These 19 genes exert critical role in regulating migration and survival of eosinophils. It has been shown that up-regulation of ADAM8, CCL15, IL-18, C3, SCG2 and down-regulation of CXCL12, IL13RA2 promote eosinophil migration into inflammatory tissues [DiScipio et al, 2007; Pardigol et al., 1998; Matsuno et al., 2008; Yasunaga et al., 2003]; while down-regulation of NR4A1, NR4A2, NR4A3, and CD69 suppress eosinophil apoptosis [Foerster et al., 2002; Hashida et al., 2007]. Moreover, up-regulation of CD86, LYN, MIF and down-regulation of NFKBIA, DUSP1 may facilitate activation signals in eosinophils [Adachi et al., 1999; Fujihara et al., 2005; Kobayashi et al., 2006]. Up-regulation of CD9, CD40, and CCL28 genes promotes migration and survival of eosinophils [John et al., 2005; Rothenberg et al., 2006].

IL-5 and CCL11 (eotaxin) have been frequently reported as the molecular markers for

eosinophils in NP [Hamilos et al. 1998; Olze et al., 2006]. IL-5 gene expression was undetectable in all samples in this study. However, IL-5Rα mRNA was significantly higher (9.85-fold by real-time PCR) in untreated NP as compared to controls, while it was not altered by GCs. Our result is in agreement with a recent study in which soluble IL-5Rα protein expression level was found to be dramatically higher (up to 1,200 times) than IL-5 concentrations in NP [Gevaert et al., 2003]. It has been found that the expression level of soluble form IL-5RA was associated with eosinophil infiltration and the concentrations of ECP and IL-5, indicating IL5RA levels were related to the disease severity [Gevaert et al. 2003]. Moreover, the expression level of IL5RA is important in relation to the sensitivity of anti-IL-5 treatment [Gevaert et al. 2003]. With regard to CCL11, its mRNA was significantly up-regulated in NP samples (14.9-fold) in comparison with the controls but it remained unchanged after oral GC therapy. It seems that the expression profiles of these two best-known gene markers of eosinophils are not in concordance to the reduction of eosinophils in NP after GC therapy, indicating other eosinophil-associated genes may respond to GCs.

6.2.6.2 Eosinophil-related genes in response to GC treatment in NP

GC is the most effective and the current standard treatment for NP [Fokkens et al., 2007]. Our data confirm that a 3-5 day oral GC treatment (30mg prednisone daily) could eventually reduce infiltration of eosinophils and tissue edema in NP. A total of 5 eosinophil-associated genes (MMP9, NR4A1, NR4A2, CD69 and DUSP1) were responsive to GC therapy (**Appendix II,** *Page 268*). These molecular changes correlate well with histological findings, such as reduced severity of tissue edema and eosinophil count in NP tissues, after oral GC treatment. In addition, a short-term oral GC therapy appears to be sufficient to normalize the expression of those genes.

In the literature, a number of research data have already confirmed the biological functions of these five genes in relation to eosinophils. MMP9 is a matrix metalloproteinase that promotes eosinophil migration and tissue edema in airway inflammation which was discussed in **Chapter 6.2.4.3** (*Page 184*). DUSP1 potently inhibits MAPK mediated inflammatory signal pathways in activated eosinophils [Lasa et al. 2002; Wong et al. 2002], and the role of DUSP1 was also discussed in **Chapter 6.2.2**.5 (*Page 165*).

NR4A nuclear receptor members are immediate-early genes, which have been shown to play a key role in regulating expression of various genes related to inflammation [Winoto et al., 2002]. Recent review suggested that the NR4A receptor family was important in causing eosinophil apoptosis, because CD30 stimulation which induced eosinophil-specific apoptosis, enhanced expression of NR4A1, NR4A2 and NR4A3 [Hashida et al., 2007]. We showed that NR4A1 and NR4A2 expression was increased after GC treatment in NPs, but NR4A3 expression was too low to detect in all NP samples. Hence, it indicates that transcription of NR4A1 and NR4A2 may be controlled by external stimuli (e.g. GCs) and it may participate in the GC-induced apoptosis of eosinophils in NP.

Although the expression of CD69 was induced by pro-inflammatory cytokines (e.g. IL-3, IL-5 and GM-CSF) [Hartnell et al., 1993] in activated eosinophils, the function of CD69 is to transduce a death signal, leading to eosinophil apoptosis [Foerster et al., 2002]. Our results showed that CD69 was up-regulated in response to GC treatment in NP, suggesting that GCs may facilitate its apoptosis effect on eosinophils via CD69

signaling transduction.

As discussed above, GCs may perform the effects on eosinophils by regulating the expression of some genes which related to eosinophil migration and survival. Although most of the other eosinophil-related markers we found did not respond to GC treatment, it does not exclude the potential effects of GCs on these markers, since the reduction of eosinophil density itself would confuse the detection of eosinophil-related genes.

6.2.6.3 Genes related to neutrophil infiltration in NP

Recently, neutrophils were detected in the tissue of NP but their pathophysiological relevance is unclear. We have reported that a combination of eosinophils, neutrophils and CD4+/CD8+ T lymphocytes is a common feature of NP and adjacent middle turbinate mucosa in Asian patients [Hao et al., 2006]. In the current study, we have assessed the expression level of important neutrophil-associated genes, together with its cellular count in NP. Our results demonstrate that: (1) infiltration of neutrophils is common (66.6%) in NP patients; (2) among the 76 important neutrophil-associated genes [Borregaard et al., 2007; Kobayashi et al., 2006; Theilgaard et al., 2005], the expression level of 14 genes (10 up-regulated and 4 down-regulated) were different in NP patients versus controls (**Table 6.7,** *Page 145*). Among those 13 differentially expressed neutrophil-associated genes, 4 genes (MMP7, MMP9, ITGB2, DEFB1 and PTX3) have been shown to promote neutrophil infiltration in NP [Baruah et al., 2007; Can et al., 2008; Chen et al., 2007; Lee et al., 2002; McNulty et al., 1999]. In addition, the up-regulation of CXCL2, CXCL6, IFNAR1, LGALS8, ITGB2 and down-regulation of CXCL12 have been suggested to promote neutrophil migration [Borregaard et al., 2007;

Kobayashi et al., 2008; Nishi et al., 2003]. The up-regulation of SERPINA1, CEACAM1, CEACAM6, MMP7 and MMP9 may facilitate neutrophil adhesion and degranulation [Borregaard et al., 2007; Kobayashi et al., 2008; Swee et al., 2008; Theilgaard et al., 2005]; DEFB1 promotes neutrophil survival [Borregaard et al., 2007]; and CRISP3 and PTX3 are associated with neutrophil-mediated innate immune defense [Baruah et al., 2007; Borregaard et al., 2007]. There is little effect of GCs on neutrophil-associated gene expression. Oral prednisone was only effective in modulating expression levels of 3 neutrophil-associated genes (MMP7, MMP9 and CXCL2) and had no significant effect on neutrophil recruitment.

