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The role of post-translation regulation of Twist expression in the tumor progression of squamous cell carcinoma of head and neck

Ying-Wen Su

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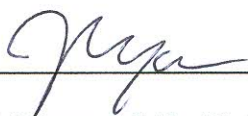
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**THE ROLE OF POST-TRANSLATIONAL REGULATION OF
TWIST EXPRESSION IN THE TUMOR PROGRESSION OF
SQUAMOUS CELL CARCINOMA OF HEAD AND NECK**

by

Ying-Wen Su, M. D.

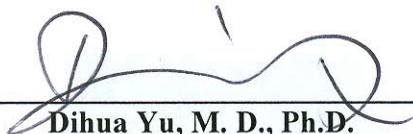
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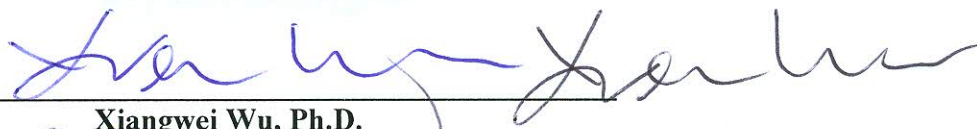
**Jeffrey N. Myers, M. D., Ph.D.
Supervisory Professor**



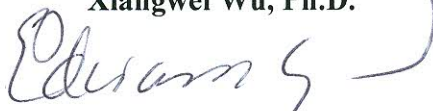
Gary E. Gallick, Ph.D.



Dihua Yu, M. D., Ph.D.



Xiangwei Wu, Ph.D.



Edward Yeh, M. D.

APPROVED:

**George M. Stancel, Ph.D.
Dean, The University of Texas
Health Sciences Center at Houston
Graduate School of Biomedical Sciences**

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TWIST EXPRESSION IN THE TUMOR PROGRESSION OF
SQUAMOUS CELL CARCINOMA OF HEAD AND NECK**

A

DISSERTATION

Presented to the Faculty of
The University of Texas
Health Science Center at Houston
and
The University of Texas
M. D. Anderson Cancer Center
Graduate School of Biomedical Sciences
In Partial fulfillment
of the Requirements
for the degree

DOCTOR OF PHILOSOPHY

By
Ying-Wen Su, M. D.

Houston, Texas

Dec , 2010

To my dear uncle Yun-Fong Su, a life-long nasopharyngeal cancer

fighter;

my previous mentor, Ming-Jer Huang, who passed away due to

metastatic cancer of unknown primary;

my dear sons Ruei-Jia & Timothy;

&

my husband & dearest friend, Li-Min

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I have to acknowledge the patience and love of my family: my six year-old son Ruei-Jia, my three-year old son Timothy, and my husband Li-Min Huang to whom this thesis is dedicated. A special thanks goes to Li-Min for being there for me throughout all of the hardships. I am grateful for all the help from my parents, through their royal prayers I can proceed to finish this project. A very special place in my heart will always hold my in-laws who supported our family to start new lives here in the US.

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who listened to my stupid ideas, spent their precious time discussing my project and helped me work on the right track.

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This work would have not been possible without support from all the members in Myers lab. Now my journey to PhD is coming to the end, however, the passion for saving lives through research will never cease here.

Go Myeritis ! Go!

Ying-Wen Su

Aug 2010

THE ROLE OF POST-TRANSLATIONAL REGULATION OF TWIST EXPRESSION IN THE TUMOR PROGRESSION OF SQUAMOUS CELL CARCINOMA OF HEAD AND NECK

Publication No. _____

**Ying-Wen Su M. D.
Supervisory Professor: Jeffrey N. Myers M.D., Ph. D.**

Squamous cell carcinoma of head and neck (SCCHN) is the tenth most common cancer in the world. Unfortunately, the survival of patients with SCCHN has not improved in the last 40 years. Therefore new targets for therapy are needed, and to this end we are studying signaling pathways activated by IL-6 which we have found stimulates cell migration and soft agar growth in SCCHN. Our data show that IL-6 increases TWIST expression in a transcription-independent mechanism in many SCCHN cell lines. Further investigation reveals TWIST can be phosphorylated upon IL-6 treatment. By computation prediction (http://scansite.mit.edu/motifscan_seq.phtml), we found that TWIST has a putative phosphorylation site for casein kinase 2 (CK2) suggesting that this kinase could serve as a link between IL-6 stimulation and Twist stability. To test this hypothesis, we used a CK2 inhibitor and shRNA to CK2 and found that these interventions inhibited IL-6 stimulation of TWIST stability. In addition, mutation of the putative CK2 phosphorylation site (S18/S20A) in TWIST decreased the amount of phospho-ATP incorporated by TWIST in an in vitro kinase assay, and altered TWIST stability. In Boyd chamber migration assay and wound-healing assay, the CK2 inhibitor, DMAT, was found to decrease the motility of IL-6 stimulated SCCHN cells

and over expression of either a wild-type or the hyperphosphorylated mimicking mutant S18/20D –Twist rather than the hypo-phosphorylated mimicking mutant S18/20A-Twist can promote SCCHN cell motility. To our knowledge, this is the first report to identify the importance of IL-6 stimulated CK2 phosphorylation of TWIST in SCCHN. As CK2 inhibitors are currently under phase I clinical trials, our findings indicate that CK2 may be a viable therapeutic target in SCCHN. Therefore, further pre-clinical studies of this inhibitor are underway.

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ABBREVIATIONS

Ab	antibody
ATP	Adenosine triphosphate
bHLH	basic helix-loop-helix
BSA	Bovine serum albumin
BSA	Bovine serum albumine
CDK	cyclin dependent kinases
cDNA	complementary DNA
CHX	Cycloheximide
CK2	casein kinase 2
DFS	disease free survival
DMAT	2-Dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotidetriphosphate
EGFR	epidermal growth factor receptor
EMT	epithelial mesenchymal transition
FBS	fetal bovine serum
FGF	fibroblast growth factor
H & E	hematoxylin-eosin
HGF	hepatocyte growth factor
IACUC	Institutional Animal Care and Use Committee

IL-6	interleukin-6
kD	kilodalton
LOH	loss of heterozygosity
mRNA	messenger Ribonucleic acid
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NLS	nucleus localization sequence
NLS	Nuclear localization signal
OD	Optical density
OS	overall survival
PCR	Polymerase chain reaction
PDGF	platelet derived growth factor
PFS	progression free survival
PMSF	Phenylmethanesulfonyl fluoride
PVDF	Polyvinylidene Fluoride
RNA	Ribonucleic acid
RNase	Ribonuclease
RR	response rate
RTPCR	Reverse transcriptase-PCR
SCCHN	squamous cell carcinoma of head and neck
SCS	Saethre - Chorsen Syndrome
SDS	Sodium Dodecylsulfate
SDS-PAGE	SDS-Polyacrylamide Gel Electrophoresis
SMA	smooth muscle actin
SMA	smooth muscle actin

SOS	son of sevenless homolog
STAT	Signal transducer and activator of transcription
TBB	4,5,6,7-tetrabromobenzotriazole
TBE	Tris-Borate-EDTA
TE	Tris-EDTA buffer
TEMED	Tetramethylethylene diamine
TGF	transforming growth factor
TNF	tumor necrotic factor
Tris	Tri-hydroxymethyl aminomethane
VEGF	vascular endothelial growth factor