6.2.6.4 Relationship between eosinophil and neutrophil infiltration in NP

Our results suggest that infiltration of eosinophils and neutrophils is a common feature in Chinese patients with NP. However, the pathophysiological interactions between eosinophils and neutrophils in NP are not fully understood. Recent results suggest that eosinophils and neutrophils may share similar pathobiological processes in inflammatory tissues and may cross-talk to facilitate the recruitment of one to the others. Neutrophil-mediated pro-inflammatory cytokines such as IL-8, IL-1β, and TNF-α may contribute to allergic inflammation by inducing the influx and degranulation of eosinophils [Borregaard et al., 2007; Kobayashi et al., 2008; Lamblin et al., 1998]. Neutrophils were suggested as a carrier of eosinophil cationic protein (ECP) and correlated with ECP level [Sur et al., 1998]. In addition, some reported eosinophil-associated genes (e.g., SELPLG, ITGB2, ADAM8, MMP9, CCL11, LTB4R, CYSLTR1, LTA4H, ALOX5AP, PLA2G4A, C3, CXCL12, NOS2A, MIF, LYN, CD69, DUSP1, NFKBIA, CD40) have also been reported to be effective on neutrophil migration and survival [Asberg et al., 2008; Corry et al., 2004; Fazal et al., 2002;

Gabbay et al., 1999; Khan et al., 2006; Kobayashi et al., 2006; Symon et al., 1996; Matsuno et al., 2008; McNulty et al., 1999; Nopp et al., 2002; Sampson, 2000; Wang et al., 2007; Ward et al., 1999]. In the future, it may be necessary to combine these two cell types in studies of inflammatory disorders (especially in NP), which have a predominant infiltration of these cells.

With a combined assessment of both cell types, our results suggest that GCs are effective in inhibiting eosinophil infiltration and decreasing the size of polyps and edema but their effect on neutrophils is not established. Our results are consistent with several independent studies in which GCs induce apoptosis in eosinophils, but have no effect on neutrophil recruitment [Burgel et al., 2004; Meagher et al., 1996; Meltzer et al., 1994; Sampson, 2000]. It raises an interesting question as to whether these unchanged expression levels of most neutrophil-associated genes underlie such phenomenon or whether neutrophils could counteract the beneficial effects of GC therapy on allergic inflammation [Benson et al., 2000]. It is an important area of research as there are quite a substantial number of NP patients who appears to be refractory to GC therapy; and therefore, the roles of those neutrophil-associated genes are worth further investigation.

6.2.6.5 Conclusion of eosinophil- and neutrophil- assocaited genes in NP

In summary, we have profiled eosinophil- and neutrophil-associated genes and their response to GC treatment in Chinese patients with NP. Clustering of a longitudinally derived data set provided a detailed picture of the gene expression patterns induced and/or suppressed in the nasal tissues during various stages of NP. In particular, the changes in expression profiles of MMP9, NR4A1, NR4A2, CD69 and DUSP1 appear to be correlated to the short-term oral GC therapy and are associated with reduction of

local tissue infiltration of eosinophils and edema in NP. This is the early step in learning the molecular events of inflammatory cell infiltrations in NP. Subsequent work is needed to localize these gene expression levels in specific cell types (e.g., eosinophils and neutrophils) and to determine their relationship with the bio-physiological functions of these infiltrating cell types.

6.2.7 Other gene families associated with pathogenesis of NP

In addition to the eosinophil/neutrophil-associated genes, the present microarray study also observed a variety of gene families which potentially contribute to the pathogenesis of NP by literature reviews. These gene families include the oxidant/antioxidant-related genes, edema-related genes, and mucin genes.

6.2.7.1 Oxidant/antioxidant-related genes in NP

Airways are exposed to high levels of environmental oxidants, yet they also have enriched extracellular antioxidants. Airways disease such as asthma, cysitic fibrosis, and chronic obstructive pulmonary disease have evidence of increased oxidative stress, suggesting that reactive oxygen (ROS) and nitrogen species (RNS) may overwhelm antioxidant defenses in these diseases. ROS include superoxide, hydrogen peroxide, and hydroxyl radical; while RNS include nitric oxide (NO) and its derivates such as nitrogen dioxide and peroxynitrite. Some key enzymes underlie the mechanisms of generation of ROS and RNS, including myeloperoxidase, NADPH oxidase, and nitric oxide synthases. While in response to oxidative stress, antioxidants are synthesized by their related enzymes including the families of superoxide dismutases (SODs), catalase, glutathione peroxidases (GPXs), glutathione S-transferasaes (GSTs), thioredoxins (TXN) and peroxiredoxins (PRDXs).

An imbalance of oxidants (e.g. ROS & RNS) and antioxidants has been regarded to play a role in the pathogenesis of NP. Eosinophils, neutrophils, macrophages, and lymphocytes are highly infiltrated in NP tissue and these inflammatory cells are considered to produce extensive ROS/RNS, which cause cellular and tissue damage [Dogru et al., 2001; Kang et al., 2004]. Dagli et al. reported a significant negative correlation between free radical-mediated peroxidation products and antioxidants in NP, suggesting high free-radical levels in NP can be related to reduced antioxidant levels or consumption of antioxidants by excess free radicals [Dagli et al., 2004]. Several oxidant/antioxidant related enzymes were found to change in GC-naïve NP as compared to controls in the current study.

(1) Oxidant-related enzymes

Our results showed up-regulation of some common oxidant-related enzymes (NOS2A, NOX4, and DUO1) in GC-naïve NP (**Appendix II,** *Page 268*). As discussed before (**Chapter 6.2.6,** *Page 189*), NO induced by NOS2A could disrupt Fas receptor-mediated apoptosis in eosinophils in NP [Hebestreit et al., 1998]. NADPH oxidase 4 (NOX4) and Dual oxidase 1 (DUO1) are the major NADPH oxidases expressed in airway epithelium and also in eosinophils/neutrophils. The principal function of NOX4/DUO1 is to catalyze transmembrane transfer of electrons form the cytosolic electron donor NADPH to the electron acceptor – O_2, leading to the production of O_2^- [Geiszt et al., 2004]. O_2^- has a very short half-life due to its rapid reaction with other radical species and its spontaneous formation of hydrogen peroxide (H_2O_2). Therefore, increases of NADPH oxidases can increase the release of ROS from epithelial cells and eosinophils/neutrophils, leading to damage of nasal

epithelium. In addition, NADPH oxidase enzymes are also involved in inflammatory signaling in airway diseases. For example, the Th2 cytokines could induce epithelial expression of DUOX1 in allergic asthma [Harper et al., 2005], and DUOX1 was associated with airway acidification [Schwarzer et al., 2004]. Both NOX4 and DUO1 have been suggested to induce overexpression of mucin 5AC (MUC5AC) in chronic inflammatory airway diseases with mucus hypersecretion [Kim et al., 2008; Shao et al., 2005].

(2) Antioxidant-related enzymes

Several antioxidant-related genes including oxidation resistance 1 (OXR1), superoxide dismutase 3 (SOD3), glutathione peroxidases 3 (GPX3) and lactoperoxidase (LPO) were down-regulated in GC-naïve NP (**Appendix II,** *Page 268*). OXR1 localizes to the mitochondria, and can prevent and repair oxidative DNA damage by detoxification of ROS, leading to protection against spontaneous mutagenesis and cell death [Volkert et al., 2000].

SOD3 belongs to the superoxide dismutases (SODs) family, which potently counteract against superoxide radicals. SODs not only catalyzes the dismutation of two superoxide radicals into H_2O_2 and O_2 [Zelko et al., 2002], but also modulates nitric oxide-mediated signaling associated with bronchodilation [Jonsson et al., 2002]. SOD3 is distributed in extracelluar regions, and abundant in airway epithelium [Su et al., 1997]. Thus, SOD3 can protect airway tissues from oxidative stress and lead to reduction of airway hyper-responsiveness [Assa'ad et al., 1998]. Decreased expression and activity of SOD3 was found in asthma and NP, indicating the excessive ROS/RNS injury in these airway diseases [Cannady et al., 2007; Comhair et al., 2005;].

Following SODs dismutase two superoxide radicals into H_2O_2, the glutathione system can decompose organic and inorganic hydroperoxides. GPX3 belongs to the GPX enzyme family which requires reduced glutathione to serve as the electron donor [Comhair et al., 2002]. GPX3 is the plasma GPX that has been detected in the epithelial lining fluid of the human lung [Comhair et al., 2001]. GPXs not only reduce ROS (e.g., H_2O_2 and O_2^-), but also protect against nitric oxide-mediated protein oxidation by regulation of RNS (e.g., peroxynitrite) levels in airway inflammatory diseases [Comhair et al., 2002].

LPO is another major contributor to H_2O_2 consumption. LPO uses H_2O_2 to oxidize the anion thiocyanate to an antibiotic compound (hypothiocyanate) that prevents growth of bacteria, fungi, and viruses in airway tissues. Unlike other members of this peroxidase group, including myeloperoxidase (MPO) (present in neutrophil and monocytes) and eosinophil peroxidase (EPO), LPO is mainly present in epithelial cells. Most importantly, LPO generated hypothiocyanate is a weak oxidizing agent and is not as toxic as the hypochlorite species produced by MPO and EPO. Hence, it may provide less damaging antimicrobial activity than the recruitment of granulocytes to prevent the airway mucosa from infection.

However, some antioxidant enzymes were up-regulated in GC-naïve NP, including GCLM, TXN, PRDX1, and PRDX5. The increase of these antioxidant enzymes in NP could be a compensatory mechanism to prevent tissue damage. However, up-regulation of only these four enzymes may not be sufficient to scavenge the increased ROS/RNS in NP as it may not compensate for the decreased levels of other

antioxidant enzymes (OXR1, SOD3, GPX3, and LPO) and increased levels of oxidases (NOS2A, DUO1, and NOX4).

(3) Oxidant/antioxidant-related enzymes in response to GC treatment

GCs appear to affect both antioxidant and oxidant levels during airway inflammation [De Raeve HR et al., 1997; Dweik et al., 1997; Rocksén et al., 2000]. However, our results demonstrated that only GPX3 responded to GC therapy, indicating the important roles of the glutathione system on prevention of oxidative stress in NP, leading to reduction of tissue damage. Whether GCs have beneficial effects on antioxidants in NP remain to be further investigated. Nevertheless, our study reviewed groups of oxidant/antioxidant related enzymes in NP, indicating the imbalance of oxidant and antioxidant levels may increase the oxidative burden in NP and then contribute to epithelial damage in NP.

6.2.7.2 Genes related to edema in NP

Stromal edema is the typical histopathological feature of NP. Two gene families were found to be associated with edema formation in NP, including bioelectric genes and angiogenesis-related genes.

(1) Bioelectric genes

Airway epithelial cells maintain the volume and composition of airway surface liquid via regulation of transepithelial ion transport [Boucher, 1994], i.e., water transport across the epithelial tissues (secretion and absorption) is regulated by anion (Cl^-) secretion and/or cation (Na^+) absorption. The permeability of Cl^- is regulated by chloride channels and their regulators; while Na^+ enters the epithelial cells via sodium

channels in the apical surface, and then it is “pumped out” via the basolateral surface by Na-K-ATPase.

As introduced in **Chapter 1.4.7** (*Page 23*), deregulation of fluid and electrolyte transport has been considered as one hypothesis of edema formation in NP [Bernstein et al., 1997]. Our results showed an up-regulation of the nonvoltage-gated sodium channel (SCNN1A, SCNN1B, and SCNN1G) and chloride intracellular channels (CLIC3, CLIC5, and CLIC6), but down-regulation of Na-K-ATPase in NP compared to controls. The sodium channels (SCNNs) were reported to be expressed higher in NP as compared to paranasal sinus mucosa and the correlation of the Na^+ absorption with mRNA expression of sodium channels was significant [Yasuda et al., 2007a]. An increase of activation of CCLs in NP would enhance anion (Cl^-) influx and consequently this hyperpolarization would increase the Na^+ absorption [Yasuda et al., 2007b]. The sodium pump (Na-K-ATPase) located on the basolateral surface of the epithelial cell transports ions by consuming ATP and pumping Na^+ out of the cell in exchange for K^+ influx to maintain Na^+ and K^+ gradients [Stern et al., 2000]. The expression patterns of these ion channels/enzymes suggest that the increase in sodium absorption and chloride permeability across the cells in NP would allow water to be absorbed through the epithelium into the interstitial space and may account for the edema in NP.

Although GC treatment significantly reduced the edema in NP samples, we did not find the expression of these ion channel-related genes was changed by GCs. It is possible that GCs may indirectly regulate the ion channel/enzyme activity instead of their expression levels or there may be an alternative way that GCs reduce tissue edema.

(2) Angiogenesis-related genes

Angiogenesis is the result of a highly orchestrated series of molecular and cellular events, resulting in the migration, proliferation, and differentiation of endothelial cells into newly formed capillaries that can subsequently develop into more mature vessels. Angiogenesis-related genes are not only associated with vascular development, but are also involved in the formation of edema in airway inflammatory diseases (such as asthma and NP) [Jones, 2003]. For example, an increase of VEGF was found to be associated with microvascular permeability in asthma and NP [Gosepath et al., 2005; Lee et al., 2008; Muluk et al., 2007]. Our results found there was a down-regulation of several angiogenesis-related genes in GC-naïve NP, including ANGPT1 and ANGPT2 (from angiopoietin family) and their receptor (TEK).

The angiopoietin family and its related receptor (TEK) are essential in regulating vascular development and maintenance of vascular integrity [Jones, 2003]. More interestingly, the ANGPTs/TEK signaling pathway plays an important role in modifying the inflammatory response in airway inflammation, especially inhibition of vascular permeability [Fiedler & Augustin., 2006; Kanazawa et al., 2007]. To reduce vascular permeability, ANGPTs/TEK interacts with integrins to stabilize tight cell-cell adhesion among endothelial cells and counteracts vascular endothelial growth factor (VEGF)-mediated leakage effects [McDonald, 2001]. Although GCs significantly relieve edema in NP, none of these angiogenesis associated genes were altered after GC treatment. Whether the GC effects on vascular permeability in NP was related to alteration of the angiogenesis genes needs to be further clarified. Nevertheless, reduction of ANGPTs/TEK may contribute to the increase of vessel leakage, leading

to mucosal edema in NP.