I. Introduction

1.1. Overview of Genetic Pathogenesis of Squamous Cell Carcinoma of Head and Neck (SCCHN)

Squamous cell carcinoma of head and neck (SCCHN) arises from the mucosa of the upper aerodigestive tract (UADT) and accounts for >90% of cancers arising from the head and neck region. The UADT consists of six major sites including the oral cavity, oropharynx, larynx, hypopharynx, the nose and paranasal sinuses, and the nasopharynx (Figure 1.1). Taking cancers arising from these sites in aggregate, SCCHN is the sixth most common cancer in the world and accounts for 3.2 percent of all malignancies in the United States. In the US, about 48,010 Americans develop SCCHN annually and about 11,260 die from the disease. Males are affected significantly more often than females with a ratio ranging from 2:1 to 4:1 (Parkin et al., 2005; Jemal et al., 2009). Despite the progress in surgery, radiation and chemotherapies that have enhanced organ preservation, survival of SCCHN patients has improved only minimally in the last 40 years. Recent advances in understanding the risk factors and molecular events involved in the pathogenesis of SCCHN have provided a foundation for development of new methods for risk stratification, patient screening, prevention and therapy.

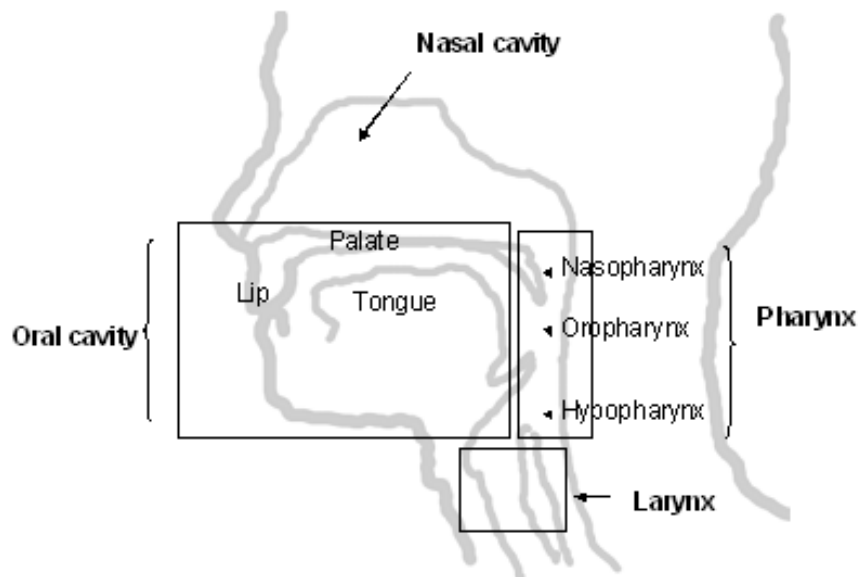


Figure 1.1 Anatomy of head and neck

Head and neck region can be further divided into several parts where SCCHN can develop: (1) The oral cavity includes the lip, floor of mouth, tongue, hard palate and buccal mucosa; (2) The pharynx includes oropharynx, nasopharynx and hypopharynx; (3) the larynx includes the supraglottic, glottic, and subglottic larynx. (4) nasal cavity.

1.1.1 Risk Factors for Development of SCCHN

Tobacco smoking and alcohol consumption are the major risk factors for developing SCCHN (Spitz, 1994). Up to 80% of SCCHN patients have been exposed to smoking or alcohol (Sankaranarayanan et al., 1998). Compared to non-smokers, there is a 5- to 25-fold increased risk in heavy smokers (Blot et al., 1988).

Betel nut chewing is another important risk factor in the certain regions of Asia, including the Indian subcontinent and Taiwan, where there is a higher SCCHN endemic rate. It can be an independent risk factor for developing SCCHN or work synergistically with smoking and alcohol (Lee et al., 2005). Chronic viral infection is also an important risk factor for head and neck cancer. Human papillomavirus (HPV) has recently been found to be associated with the development of SCCHN arising within the tonsil and base of tongue subsites of the oropharynx (D'Souza et al., 2007). There are emerging data showing HPV-related SCCHN tends to have better prognosis relative to HPV-negative cancers (Fakhry et al., 2008). The vast majority of nasopharyngeal cancers are associated with the Epstein-Barr Virus (EBV) and these tumors are major causes of cancer morbidity and mortality in Southern China, Taiwan, and Hong Kong (Young & Rickinson, 2004). Other risk factors include occupational exposure to chemicals, prior radiation for malignant or benign disease (Sale et al., 2004), genetic factors, poor oral hygiene and periodontal disease (Tezal et al., 2007; Tezal et al., 2009).

1.1.2 SCCHN Carcinogenesis

The chronic exposure of the upper aerodigestive tract to the aforementioned risk factors leads to accumulation of genetic abnormalities in the epithelial cells that line the mucosal surfaces of head and neck region, leading to the transformation of normal squamous mucosa to pre-malignant dysplastic lesions and eventually invasive cancers.

Pathologically, the process can be divided into several steps including normal mucosa, hyperplasia, dysplasia, carcinoma in situ and cancer as illustrated in Figure 1.2. A retrospective study of 97 cases of with epithelial dysplasia in the head and neck mucosa demonstrated that 50% of patients developed carcinoma within a 30-month of follow-up, lending support to the multi-step carcinogenesis model. (Partridge et al., 2001; Bosatra et al., 1997). Cells injured by exposure to carcinogens may undergo an irreversible genetic damage and cell death or undergo DNA repair and survive and propagate these genetic abnormalities. The surviving cells often acquire increased resistance to apoptosis, continue proliferation, migrate and heal the area of injury. With repeated injury, the proliferating cells may accumulate to produce a thickened epithelium, termed hyperplasia. If the mutagen exposure persists, further irreversible genetic mutations and epigenetic changes may occur with changes of not only the number but also the morphology of proliferating cells, which result in dysplasia or carcinoma *in situ* (CIS). Dysplasia and CIS lesions are premalignant. With continued growth, the transformed cells can acquire the ability to invade and migrate through the basement membrane into the stroma, which defines the lesion as an invasive carcinoma.

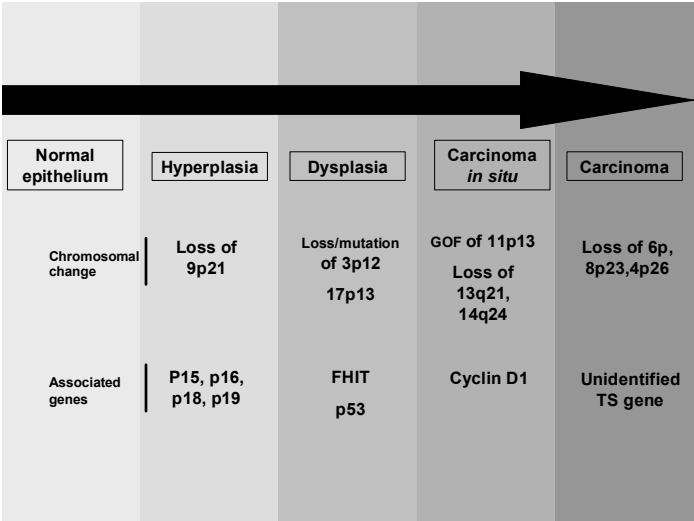
Hyperplasia, dysplasia, or CIS may be accompanied by hyper-keratinization, which may be visible clinically as leukoplakia. When they are accompanied with a progressive increase in inflammation and proliferation of fibrous stroma, which may be visible clinically as induration and vascular erythema, called erythroplasia.