6.2.7.3 Mucin genes in NP

Mucus is the layer that covers, protects, and lubricates the luminal surfaces of epithelial respiratory, gastrointestinal, and reproductive tracts. Airway mucus is composed of water, ions, lung secretions, serum protein transduates, anti-microbial proteins and mucus glycoproteins (mucins). The viscoelastic properties of mucus are mainly determined by the presence of mucins that are high molecular weight proteins extensively synthesized and secreted by submucosal glands and epithelial goblet cells [Rose et al., 2001]. Genes encoding for 19 human mucin proteins and the mucins have been categorized in two distinct families [Moniaux et al., 2001]: (i) secreted mucins (e.g. MUC2, MUC5AC, MUC5B, and MUC7); (ii) membrane-bound (surface) mucins (e.g. MUC3, MUC4, MUC16, and MUC20).

Mucus hypersecretion as well as mucus obstruction is one of the main symptoms of NP. Several mucin genes such as MUC4 and MUC5 which have been studied in NP and submucosal glands have been considered to play a major role in mucin gene expression in NP [Ali et al., 2005; Ali and Pearson, 2007; Martínez-Antón et al., 2006]. In our study, we found three cell surface-associated mucin genes (MUC4, MUC16, and MUC20) were up-regulated, but one secreted mucin gene (MUC7) was down-regulated in GC-naïve NP compared to controls.

MUC4 is commonly expressed in airway epithelium from both healthy and diseased tissues [Ali et al., 2005; Martínez-Antón et al., 2006]. However, Ali *et al.* showed that MUC4 was rarely expressed in submucosal glands from control nasal mucosa, but was

extensively expressed in enlarged glands from NP [Ali et al., 2005]. MUC16 has been well known in epithelial origin cancer (such as ovarian carcinoma) [Yin and Lloyd, 2001] and been found to be expressed in tracheal surface epithelium and submucosal glands in the bronchial epithelial cells [Hattrup and Gendler, 2008]. MUC20 is a novel mucin marker and up-regulation of MUC20 was involved in renal injuries [Higuchi et al., 2004]. Our H&E staining results showed clearly different patterns of gland structure between NP (dilated glands) and controls (small branched glands) (**Figure 6.1,** *Page 91*) and thus increase of MUC4, MUC16, and MUC20 expression may be attributed to this histological difference.

In contrast to those membrane-bound mucins, MUC7 is an unusually small secretory mucin, sharing no homology with other MUC proteins [Bobek et al., 1993]. Previous studies reported that MUC7 was exclusively detected in submucosal glands from healthy nasal mucosa but not from NP. MUC7 was not expressed in the epithelial region of airway tissues [Ali et al., 2005; Martínez-Antón et al., 2006]. Therefore, the reduced MUC7 content in NP may be associated with the increase of mucin secretion, indicating the enhancement of mucus in the lumen of hypersecretion glands.

Although GCs significantly suppress eosinophil infiltration in NP and effectively decrease polyp size, we did not find that GCs have any effect on mucin expression. Our results are in line with the previous study which also found no response of MUC5AC to GCs in NP [Burgel et al., 2004]. We found that the extent of submucosal glands was not significantly different in NP before and after GC treatment, indicating GCs may not inhibit mucus hypersecretion in NP. Therefore, the unchanged expression of mucins may be due to the lack of effect by GCs on the extent of glands

in NP.

Part III Conclusion

We have explored the molecular mechanisms underlying the pathogenesis of NP and its response to GC treatment. Utilizing the pathway analysis, down-regulation of the AP-1 network, anti-inflammatory gene network, EGF/EGFR signaling, PGE_2 signaling, IL-6 signaling, up-regulation of apoptosis signaling, complement system, leukotriene signaling, ERK/MAPK signaling, and NF-kappaB signaling have been considered to contribute to NP pathogenesis.

Our data suggest that AP-1 (especially c-Jun) and its related gene network are central molecular effectors of epithelial damage and repair in NP, which can be modulated by GC treatment. Moreover, the anti-inflammatory effect of GCs is considered to up-regulate the expression level of negative regulators among the inflammatory signaling pathways and down-regulate the pro-inflammatory genes in NP. The interaction and relation between AP-1/AP-1 related genes and anti-inflammation-related genes are documented. The dramatic modulatory effects of GCs on NP epithelium could also be readily demonstrated in histological observation. Hence, we provided *in vivo* evidence to support the notion that the beneficial effects of GCs in NP may be related to its ability to promote epithelial repair without uncontrolled remodeling (i.e., anti-inflammatory function), potentially via its regulation of the AP-1 gene network.

Combined infiltration of esoinophils and neutrophils as well as edema is prominent in the stroma of NP from Chinese patients. GCs potently suppress eosinophils and

edema in NP, but have no effect on neutrophil infiltration. We have profiled eosinophil- and neutrophil-associated genes based on reports in the literature. Although only a few of these genes responded to GC treatment, their expression patterns are thought to be associated with eosinophil- and neutrophil-infiltration in NP. In addition, the cross-relationship of these genes in eosinophil and neutrophil biological process indicates the potential interaction of these two common inflammatory cell types in NP. Other gene families such as oxidant/antioxidant-related genes, edema-related genes, and mucin genes have also been reviewed in this study. These genes are regarded to be associated with some histological features of NP, such as tissue damage, edema, and mucus hypersecretion.

Chapter 7. Conclusions and Suggested Future Studies

7.1 Summary of important findings

This thesis studies the molecular mechanisms underlying the pathogenesis of NP and its response to GC treatment. Although negative results which were not anticipated initially came out in the superantigen and methylation studies, they eventually provided valuable information as to the pathogenesis of NP. The presence of *S. aureus* as well as its superantigens may not be significant in Asian NP, indicating the different pathological profiles underlying Asian and Caucasian NP. Our study demonstrates for the first time that methylation of common TSGs can be detected in NP as well as control nasal mucosa, indicating that the role of methylation of these TSGs in nasal mucosal inflammation appears to be minimal.

The most significant findings in this thesis are the identification of NP associated genes and the GC responsive genes as well as their related network pathways in NP based on the advanced microarray analysis methods. As far as we know, it is the first study to investigate the gene expression profiles and their interactive networks in NP before and after oral GC treatment and to systemically explore the NP associated genes in Chinese NP in a genome-wide manner. There are two main approaches to identify the interested genes, the IPA pathway analysis and literature review.

Pathway analysis revealed that down-regulation of AP-1 network, anti-inflammatory gene network, EGF/EGFR signaling, PGE_2 signaling, and IL-6 signaling and up-regulation of apoptosis signaling, complement system, leukotriene signaling, ERK/MAPK signaling, and NF-kappaB signaling would be involved in NP pathogenesis. GCs are considered to perform two major beneficial effects in NP:

epithelial repair and anti-inflammatory function, leading to a proper epithelial restitution. AP-1/AP-1 related genes are supposed to promote epithelial remodeling from the damaged NP epithelium; while the regulation of inflammatory related genes (especially the negative regulators of inflammatory signaling) are supposed to control aberrant epithelial remodeling. So these two types of the genes would contribute to the regulatory effect of GCs on epithelial repair. AP-1 (especially c-Jun) is considered the core gene underlying these GC-mediated beneficial effects. Although the actual role of these genes and gene interactions in the pathway picture requires careful functional evaluation, the network may contribute to understanding the key biological functions and pathways that are altered during GC treatment in NP.

Based on the literature review, eosinophil- and neutrophil-associated genes were applicable to differentiate eosinophilia and neutrophilia in nasal samples, and were proposed to participate in the biological process of these two major inflammatory cell types in Chinese NP. In addition, the histopathological features of NP are also attributed to the change of other genes/gene families in NP, such as oxidant/antioxidant related genes, edema related genes, and mucin genes. Therefore, these disease-related genes supply a comprehensive molecular profile for Chinese NP, and such information will ultimately prove useful for researchers in providing new insight into the pathogenesis and treatment of NP.

7.2 Limitation of the current study

Although we tried to reduce the potential confounding/variation factors during the experiment, there are several limitations to this thesis that could be rectified in future investigations. We have recruited a relative small number of NP and control subjects

in this gene expression study. Subsequent follow-up studies using a large pool of patients are needed to verify the important findings. Another potential shortcoming is the analysis of NP versus nasal mucosal control. Since there are various cell types in NP but not in control tissues, some of the findings may reflect the differences in the cellular makeup of the tissues. Such limitation can be improved by using the advanced microdissection technique to isolate the cell type of interest. An additional limitation of the current study is that the functional networks and their essential gene-gene interaction is deduced by an array modeling approach, which is based on a predetermined database knowledge and can only be considered as source of hypotheses. Therefore, functional testing will be needed for the rigorous evaluation of individual molecular interactions inferred by our database approach.

7.3 Suggestions for the future work

Our gene expression study provided a large amount of valuable information potentially associated with NP pathogenesis and the response of NP to GC treatment. Considering that the current study is a descriptive observation, future studies (especially functional studies) should be used to follow up this project.

7.3.1 Future study for gene expression profiles in NP in response to nasal GCs

Nasal (topical) GC is recommended the first-line treatment of NP [Fokkens et al., 2007]. Data generated from this study (with oral GCs) provide reference information for the evaluation of the effect of nasal GCs in the treatment of NP. Therefore, a study with similar design using nasal GCs will be performed in future. The comparison of the gene expression profile between oral and nasal GCs in NP treatment will give useful information to identify drug target genes.

7.3.2 Future study for AP-1 and AP-1 gene network

Our study identified AP-1 and its gene network as the central effectors underlying the GC effects on NP, which mainly contribute to proper epithelial repair. To specifically target the epithelial cells, epithelium of NP could be microdissected from histological sections. Then expression of AP-1/AP-1 genes could be determined in a cell specific manner. *In vitro* functional study is also worth carrying out regarding the response of AP-1 and its mediated network to GC treatment. Epithelial cells can be isolated from fresh NP tissues and then treated with GCs; consequently expression of AP-1 genes can be determined at different time points. Moreover, those known AP-1 upstream activators/inhibitors and AP-1 downstream target genes along the AP-1 signaling pathways can be also evaluated. Factors related to epithelial repair (e.g., cell proliferation and migration) are evaluated. If the correlation between epithelial cell and AP-1/AP-1 related genes is confirmed, RNA interference technique can be applied to specifically knock down the expression of AP-1 genes and confirm the necessary roles of AP-1 in epithelial biological process.

7.3.3 Future study for the anti-inflammatory genes in NP

In response to GC treatment, a group of negative regulators (e.g., DUSPs, SPRYs, IKappaB, SOCS3, ANXA1, ZFP36, and SCGB1A1) of the inflammatory signaling pathways have been identified in our array study. This finding indicates one important mechanism of GCs in controlling the inflammatory response in NP. Again, a future study could target on this epithelial region. Epithelium can be microdissected from NP and expression level of these anti-inflammatory genes can be determined. Epithelial cells also can be isolated from NP for *in vitro* functional analysis. Epithelial cells can

be treated with GCs and the expression levels (both RNA and protein level) of these genes can be detected. Moreover, the expression and activity (or say phosphorylation status) of those inflammatory signaling factors (such as MAPKs, NFkappaB) can also be evaluated. Therefore, the activators and regulators in these major inflammatory pathways in response to GC treatment can be profiled in an *in vitro* cell model.

7.3.4 Future study for eosinophil- and neutrophil-associated genes

Our study reviewed series of eosinophil- and neutrophil-associated genes in NP. Some of them have not been reported in previous NP study. We will confirm the protein expression level of these genes first and try to localize these genes in specific cell types (e.g., eosinophils and neutrophils). In *in vitro* analysis, the cells can be isolated from NP tissues and the expression levels (both RNA and protein) can be assessed. Among the eosinophil- and neutrophil-associated genes of interest, specific gene knock-down methods (e.g., RNAi) for the over-expressed ones or gene induction methods for the under-expressed ones in NP can be performed in the cell model. Consequently, correlation analysis can be applied to evaluate the relation between eosinophil/neutrophil amount and the change of gene expression level in a functional manner.

7.3.5 Future study for cancer-related genes in NP

In the functional analysis by IPA, the most significant disease associated with NP related genes is cancer. Since some of the clinical characters of NP (e.g., high recurrence rate) is similar to tumor growth, whether NP is a benign neoplasm or only an inflammatory lesion has been discussed for a long time. Therefore, the observation of numerous cancer-related genes in NP in our current study indicates there could be

some potential link between the NP and tumor with regard to their pathogenesis. To study some cancer related mechanism in NP, methylation of TSGs is considered an interesting area to start. This is because we already found methylation of some TSGs in NP. In future work, we will focus on the epithelial region due to two reasons: (i) hyperplasia of NP epithelium (e.g., squamous metaplasia) may have some similar properties to the epithelium from tumors; (ii) epithelial cells are available to microdissect for downstream analysis in a cell-specific manner. In this case, DNA can be extracted from the microdissected epithelial cells, and the methylation profile of cancer-related genes can be assessed by using an array-based platform. The genes with different methylation status in NP as compared to controls are selected. The expression level of these interested genes is consequently evaluated. If the negative correlation between expression level and methylation status of the indicated gene is determined, this gene is regarded as a methylation candidate in NP. Furthermore, the relation between these methylation candidates and histopathological features or even prognosis (such as recurrence rate) of NP can be evaluated.

In summary, the follow-up studies proposed here will open a new area in investigating NP pathogenesis and its response to GC treatment. It is ultimately useful to understand the beneficial roles of GCs in NP and develop some novel therapeutic approach for the treatment of NP.

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Appendices

Appendix I Significant functions of the datasets

GC-naïve NP versus control

Category	Significance *p*-value*	Genes [n]
Diseases & Disorders		
Cancer	2.82E-25 to 1.26E-03	554
Developmental Disorder	2.08E-11 to 3.46E-04	135
Inflammatory Disease	1.30E-09 to 1.26E-03	188
Connective Tissue Disorders	3.59E-09 to 8.06E-04	168
Cardiovascular Disease	1.51E-08 to 1.26E-03	130
Immunological Disease	6.29E-06 to 1.21E-03	152
Respiratory Disease	1.47E-05 to 1.26E-03	112
Organismal Injury and Abnormalities	4.73E-05 to 8.01E-04	61
Genetic Disorder	4.86E-05 to 1.26E-03	160
Physiological System Development & Function		
Tissue Development	2.59E-15 to 1.26E-03	270
Organismal Development	3.70E-12 to 5.66E-04	210
Cardiovascular System Development and Function	6.97E-12 to 1.26E-03	154
Tissue Morphology	4.39E-08 to 1.26E-03	227
Organismal Survival	1.51E-06 to 5.82E-06	140
Organ Development	8.95E-06 to 7.63E-04	145
Tumor Morphology	1.47E-05 to 1.26E-03	66
Immune Response	2.20E-05 to 9.57E-04	106
Connective Tissue Development and Function	2.96E-05 to 1.26E-03	142
Organismal Functions	2.45E-04 to 2.45E-04	19
Molecular & Cellular Functions		
Cellular Movement	6.51E-15 to 8.89E-04	282
Cellular Growth & Proliferation	9.88E-13 to 1.24E-03	456
Cell Death	6.74E-12 to 1.19E-03	411
Cellular Development	1.41E-10 to 1.26E-03	336
Cell morphology	1.56E-08 to 1.26E-03	230
Cell Cycle	4.14E-09 to 1.26E-03	197
Cellular Assembly and Organization	9.50E-08 to 1.26E-03	155
Gene Expression	1.80E-06 to 1.26E-03	268
Cell-to-Cell Signaling and Interaction	1.94E-06 to 6.87E-04	122
Cellular Function and Maintenance	2.26E-04 to 1.21E-03	45