1.1.3 The Multi-step Model of SCCHN Carcinogenesis

Carcinogenesis occurs as a result of multiple sequential alterations to the DNA of epithelial cells that impact the quantitative and qualitative expression of multiple genes involved in regulation of cell proliferation, survival, invasion and other hallmarks of cancer (Hanahan and Weinberg, 2000). An “allelogram” model of genetic progression in SCCHN carcinogenesis was proposed by Califano et al (Figure 1.2), which was based on microsatellite analysis of patient specimens in which the abnormal mucosa cells surrounding invasive cancers harbored similar genetic losses to the tumor cells (Califano et al., 1996; Califano et al., 2000). In this model, loss of 9p, which results in inactivation of the p16 and p14 ARF tumor suppressor genes, was a very early event, during the transition from normal mucosa to benign squamous hyperplasia. This was frequently followed by loss of 3p (the site of potential tumor suppressor genes, including FHIT [fragile histidine triad] and RASSF1A) and 17p (site of the p53 tumor suppressor gene) with the transition at the histologic level to dysplasia. At later stages, the transition from dysplasia to carcinoma *in situ* was commonly associated with specific additional losses (11q, 13q, 14q) and further losses at (6p, 8p, 4q) were found in the transition to invasive

squamous cell carcinoma. This model were confirmed and expanded upon by other researchers using different techniques such as comparative genomic hybridization (Bockmuhl et al., 1998). In summary, the most frequent cytogenetic alterations in SCCHN are gains at the 3q,8q,9q, 20q, 7p,11q13 and 5p chromosomal regions and losses of 3p,9p,21q,5q,13q,18q and 8p (Gollin, 2001). Several findings observed in the first models such as 17p13 loss, 14q24 loss and 6p loss have not been observed to occur at high frequency in classical or molecular cytogenetic methods. Recent studies of chromosomal gain and loss in SCCHN are summarized in Figure 1.2. Despite the discrepancies between studies and controversies, the classical view of genetic progression provided useful information to examine the tumor biology and molecular events in SCCHN.

Figure 1.2
Summary of the pathologic and molecular carcinogenesis of SCCN



1.2 A contemporary overview of the molecular pathogenesis of SCCHN and a brief review of molecularly targeted therapy of SCCHN

The development of cancer from normal epithelial requires the acquisition of 6 hallmarks including (1) self-sufficiency in growth signals, (2) insensitivity to antigrowth signals, (3) evading apoptosis, (4) limitless replicative potential, (5) sustained angiogenesis, and (6) tissue invasion and metastasis (Hanahan and Weinberg, 2000). These hallmarks lead to dysregulation of gene expression profiles and disruption of molecular networks. Weinberg has shown that molecular alterations in the expression or function of at least two genes is required to alter important cellular functions and transform normal rodent cells to tumors under experimental conditions, while the human counterparts are more difficult to transform (Hahn and Weinberg, 2002). One important requirement for neoplastic transformation is to acquire the ability of uncontrolled cell proliferation. In the past decades, numerous efforts have been spent to study the SOS-Ras-Raf-MAP kinase mitogenic cascade (SOS: son of sevenless homolog), the central pathway controlling cell proliferation. In the model, the increased proliferation and concomitant cell-cycle progression can result from activation of Ras, which can be activated by viral infection, mutation, or by upstream signal activation due to over expression of the growth factors or receptors, such as epidermal growth factor receptor (EGFR). Upon Ras activation, many of downstream proteins related to cell proliferation or the cell-cycle entry such as MAPKs (mitogen activated protein kinases), AP-1 (transcription factor activator protein-1) and cyclin D1 are activated. However, Ras

activation and increased proliferation alone are not sufficient to cause tumor transformation because these proliferating cells eventually undergo apoptosis or senescence (Bennecke et al., 2010). Additional alterations, leading to the inactivation of tumor suppressor genes (TSG) such as p14ARF, p16INK4a or p53, are needed. TSGs usually serve as a “gatekeepers” of cellular proliferation, and inactivation of TSG can result in the continuous replication of cells with DNA mutations and damage and further changes in the expression and function of additional genes, leading to further increases in malignant behavior. The losses of TSGs occur from chromosomal mutation, deletion or epigenetic changes such as methylation. The relatively high frequency of chromosomal losses in SCCHN compared to allelic gains highlights the the important role for loss of TSGs in carcinogenesis and tumor progression (Gollin, 2001).

The genetic or epigenetic changes in SCCHN can lead to altered activation of several signaling and transcription factor pathways that in turn regulate many of the several hundred genes and proteins altered in cancer. These common pathways may provide an insight to the tumor biology and may provide useful markers for molecular diagnosis and targets for therapy. The signaling pathways that have been implicated in the development of SCCHN are briefly reviewed in the following sections and are summarized in Figure 1.3. In the following sections, several important molecules in SCCHN tumorigenesis are briefly reviewed in addition to their potential utility as biomarkers and/or targets for therapy.

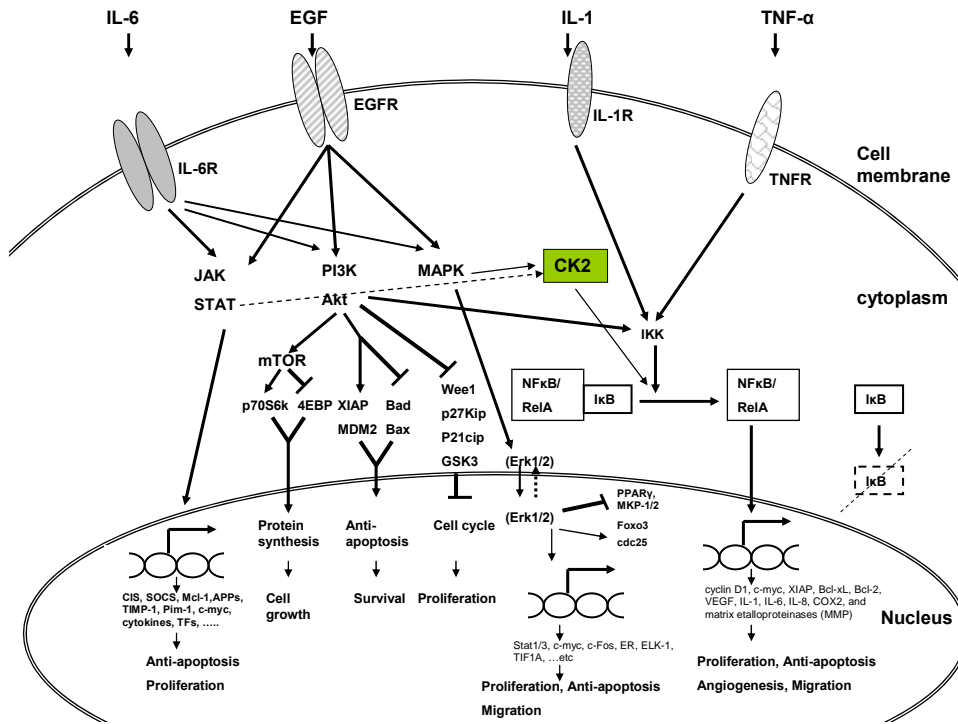


Figure 1.3 Summary of the major signaling pathways involved in the development of SCCHN