GC-treated NP versus control

Category	Significance *p*-value*	Genes [n]
Diseases & Disorders		
Cancer	2.14E-12 to 1.38E-02	317
Developmental Disorder	2.83E-11 to 9.51E-03	98
Cardiovascular Disease	8.28E-06 to 1.20E-02	72
Genetic Disorder	4.57E-05 to 1.38E-02	120
Respiratory Disease	3.36E-04 to 1.38E-02	60
Organismal Injury and Abnormalities	1.27E-03 to 1.38E-02	30
Inflammatory Disease	4.73E-03 to 1.00E-02	101
Immunological Disease	5.18E-03 to 5.18E-03	4
Physiological System Development & Function		
Tissue Morphology	1.03E-08 to 1.18E-02	75
Tissue Development	1.07E-06 to 1.38E-02	165
Cardiovascular System Development and Function	1.03E-04 to 1.38E-02	91
Organismal Development	1.23E-04 to 1.29E-02	122
Organ Development	2.22E-04 to 1.38E-02	132
Immune Response	3.03E-04 to 1.38E-02	65
Immune and Lymphatic System Development	3.03E-04 to 1.38E-02	75
Organ Morphology	7.93E-04 to 1.38E-02	48
Connective Tissue Development and Function	1.01E-03 to 1.18E-02	64
Tumor Morphology	1.55E-03 to 1.33E-02	22
Molecular & Cellular Functions		
Cellular Movement	6.23E-08 to 1.38E-02	176
Cell Morphology	8.00E-06 to 1.38E-02	151
Cellular Assembly and Organization	2.92E-05 to 1.38E-02	112
Cell Signaling	3.38E-05 to 1.38E-02	73
Cellular Growth & Proliferation	5.00E-05 to 1.38E-02	266
Cellular Development	6.93E-05 to 1.38E-02	190
Cell-to-Cell Signaling and Interaction	7.56E-05 to 1.38E-02	101
Lipid Metabolism	1.1E-04 to 9.51E-03	15
Small Molecule Biochemistry	1.1E-04 to 1.38E-02	64
Cell Death	1.30E-04 to 1.33E-02	241

GC-treated versus steroid naïve NP

Category	Significance *p*-value*	Genes [n]
Diseases & Disorders		
Inflammatory Disease	5.43E-15 to 1.86E-04	33
Cancer	1.24E-12 to 2.58E-04	51
Cardiovascular Disease	2.37E-12 to 1.73E-05	23
Connective Tissue Disorders	8.03E-12 to 1.86E-04	33
Immunological Disease	1.78E-10 to 1.23E-04	29
Organismal Injury and Abnormalities	1.47E-08 to 2.39E-04	16
Genetic Disorder	9.19E-07 to 9.19E-07	7
Respiratory Disease	3.44E-05 to 1.15E-04	6
Developmental Disorder	1.10E-04 to 1.10E-04	14
Physiological System Development & Function		
Organismal Functions	6.90E-10 to 1.27E-05	9
Tissue Morphology	9.30E-10 to 2.28E-04	26
Tissue Development	2.24E-08 to 2.32E-04	34
Immune Response	3.54E-08 to 1.55E-04	29
Organismal Development	6.69E-08 to 2.77E-05	25
Tumor Morphology	2.67E-07 to 2.28E-04	11
Organ Morphology	9.54E-07 to 8.06E-05	8
Connective Tissue Development and Function	1.09E-06 to 2.46E-04	23
Organ Development	1.13E-06 to 1.13E-06	18
Cardiovascular System Development and Function	1.20E-06 to 2.52E-04	19
Molecular & Cellular Functions		
Cell Death	1.83E-14 to2.15E-04	47
Cellular Growth & Proliferation	1.08E-13 to 2.41E-04	53
Cellular Development	9.50E-13 to 2.46E-04	41
Cellular Movement	1.59E-12 to 2.52E-04	37
Cell Cycle	3.63E-09 to 2.58E-04	25
Gene Expression	3.94E-09 to 2.58E-04	31
Cell-to-Cell Signaling and Interaction	2.15E-08 to 2.28E-04	27
DNA Replication, Recombination, and Repair	1.38E-06 to 1.38E-06	13
Cell Morphology	1.68E-06 to 1.82E-04	22
Cellular Function and Maintenance	3.84E-05 to 2.28E-04	5

* *p*-value is determined by right-tailed Fischer's exact test.

Appendix II Fold change of interested genes in three datasets (measured by microarray and quantitative PCR)

Gene symbol	Fold difference assessed by means of microarray			Fold difference assessed by means of quantitative PCR		
	GC-naïve NP vs. control	GC-treated NP vs. control	GC-treated vs. GC -naïve NP	GC-naïve NP vs. control	GC-treated NP vs. control	GC-treated vs. GC -naïve NP
ADAM8	1.61	*NS*	*NS*	*N.A.*	*N.A.*	*N.A.*
ALOX5AP	2.61	*NS*	*NS*	*N.A.*	*N.A.*	*N.A.*
ANGPT1	0.27	0.29	*NS*	*N.A.*	*N.A.*	*N.A.*
ANGPT2	0.34	0.36	*NS*	*N.A.*	*N.A.*	*N.A.*
ANXA1	*NS*	*NS*	5.91	*N.A.*	*N.A.*	4.55
AREG	0.22	*NS*	6.47	0.01	*NS*	6.7
ATP1A2	0.34	0.35	*NS*	*N.A.*	*N.A.*	*N.A.*
Bid	1.65	*NS*	*NS*	*N.A.*	*N.A.*	*N.A.*
C1QB	4.73	2.67	*NS*	*N.A.*	*N.A.*	*N.A.*
C3	2.64	2.21	*NS*	5.75	*N.A.*	*N.A.*
C4A	5.54	3.97	*NS*	*N.A.*	*N.A.*	*N.A.*
CASP3	1.73	1.60	*NS*	*N.A.*	*N.A.*	*N.A.*
CASP7	1.85	1.70	*NS*	*N.A.*	*N.A.*	*N.A.*
CCL11	3.87	*NS*	*NS*	14.92	*NS*	*N.A.*
CCL15	4.68	3.76	*NS*	*N.A.*	*N.A.*	*N.A.*
CCL28	0.18	0.22	*NS*	*N.A.*	*N.A.*	*N.A.*
CD40	0.51	*NS*	*NS*	*N.A.*	*N.A.*	*N.A.*
CD69	0.41	*NS*	2.99	0.05	*NS*	4
CD86	3.39	2.38	*NS*	*N.A.*	*N.A.*	*N.A.*
CD9	0.51	*NS*	*NS*	*N.A.*	*N.A.*	*N.A.*
CEACAM1	0.57	*NS*	*NS*	*N.A.*	*N.A.*	*N.A.*
CEACAM6	6.06	4.68	*NS*	*N.A.*	*N.A.*	*N.A.*
CFH	0.49	*NS*	*NS*	*N.A.*	*N.A.*	*N.A.*
c-Fos	0.08	*NS*	14.85	0.02	*NS*	14.2
c-Jun	0.29	*NS*	3.39	0.06	*NS*	4.9
CLIC3	2.03	*NS*	*NS*	*N.A.*	*N.A.*	*N.A.*
CLIC5	1.85	*NS*	*NS*	*N.A.*	*N.A.*	*N.A.*
CLIC6	3.57	4.41	*NS*	*N.A.*	*N.A.*	*N.A.*
COX-2	0.38	NS	3.47	0.17	*NS*	5.51
CRISP3	0.01	0.14	*NS*	*N.A.*	*N.A.*	*N.A.*
CXCL11	*NS*	*NS*	0.4	*N.A.*	*N.A.*	0.35
CXCL12	0.34	0.32	*NS*	*N.A.*	*N.A.*	*N.A.*
CXCL2	0.61	*NS*	5.42	0.14	*NS*	7.45
CXCL6	7.65	6.09	*NS*	*N.A.*	*N.A.*	*N.A.*
CXCL9	*NS*	*NS*	0.4	*N.A.*	*N.A.*	0.4