1.2.1 Disruption of Mitogenic Signaling Pathways in SCCHN and Current Roles in Targeted Therapy

1.2.1.1 EGFR

The EGF receptor (EGFR), also known as ErbB1 and HER1, is a receptor tyrosine kinase (RTK) belonging to the EGFR RTK family, which also includes ErbB2 (also known as HER2), ErbB3 and ErbB4. Activation of the 170kD transmembrane glycoprotein receptor by binding of its ligands including EGF and TGF- α , etc, can lead to activation of many intracellular signaling cascades, including MAPK, signal transducer and activator of transcription (STATs) and Akt anti-apoptotic kinases pathways which in turn contributes to cell survival, proliferation, invasion, cell motility, angiogenesis & tumorigenesis (Ang et al., 2002; Robinson et al., 2000; Kim et al., 2008).

Over-expression of the EGFR protein is frequently (80-100%) observed in tumor specimens from SCCHN patients. This over expression of EGFR is often accompanied by the over expression of one of its ligand, such as TGF- α (Grandis et al., 1998; Dasonville et al., 1993). Over-expression of TGF- α has been found to occur early in carcinogenesis as it has been detected in mild dysplasia, with no further increases in advanced dysplasia or carcinoma. In contrast, EGFR over expression increases progressively with increasing degree of dysplasia and is markedly elevated in many fully transformed SCCHNs (Shin et al., 1994b).

However, the mechanisms of EGFR over-expression are still unclear. Somatic mutations in EGFR in SCCHN are less common than in lung adenocarcinomas (Lee et al., 2005), and gene amplification of EGFR is only detected as many as 15% of tumors (Temam et al., 2007). EGFR vIII, a constitutively activated EGFR variant resulting from truncation of the extracellular domain, has been found infrequently in SCCHN (Zhu et al., 2003; Kim et al., 2008). Sustained autocrine stimulation may be responsible for the activation of EGFR in SCCHN since over expression of EGFR is often accompanied with over expression of its ligand, TGF- α expression, and this association is often correlated with decreased disease-specific survival (Grandis and Tweardy, 1993).

High expression levels of EGFR correlates with a more advanced stage of the disease, a poor prognosis and worse response to chemotherapy (Rubin et al., 1998; Temam et al., 2007). Multiple Phase II and III clinical trials have demonstrated the therapeutic importance of targeting EGFR. Therapeutic approaches to target EGFR signaling include anti-EGFR monoclonal antibodies (mAbs) or small molecule tyrosine kinase inhibitors (TKIs). TKIs are administered as oral pills and convenient for outpatient treatment. MAb can only be administered parentally but are more specific than TKI in target inhibition.

Cetuximab, a monoclonal antibody to EGFR's extra cellular domain, is currently the only molecular targeted agent approved by FDA based on the phase III clinical trial in newly diagnosed SCCHN comparing cetuximab plus radiation versus radiation alone (Bonner et al., 2006; Bonner et al., 2010). The response rate (RR) of single agent cetuximab in SCCHN patients who had failed treatment with cisplatin was 13% (Vermorken et al., 2007), however, the addition of cetuximab to front-line platinum-containing chemotherapy did not lead to either improved OS (overall survival) or DFS (disease-free survival) (Burtneiss et al., 2005; Vermorken et al., 2007). The beneficial effects of cetuximab plus chemotherapy in SCCHN were not confirmed until the Extreme Phase III trial, which randomized 442 recurrent/metastatic & chemotherapy naive patients to first-line PF (cisplatin & 5-fluorouracil) +/- cetuximab treatment (Vermorken et al., 2008). In this study, both PFS and OS were improved from 3.3 to 5.6 and 7.4 to 10.1 months respectively. The result shows cetuximab is active in PF but not carboplatin-based chemotherapy and does not impair the tolerability of standard chemotherapy.

Gefitinib and erlotinib are two of the most widely used oral EGFR TKI clinically. As single agent treatment in recurrent and metastatic SCCHN, patients receiving gefitinib at higher dose (500 mg/m²) had higher RR (10.6 %) than those (1.4%) at lower dose (250 mg/m²). (Cohen et al., 2005; Cohen et al., 2003). However, the IMEX (iressa versus methotrexate) study failed to demonstrate a survival advantage in single agent gefitinib

(250 mg/m² or 500 mg/m²) versus methotrexate (Stewart et al., 2009). The overall RR in erlotinib in the first- or second-line setting in SCCHN was 4.3% (with PFS 9 weeks and OS 6 months) (Soulieres et al., 2004). Two Phase II studies using either gefitinib or erlotinib plus chemotherapy as front-line treatment in recurrent/metastatic patients showed RR from 67-75%, and OS around 11 months (Belon J, 2010; Kim ES, 2010), suggesting a benefit to the addition of the agents to chemotherapy. These observations are awaiting further confirmation in randomized phase III trials.

In summary, the response rates for single agent cetuximab or EGFR TKI usage in SCCHN are generally low. However, cetuximab has additive or synergistic effects when used in combination with systemic chemotherapy or radiation. The benefits of treatment with EGFR TKIs are still under investigation. Targeting EGFR has shown some anti-tumor effects in SCCHN, however, only a minority of patients benefit from the treatment. High levels of EGFR tumor expression is associated with poor prognosis, but does not correlate with tumor response to EGFR-targeted therapy (Kies M, 2007).

1.2.1.2 HER2/neu

The HER2/neu protooncogene is also encodes a transmembrane protein with protein kinase activity, and the HER2 gene product (also known as c-erbB-2) has extensive structural

homology to EGFR and is over expressed in many solid tumors, including breast, lung, ovarian, and gastric cancers. In all of these sites, HER2 over expression has been identified as a negative prognostic factor. However, the results of HER2 expression in SCCHN are conflicting. The expression of HER2 in SCCHN ranges from <10% to 70% in some large series (Khademi et al., 2002; Akamatsu et al., 2003; Craven et al., 1992). In one study, the expression of HER is negative in normal oral mucosa and increased from dysplastic to malignant oral squamous cell carcinoma, indicating a dynamic change in the development of SCCHN (Fong et al., 2008). Some studies have shown that HER2 expression correlates with decreased overall survival and can serve as a poor prognostic factor in SCCHN (Chen et al., 2003); however, some other studies report a lack of correlation of HER2 expression with tumor grade, lymph node metastases and survival (Giatromanolaki et al., 2000; Khan et al., 2002).

Trastuzumab (Herceptin) is a humanized monoclonal antibody targeting the extracellular domain of HER-2. In breast cancer patients over expressing HER-2, it has shown significant anti-cancer activity (Pegram et al., 1998). For cancers in the head and neck region, only a small subset of salivary ductal carcinomas have been reported to respond to trastuzumab therapy (Prat et al., 2008; Nabili et al., 2007). The effect of trastuzumab in the treatment of SCCHN has not been reported yet.

Several studies have shown that the presence of HER-2 overexpression may contribute to the resistance of cancer cells to EGFR targeting therapy, providing a rationale for combination therapy with EGFR and HER-2 inhibition (Christensen et al., 2001). HER-2 overexpression has also been shown to reduce the EGFR internalization rate and thereby results in an increase in the overall level of activated EGFR (Hendriks et al., 2003). Several studies have shown that the combination of Herceptin with anti-EGFR treatment can overcome the EGFR resistance. In SCCHN, tumor growth inhibition is also observed when gefitinib and pertuzumab (targeting HER-2 heterodimerization) or trastuzumab are combined in vitro (Erjala et al., 2006; Kondo et al., 2008). Since the levels of HER-2 over expression in SCCHN tumors vary in different studies and no synergistic effects have been observed in the combination anti-HER-2 treatment and anti-EGFR treatment, more preclinical and clinical trials to confirm whether this type of combination treatment is of clinical benefit to SCCHN patients.

1.2.1.3 *Src* kinase

Although EGFR targeted therapy has shown some anti-tumor effects clinically, only a minority of patients benefit from the treatment. Therefore, many research efforts have been

focused on the identification of strategies to overcome the resistance of EGFR targeted therapy. Another emerging target for cancer therapy is the non-receptor kinase, *src*, a downstream molecule in the EGFR signaling pathway. Elevated c-*src* activity and/or its protein level has been reported in many cancers, including SCCHN (Xi et al., 2003). C-*src* over expression in SCCHN promotes invasion and tumor growth. Constitutive activation of *src* may result in resistance to EGFR inhibitors, and thus EGFRi resistance might be overcome by combined inhibition of both targets: *src* and EGFR. *In vitro*, combined treatment with the c-*src* inhibitor AZD0530 and gefitinib resulted in greater inhibition of SCCHN cell growth and invasion compared with either agent alone (Koppikar et al., 2008). However, in an *in vivo* study performed in mice, sustained *src* inhibition can lead to activation of STAT3 which may counteract its anti-tumor effects (Sen et al., 2009).

1.2.2 Disruption of Cell Cycle Control in SCCHN and Its Impact on Targeted Therapy

In contrast to the over expression of genes that “turn on” or promote cell proliferation, the decreased inhibition of cell growth, which occurs commonly through the loss of tumor suppressor genes can also contribute to the tumorigenesis of SCCHN (Hanahan and Weinberg, 2000). Many tumor suppressor genes regulating the cell cycle

and other cellular processes are lost in SCCHN tumor development and progression including cyclin D1, p16, p53, *etc.*

1.2.2.1 p16, CDK 4/6-Cyclin D1 and RB

The p16 pathway regulates cell-cycle entry and progression at the G1/S transition. (Figure 1.4) (Chiocca, 2002) as it is a potent inhibitor of the cyclin-dependent kinases 4 and 6 (CDK4/6) –cyclin D complex. This complex increases the phosphorylation of the retinoblastoma gene product, pRB, leading to the release of the transcription factor E2F, which mediates the functions of many cellular proteins leading to progression through G1/S. Therefore, the inactivation of p16, or overexpression of cyclin D1, increases CDK4/6 activity and promotes cell proliferation.

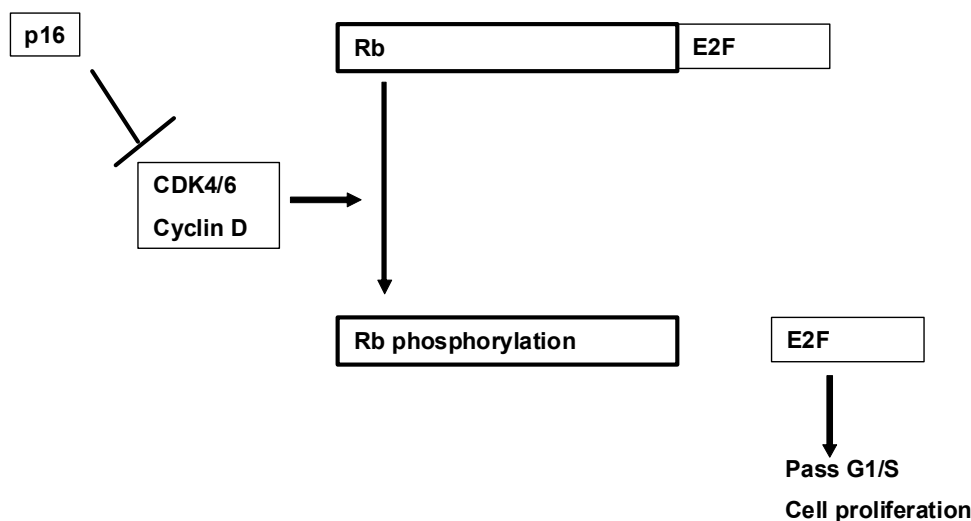


Figure 1.4 The p16 pathway.

The cyclin D1 gene is located on chromosome 11q13, the amplification of which is one of the most common cytogenetic alterations seen in carcinoma in situ lesions through SCCHNs (Gollin, 2001). Amplification and over expression of cyclin D1 has been described in up to 40% of cases of oral early premalignant lesions, including oral squamous dysplasia, or mild dysplasia (Rousseau et al., 2001). In tumors, this genetic alteration is seen in 30–60% of HNSCC cases and has been correlated with tumor grade, recurrence, an increased lymph node metastases and poor prognosis (Perez-Ordenez et al., 2006).

The tumor suppressor genes, p14 and p16, are both encoded by CDKN2A gene through use of alternative first exons. CDKN2A gene is located on chromosome 9p21 and loss of heterozygosity (LOH) of 9p21 has been proposed as an early event and is seen in 30% of the cases of squamous hyperplasia of the upper aerodigestive track mucosa. The loss of the chromosomal region 9p21 is found in 70–80% of all SCCHN cases, making it the most common genetic alteration (Van der et al., 1994). Re-expression of the wild type p16 protein in SCCHN cells, in turn, leads to significant anti-tumor effects both *in vitro* and in animal models, indicating the importance of loss of p16 expression /function in SCCHN tumorigenesis (Liggett, Jr. et al., 1996; Rocco et al., 1998).

Since the loss of p16 expression in dysplastic lesions of the oral mucosa is associated with an increased risk of progression SCCHN, the presence of 9p loss by LOH analysis is currently being used as a selection criteria for the chemopreventive treatment of patients with oral dysplasia with erlotinib in an NCI sponsored (EPOC trial).

1.2.2.2 p14, MDM2 and p53

The major function of tumor suppressor gene p14^{ARF} is to neutralize MDM2, an inhibitor of p53. Thus it subsequently blocks the association of p53 and MDM2 and leads to p53 stabilization and activation. Loss of p14 results in indirect inhibition of p53 function. p53 is critically important for suppressing aberrant cell proliferation through inhibition of cell cycle progression or induction of apoptosis (Gasco et al., 2002).