Gene symbol	Fold difference assessed by means of microarray			Fold difference assessed by means of quantitative PCR		
	GC-naïve NP vs. control	GC-treated NP vs. control	GC-treated vs. GC -naïve NP	GC-naïve NP vs. control	GC-treated NP vs. control	GC-treated vs. GC -naïve NP
CYSLTR1	2.92	3.05	*NS*	*N.A.*	*N.A.*	*N.A.*
DEFB1	3.93	2.53	*NS*	*N.A.*	*N.A.*	*N.A.*
DUOX1	6.67	2.56	*NS*	*N.A.*	*N.A.*	*N.A.*
DUSP1	0.1	*NS*	4.98	0.12	*NS*	8.35
DUSP2	0.18	*NS*	3.19	0.06	*NS*	2.8
DUSP4	0.21	0.32	*NS*	*N.A.*	*N.A.*	*N.A.*
DUSP5	0.22	*NS*	*NS*	*N.A.*	*N.A.*	*N.A.*
DUSP6	0.34	*NS*	2.08	0.16	*NS*	2.5
EGF	0.25	*NS*	NS	*N.A.*	*N.A.*	*N.A.*
EGR1	0.08	0.36	7.81	0.01	0.12	12.3
ERBB4	0.47	*NS*	*NS*	*N.A.*	*N.A.*	*N.A.*
FosB	0.05	*NS*	16.67	0.01	*NS*	15.3
GCLM	3.65	3.3	*NS*	*N.A.*	*N.A.*	*N.A.*
GPX3	0.38	*NS*	1.59	*N.A.*	*N.A.*	*N.A.*
GRα	*N.A.*	*N.A.*	*N.A.*	0.31	0.34	1.2
GRβ	*N.A.*	*N.A.*	*N.A.*	*UDab*	*UDab*	*UDab*
HBEGF	0.21	*NS*	5.45	0.06	NS	5.05
IFNAR1	1.56	*NS*	*NS*	*N.A.*	*N.A.*	*N.A.*
IL13RA2	0.49	*NS*	*NS*	*UDa*	*N.A.*	*N.A.*
IL18	3.03	2.9	*NS*	4.37	*N.A.*	*N.A.*
IL5Ra	1.58	*NS*	*NS*	9.85	*NS*	*N.A.*
IL-6	0.16	0.42	7.87	0.01	0.2	9.85
IL6ST	0.59	*NS*	*NS*	*N.A.*	*N.A.*	*N.A.*
ITGB2	2.62	*NS*	*NS*	*N.A.*	*N.A.*	*N.A.*
JunB	0.24	0.37	3.37	0.06	0.15	3.15
LGALS8	1.64	*NS*	*NS*	*N.A.*	*N.A.*	*N.A.*
LGALS9	1.69	*NS*	*NS*	*N.A.*	*N.A.*	*N.A.*
LPO	0.03	0.23	*NS*	*N.A.*	*N.A.*	*N.A.*
LTA4H	1.53	*NS*	*NS*	*N.A.*	*N.A.*	*N.A.*
LTB4R	1.66	2.35	*NS*	*N.A.*	*N.A.*	*N.A.*
LYN	1.97	*NS*	*NS*	*N.A.*	*N.A.*	*N.A.*
MIF	1.75	*NS*	*NS*	*N.A.*	*N.A.*	*N.A.*
MMP7	6.43	2.48	0.47	8.62	4.05	0.5
MMP9	3.48	*NS*	0.44	*UDb*	*UDb*	0.45
MUC16	11.12	11.21	*NS*	*N.A.*	*N.A.*	*N.A.*
MUC20	4.51	3.58	*NS*	*N.A.*	*N.A.*	*N.A.*
MUC4	5.47	5.48	*NS*	*N.A.*	*N.A.*	*N.A.*
MUC7	0.02	0.06	*NS*	*N.A.*	*N.A.*	*N.A.*