In a high proportion of cancers, p53 function is compromised without p53 mutation. Mechanisms include viral oncoproteins, loss of p14, increased expression of mdm-2, *etc.* This observation has taken on greater significance now that SCCHN of the tonsil and tongue base have been found to be strongly associated HPV infection (D'Souza et al., 2007; Fakhry et al., 2008), The E6 and E7 proteins encoded by high risk HPV types 16 and 18 bind to and induce the degradation of p53 and pRB respectively. E7 is found to be the major transforming oncogene during the early stage of carcinogenesis of SCCHN as compared to E6, which appears to function at later stage (Chung and Gillison, 2009). The loss of p14 has been reported in several human cancers, particularly those with wild-type

p53 (Vogelstein et al., 2000). In SCCHN tumors, inactivation of p14 has been reported to occur in 26.5% of cases (Shintani et al., 2001).

Mutation of the p53 gene is the most common genetic change in human cancer (Farnebo et al., 2010), and is estimated to occur in 50% of all cancers. P53 mutations are most often missense mutations that adversely impact its DNA binding function and, therefore its potential to transactivation of genes upregulated by the wild-type protein (Sigal and Rotter, 2000). The proportion of missense mutations in p53 is higher than that seen in other tumor suppressor genes, indicating that expression of p53 mutants may possess additional functions and can confer selective advantages over wild-type p53 (Hussain and Harris, 2000). In SCCHN, p53 is found inactivated or mutated in 50-80% of patients' tumors (Shin et al., 1994a; Balz et al., 2003). Although some reports have shown that these mutations can be identified as early as dysplasia, other studies have found that p53 mutation may be a late event in tumor evolution (Boyle et al., 1993).

Identification of p53 mutation is of prognostic value in SCCHN patients. Patients with a p53 mutation were associated with reduced post-op survival and less chance to responding to neoadjuvant chemotherapy (Poeta et al., 2007; Temam et al., 2000). As a result of the high incidence of p53 mutations in SCCHN, several clinical trials targeting p53 have been undertaken (Khuri et al., 2000; Clayman et al., 1998). However, treatment

with single agent ONYX-015 , an engineered adenovirus that does not express E1B protein, only has marginal effect in SCCHN patients.

1.2.3 VEGF

Overexpression of vascular endothelial growth factor (VEGF) and interleukin-8 (IL-8) have been reported in SCCHN (Howell and Grandis, 2005). VEGF is over expressed in SCCHN tumor expression and this overexpression is correlated with poor prognosis in SCCHN patients (Mineta et al., 2000). It has been shown that VEGF is upregulated by EGFR activation and confers resistance to anti-EGFR therapy (Charoenrat et al., 2000). In addition, combination treatment with bevacizumab, a monoclonal antibody to VEGF, and chemotherapy has been also shown more active than chemotherapy alone in many cancers types (Hurwitz et al., 2004).

Based on the pre-clinical/ clinical observations, several clinical trials using combination anti-VEGFR and anti-EGFR therapy or chemotherapy in SCCHN are under investigation for patients with SCCHN.

1.2.4 Conclusion

Over the preceeding decades, surgery, radiation and chemotherapy have been the major modalities used for primary SCCHN treatment,. In patients with recurrent and/or

metastatic disease systemic chemotherapy plays a more prominent role. However, treatment in this setting has been characterized by limited survival benefit and significant toxicity. This is true even with more contemporary combination therapy with taxanes, such as cisplatin and 5-FU (PF). The advent of molecularly targeted agents has brought more hope for SCCHN patients. For example in the Extreme trial, improvements in major response rate (RR) overall survival (OS) & progression free survival (PFS) were seen with the addition of cetuximab, an EGFR monoclonal antibody (mAb) to classical PF.

In contrast, the current results from EGFR-specific tyrosine kinase inhibitors (TKI) have been disappointing, as only a small number of patients benefit from EGFR targeting therapy. High costs of mAbs and the identification of predictive factors have become important issues to explore. On the other hand, many efforts have been spent to find ways to overcome the resistance to EGFR targeting therapy including HER2, VEGFR, *src*, etc. With the explosive accumulation of knowledge in understanding the carcinogenesis of SCCHN, we expect to see more and more SCCHN patients will benefit from the risk factor-stratified as well as specific targeted therapeutic approaches.

1.3 The Role of Inflammation, Inflammatory Cytokines IL-6 and Casein Kinase 2 in SCCHN

Inflammation is a protective reaction to tissue injury, irritation, or disease, and is an important part of innate immunity. It has been noted that many chronic inflammatory conditions increase the incidence of cancer development (Gonda et al., 2009). Examples of this include ulcerative colitis and colon cancer; chronic hepatitis/cirrhosis and hepatocellular carcinoma; HPV infection and cervical cancer; and *Helicobacter pylori* infection and gastric carcinoma. (Hagemann et al., 2007). In SCCHN carcinogenesis, many important and commonly cited risk factors such as smoking, betel nut chewing, poor oral hygiene, and HPV infections often contribute to increased proinflammatory cytokine expression and aberrant signaling through inflammatory pathways (Allen et al., 2007b; Molinolo et al., 2009).

The growth of SCCHN is generally associated with an inflammatory component, however, many inflammatory cytokines such as interferons, tumor necrosis factors, interleukin-6 have been found to be largely responsible for suppressing proliferation of normal epithelial cells in association with inflammation (Grohmann and Puccetti, 2002; Hernberg et al., 2003; Grant and Begley, 1999). Therefore in the presence of a background, inflammation SCCHN cancer cells must develop molecular strategies to escape anti-tumor immune responses (Lu and Kerbel, 1994). Furthermore, the presence of cells involved in innate immunity, including macrophages, can contribute to malignant progression

via the production of proinflammatory mediators such as tumor necrosis factor (TNF)- α and interleukin (IL)-6 (Greten et al., 2004; Maeda et al., 2003), therefore, attention has been focused on the role of NF- κ B and its upstream activator, IKK β , in the link between inflammation and cancer. In this section, we will review the current studies regarding NF- κ B in SCCHN and then explore the roles of major cytokines involved in the tumorigenesis and tumor progression of SCCHN.

1.3.1 Aberrant Activation of the NF- κ B Pathways in SCCHN

NF- κ B is a transcription factor found to play an important role in the expression of genes involved in inflammation and immune responses. The signaling pathway controlling the activity of NF- κ B is summarized in Figure 1.5. NF- κ B acts as a heterodimer and the most common form of this is p50/RelA. In the resting state, NF- κ B is inactive and retained in cytoplasm by binding to its inhibitory proteins including I κ B α . In response to inflammatory signals, I κ B kinase (IKK) complexes are activated leading to phosphorylation of I κ B α , and the subsequent release of NF- κ B with translocation to nucleus. This translocation enables NF- κ B to activate the transcription of its target genes including cyclin D1, c-myc, *XIAP*, *Bcl-xL*, *Bcl-2*, VEGF, IL-8, COX2, and matrix metalloproteinases (MMP) (Chen and Castranova, 2007).