Gene symbol	Fold difference assessed by means of microarray			Fold difference assessed by means of quantitative PCR		
	GC-naïve NP vs. control	GC-treated NP vs. control	GC-treated vs. GC -naïve NP	GC-naïve NP vs. control	GC-treated NP vs. control	GC-treated vs. GC -naïve NP
NFKBIA	0.65	*NS*	*NS*	0.45	*N.A.*	*N.A.*
NFKBIZ	0.46	*NS*	2.22	0.23	*NS*	2.2
NOS2A	3.96	6.76	*NS*	*N.A.*	*N.A.*	*N.A.*
NOX4	3.03	*NS*	*NS*	*N.A.*	*N.A.*	*N.A.*
NR4A1	0.14	*NS*	4.36	0.03	*NS*	9.15
NR4A2	0.11	*NS*	7.02	0.08	*NS*	6.25
NR4A3	0.11	0.28	*NS*	*N.A.*	*N.A.*	*N.A.*
NRG3	0.23	0.29	*NS*	*N.A.*	*N.A.*	*N.A.*
OXR1	0.42	0.39	*NS*	*N.A.*	*N.A.*	*N.A.*
PLA2G10	2.51	2.53	*NS*	*N.A.*	*N.A.*	*N.A.*
PLA2G4A	2.61	3.28	*NS*	*N.A.*	*N.A.*	*N.A.*
PRDX1	1.62	*NS*	*NS*	*N.A.*	*N.A.*	*N.A.*
PRDX5	1.79	*NS*	*NS*	*N.A.*	*N.A.*	*N.A.*
PTGER2	1.99	*NS*	*NS*	*N.A.*	*N.A.*	*N.A.*
PTGER3	0.15	0.29	*NS*	*N.A.*	*N.A.*	*N.A.*
PTGIS	0.26	0.25	*NS*	*N.A.*	*N.A.*	*N.A.*
PTX3	0.12	*NS*	*NS*	*N.A.*	*N.A.*	*N.A.*
SCGB1A1	*NS*	*NS*	5.51	*N.A.*	*N.A.*	4.9
SCNN1A	1.96	2.11	*NS*	*N.A.*	*N.A.*	*N.A.*
SCNN1B	4.93	6.04	*NS*	*N.A.*	*N.A.*	*N.A.*
SCNN1G	5.65	7.34	*NS*	*N.A.*	*N.A.*	*N.A.*
SELPLG	2.04	*NS*	*NS*	*N.A.*	*N.A.*	*N.A.*
SERPINA1	3.18	3.48	*NS*	*N.A.*	*N.A.*	*N.A.*
SOCS3	0.31	*NS*	4.2	0.21	*NS*	3.8
SOD3	0.39	0.47	NS	*N.A.*	*N.A.*	*N.A.*
SPRY1	0.33	*NS*	2.49	0.19	*NS*	2.1
SPRY2	0.62	*NS*	2.11	0.51	*NS*	2.21
SPRY4	0.62	*NS*	2.13	0.42	*NS*	2.03
STAT3	0.58	*NS*	*NS*	N.A.	*N.A.*	*N.A.*
TEK	0.31	0.41	*NS*	N.A.	*N.A.*	*N.A.*
THBD	0.22	*NS*	2.63	0.09	*NS*	2.7
TXN	1.64	*NS*	*NS*	*N.A.*	*N.A.*	*N.A.*
ZFP36	0.17	*NS*	3.77	0.11	*NS*	5.35

- *NS*, No statistical difference;
- *UDa*, Undetectable in NP (either GC-naïve or GC-treated);
- *Udb*, Undectable in control;
- *Udab*, Undectable in both NP and control;
- *N.A.*, Not applicable.

Appendix III Relative expression level of selected genes by real-time RT PCR

Gene symbol	Median of Δ Ct ($Ct_{target} - Ct_{GAPDH}$)		
	GC-naïve NP (n=10)	GC-treated NP (n=10)	Control (n=6)
ANXA1	5.45	3.50	*N.A.*
AREG	12.85	8.98	6.59
C3	2.15	*N.A.*	4.68
CCL11	5.56	*N.A.*	9.52
CD69	11.94	9.46	7.59
c-Fos	5.74	0.97	0.13
c-Jun	7.67	*5.26*	3.6
COX-2	9.26	6.8	6.72
CXCL11	*8.53*	9.8	*N.A.*
CXCL2	8.75	6.35	5.67
CXCL9	5.45	6.32	*N.A.*
DUSP1	4.52	1.00	1.47
DUSP2	11.98	9.92	7.97
DUSP6	5.75	4.57	3.06
EGR1	5.31	1.86	-0.56
FosB	10.59	5.31	4.52
GRα	8.10	7.97	6.41
GRβ	*UD*	*UD*	*UD*
HBEGF	8.45	5.66	4.61
IFNAR1	*N.A.*	*N.A.*	*N.A.*
IL13RA2	*UD*	*UD*	*UD*
IL18	10.37	*N.A.*	12.36
IL5Ra	9.67	*N.A.*	12.22
IL-6	13.24	9.12	6.82
JunB	5.19	3.98	1.01
MMP7	6.41	7.51	9.18
MMP9	5.89	7.33	*UD*
NFKBIA	3.40	*N.A.*	2.18
NFKBIZ	7.85	6.23	5.67
NR4A1	7.43	3.26	2.19
NR4A2	7.82	5.19	4.09
SCGB1A1	*11.72*	*9.29*	11.99
SOCS3	5.68	4.05	3.40
SPRY1	9.79	9.15	7.47
THBD	8.45	7.09	5.05
ZFP36	5.09	3.21	1.88

- *UD*, Undetectable
- *N.A.*, Not applicable

Curriculum Vitae

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Education

01/2003-Present	➢ PhD candidature in Department of Otolaryngology, National University of Singapore, *Singapore*
09/1997-07/2002	➢ Bachelor of Medicine in Sun Yat-sen University, *P.R. China*

Working Experience

01/2007-Present	➢ Research assistant in Department of Otolaryngology, National University of Singapore, *Singapore*
02/2002-07/2002	➢ Internship in the Center of Disease Control in Guangzhou, *P.R. China*
09/2001-02/2002	➢ Internship in the Faculty of public health of Sun Yat-sen University, *P.R. China*
11/2000-08/2001	➢ Internship in The First affiliated hospital of Sun Yet-sen University, *P.R. China*

Scholarship and Award

01/2003-01/2007	➢ NUS Research Scholarship, National University of Singapore, Singapore
06/2005	➢ Travel grant of 2005 Wordl Allergy Congress, Munich, Germany
06/2008	➢ Travel grant of XXVII Congress of the European Academy of Allergology and Clinical Immunology, EAACI 2008, Barcelona, Spain

Membership

01/2005-Present	➢ Junior member of European Academy of Allergology and Clinical Immunology

Skills profile

- Be familiar with molecular and cell biology techniques, such as DNA/RNA purification, microarray, Real-time PCR, immunohistochemistry and cell culture work.
- Be familiar with biostatistics work and related software.

Publications

- Li CW, Cheung W, Lin ZB, Li TY, Lim JT, Wang DY. **Oral steroids enhance epithelial repair in nasal polyposis via up-regulation of AP-1 gene network.** *Thorax.* 2009 Jan 21. [Epub ahead of print]. (***Impaction Factor: 7.06***

- Liang XH, Cheung W, Heng CK, Liu JJ, Li CW, Lim B, Wang DY. **CD14 promoter polymorphisms have no functional significance and are not associated with atopic phenotypes.** *Pharmacogenet Genomics.* 2006 Apr;16(4):229-36. (***Impaction Factor: 4.14***)

- Wang DY, Li CW. **Control of nasal obstruction in patients with persistent allergic rhinitis.** *Zhonghua Er Bi Yan Hou Tou Jing Wai Ke Za Zhi.* 2006 Sep;41(9):716-20.

- Li CW, Cheung W, Li TY, Lin ZB, Lim JT, Wang DY. **Expression profile of eosinophil- and neutrophil-associated genes in patients with nasal polyposis.** Article submitted.

- Li CW, Cheung W, Pang YT, Wang DY. **Low level methylation of some tumor suppressor genes in nasal polyps and normal nasal mucosa.** Article in preparation.

Presentations at conferences

- Li CW, Pang YT, Tao Q, Wang DY. **Promoter methylation status of multiple genes in nasal polyps.** Poster presentation in 2005 World Allergy Congress, *Munich, Germany*. Poster No. 751.

- Li CW, Cheung W, Lin ZB, Li TY, Lim JT, Wang DY. **Glucocorticoids promote epithelial repair in nasal polyps via upregulating Activator protein-1.** Oral presentation in XXVII Congress of the European Academy of Allergology and Clinical Immunology, EAACI 2008, *Barcelona, Spain*. Abstract No. 119. (Awarded with the best oral presentation in the session of "Inflammatory Mechanisms in Rhinosinusal Disease".)