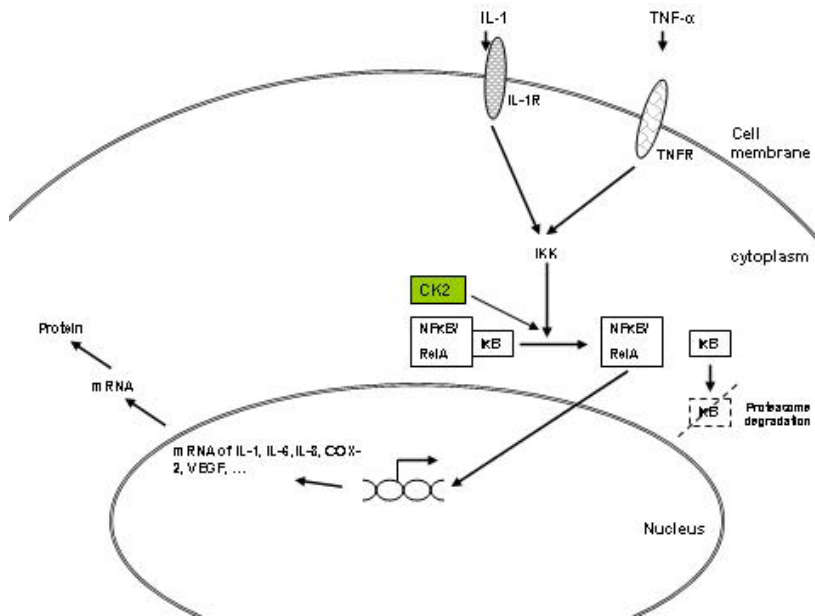


Figure 1.5 The mechanisms of NF-κB activation and the signaling pathway

NF-κB is induced by nicotine and other carcinogens contained in tobacco and betel nut, two of the most important carcinogenic agents that promote development of SCCHN (Zhang et al., 2006; Anto et al., 2002). Several studies have shown that NF-κB is aberrantly activated in human SCCHN cell lines (Ondrey et al., 1999; Arun et al., 2009). Increased nuclear staining of the phosphoactivated form of p65 has been shown to occur in premalignant dysplastic lesions and ~85% of SCCHN specimens, and correlated with

decreased survival, indicating that it is an early event and associated with tumor development (Zhang et al., 2005; Arun et al., 2009). Since NF- κ B was found to be constitutively activated in SCCHN cell lines and tumors, several mechanisms contributing the activation have been proposed such as genetic alterations already present in the epithelium and autocrine or paracrine activation by cytokines from tumors themselves or microenvironment. Kinases/ Cytokines/ growth factors that phosphorylate I κ B and can lead to activation of NF- κ B include IKKs (I κ B kinases), CK2 kinase, IL-1 α , tumor necrotic factor- α (TNF), and EGF (Wolf et al., 2001; Helms et al., 2001; Yu et al., 2006; Biswas et al., 2000). Interfering with NF κ B function in SCCHN results in down regulation of many cytokines and chemokines such as IL-2, IL-5, IL-8, IL-6, IL-10, IL-12, IL-13, IL-17, GM-CSF, and G-CSF as well as a remarkable reduction in cell survival and tumor growth (Molinolo et al., 2009). In addition, aberrant NF κ B activation leads to activation of STAT3 independent of EGFR (Squarize et al., 2006), which may contribute to EGFR inhibitor resistance.

Recently, NF- κ B and its upstream activating signals have been proposed as a potential target in cancer treatment. In SCCHN, bortezomib, an inhibitor of proteasome and I- κ B degradation, has shown to have anti-tumor effects in preclinical and phase I studies (Roccaro et al., 2006). However, the anti-tumor effects of bortezomib are limited by the achievable dose within tumors and toxicity. In addition, many studies found that

NF- κ B also has tumor suppressor activity in several specific cell types including mouse embryonic fibroblasts (Chen et al., 2006), human skin cancer models (Seitz et al., 1998) and mouse liver cancer models (Chen and Castranova, 2007). These findings suggest that caution is needed in inhibiting NF- κ B pathways as a broad strategy in cancer treatment.

1.3.2 The Role of IL-6 in the Pathogenesis and Tumor Progression of SCCHN

Given that NF- κ B activates different proinflammatory cytokines and chemokines and is commonly activated in many types of cancers including SCCHN, it is not surprising that the expression of many cytokines and growth factors including IL-6, IL-8, and VEGF-A, is upregulated in SCCHN (Pries et al., 2006).

IL-6 is a major mediator of inflammatory response and its expression is under NF- κ B's control. It is involved in embryonic development, organogenesis, differentiation as well as tumor growth (Yoshida et al., 1996). IL-6 is upregulated in many epithelial cancer cells such as those breast and prostate (Sasser et al., 2007; Wegiel et al., 2008). In SCCHN patients, IL-6 can be detected in the serum and saliva as well as the tumors of patients. In a small prospective clinical study, the plasma level of IL-6 was found to correlate with the response of SCCN tumors to treatment (Allen et al., 2007a), indicating its potential role in SCCHN tumor progression. Results from a large (N=444) prospective study in SCCHN patients, indicate that IL-6 expression could be a valuable biomarker for predicting recurrence and overall survival among HNSCC patients (Duffy et al., 2008).

There are 3 members of the IL-6 subfamily (or the gp130 sharing cytokine family); IL-6, leukemia inhibitory factor (LIF) and oncostatin M (OSM) (Auernhammer and Melmed, 2000; Gadiant and Patterson, 1999; Grenier et al., 1999; Klausen et al., 2000). These factors are released at the sites of injury by activated monocytes, neutrophils, mast cells, dendritic cells and lymphocytes. Through a paracrine loop, these factors can act on adjacent endothelial, epithelial and stromal cells. The receptors for IL-6 subfamily cytokines include non-signaling α -receptors (e.g. IL-6R α , IL-11R α , and CNTFR α) and signal transducing receptors (e.g. gp130, LIFR, and OSMR) (Heinrich et al., 2003). The latter associate with JAKs and become tyrosine phosphorylated in response to cytokine stimulation. Each of the IL-6-type cytokines is recognized by a specific receptor subunit in combination with the shared gp130.

The canonical signaling pathways following IL-6 treatment involve (1) JAK-STAT (2) PI3K-AKT and (3) Ras-Raf-MAPK, which are summarized in Figure 1.6 (Heinrich et al., 2003). In addition to MAPK cascades, IL-6 also activates the stress-activated members of the MAPK family: p38 and JNK (Bode et al., 2001; Zauberman et al., 1999) through JAK phosphorylation, which creates docking sites for STAT and adaptor proteins. Following the signaling events, genes involved in acute phase reaction and inflammation are activated.

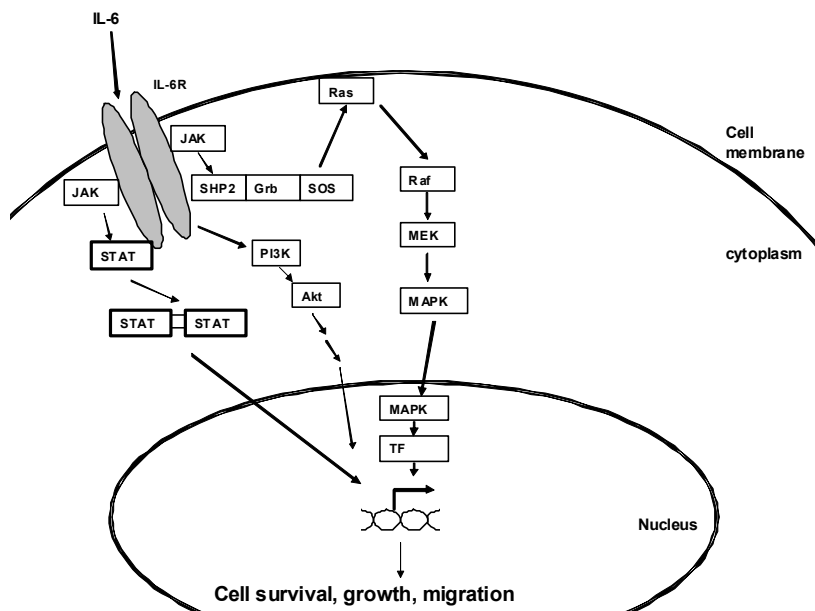


Figure 1.6 Major IL-6 signaling pathways (TF: transcription factor)

Since decreasing IL-6 in serum is associated with response to therapy, while increasing its level is related to cancer progression and recurrence, many efforts have been made to better determine the role of IL-6 in tumor progression. In addition to the contribution to proliferation and invasiveness of SCCHN cancer cells through the JAK-STAT pathway, IL-6 treatment can increase HGF and VEGF expression by SCCHN cells (Kanazawa et al., 2007). A recent study using tocilizumab, a monoclonal antibody against IL-6, reduced tumor growth and angiogenesis in of SCCHN tumor cells in mice (Shinriki et al., 2009), supporting the concept that IL-6 is important for SCCHN progression.

Although the link between IL-6 and cancer is strong from clinical observations, a discrepancy between *in vitro* and *in vivo* studies remains. Several *in vitro* studies have shown that IL-6 treatment can inhibit SCCHN growth, whereas, IL-6 appears to stimulate *in vivo* tumor growth (Knupfer and Preiss, 2007). A recent study showed Ras^{G12V} induced IL-6 expression is required for tumor growth of human kidney cells *in vivo*. However, another study using primary human fibroblasts IL-6 treatment results in senescence (Ancrile et al., 2007; Kuilman et al., 2008). One potential clue to solve this discrepancy may be the presence or absence of the CDK inhibitor p15^{INK4B}. Using MCF10A cells which have deletion of chromosome 9p21, that encodes the INK4-ARF (Iliopoulos et al., 2009), or human kidney cells over expressing SV40 T antigen, which

inactivates the ARF-p53 and INK/Rb (Ancrile et al., 2007), researchers observe a tumor promoting role for IL-6; while in the study using human fibroblasts, in which the locus is intact, IL-6 induced anti-proliferation effect is observed. These studies indicate that of the cellular response to IL-6 response depends at least in part on the status of the INK4/ARF locus (Houk et al., 2009). Since deletion at chromosome 9p21 is commonly observed in oral premalignant lesions and SCCHN, this may strengthen the oncogenic role of IL-6 in the tumor progression in SCCHN if the concept can be experimentally validated.

1.3.3 The Role of Casein Kinase 2 (CK2) in SCCHN

1.3.3.1 Overview of the Structure of CK2

Casein kinase 2 (CK2) was first discovered in 1954 by Burnett and Kennedy (Pinna, 1994; Burnett and Kennedy, 1954):

“An enzyme capable to catalyzing the transfer of phosphate from ATP to protein has been discovered in the rat liver mitochondria. Of a number of proteins tested, only casein is phosphorylated at an appreciable rate...”

Based on this ability to phosphorylate β -casein at its discovery this protein was initially referred to as casein kinase 2 (CK2), which represents a distinct protein family from CK1 serine/threonine kinase superfamily in terms of the structure, substrates and phosphorylation consensus motifs. Fifty-six years after its first description, there have been over 2000 publications describing its activity and biological functions and more than 300 substrates for this kinase have been identified (Cozza et al., 2010; Meggio and Pinna, 2003; Litchfield, 2003).

There are three different subunits that comprise the CK2 hetero-tetrameric complex: α (MW:41KD), α' (MW: 38KD), and β (MW: 28KD). Two catalytic ($\alpha\alpha$, $\alpha'\alpha'$, or $\alpha\alpha'$) and two β regulatory subunits make a complete CK2 heterotetramer ($\alpha\alpha\beta\beta$, $\alpha'\alpha'\beta\beta$, or $\alpha\alpha'\beta\beta$) (Pinna, 2002). The sequence similarity between the catalytic subunit of CK2, α and α' , is about 75% (Cozza et al., 2010). The most important primary structural differences are in the C-terminal region, however, these do not significantly impact the kinase activities (Cozza et al., 2010). CK2 α' is highly expressed in mouse testis and brain. In testis, CK2 α' protects developing spermatocytes from apoptosis while CK2 α' deficiency leads to oligospermia and abnormal development of the sperm head (Seldin et al., 2008). The deletion of CK2 α' (CK2 $\alpha'^{-/-}$) is well tolerated but the males are infertile. (Xu et al., 1999). Mice lacking CK2 α subunit (CK2 $\alpha^{-/-}$) die in mid-gestation, with structural defects in the heart and neural tube (Lou et al., 2008), indicating that CK2 α and CK2 α' are not redundant. The CK2 β subunit is well known to enhance the stability and the catalytic activity of CK2 and function as an acceptor site for intramolecular autophosphorylation (Ackerman et al., 1990). However, as many cellular proteins such as p53, DNA topoisomerase II, FGF-2, and eIF2 β identified in recent studies directly interact with CK2 β , this subunit is believed to provide a docking site or a recruitment surface for substrates or potential regulatory proteins (Filhol et al., 1992; Bojanowski et al., 1993; Bonnet et al., 1996; Poletto et al., 2008). Of note, deletion of the CK2 β subunit in mice is also embryonally lethal (Buchou et al., 2003). All of these studies indicate that CK2 plays a in cell survival.

1.3.3.2 Major Cellular Functions of CK2

Protein kinase CK2 is a ubiquitously expressed and highly conserved serine-threonine kinase, which is localized in the cell nucleus and cytoplasm. It is intimately involved in the control of cell growth, proliferation and differentiation through the regulation of DNA transcription, protein translation, and stability (Guerra and Issinger, 1999; Ahmed, 1994; Zhu et al., 2010; Poletto et al., 2008; Kato, Jr. et al., 2003). To date, many important CK2 substrates are nuclear proteins such as RNA polymerase I and II, DNA topoisomerase I and II, nucleolin, and nucleolar proteins B23 as well as the cellular oncoproteins *myc*, *myb* and *jun* (Pinna, 1990; Issinger, 1993; Bousset et al., 1993; Tawfic et al., 1999), which may emphasize its role in DNA replication and cell proliferation. Of note, the ubiquitin/proteasome degradation of many cellular proteins such as I κ B α and PML are mediated through CK2 phosphorylation (Scaglioni et al., 2008; Poletto et al., 2008; Kato, Jr. et al., 2003). Since most of the CK2 substrates identified so far are linked to fundamental cellular processes, it is not surprising that attempts to knock out the catalytic subunit of CK2 (CK2 α) in mice resulted in embryonic death (Lou et al., 2008) and aberrant activation of CK2 function in the cell results in malignant transformation (Faust et al., 1996).

In addition to cell survival, dysregulation of CK2 is also involved in many inflammatory or viral infectious diseases such as glomerulonephritis and SLE (systemic lupus erythematosus) (Yamada et al., 2005). Many proteins involved in the signaling

pathways implicated in inflammation and viral infection have been shown to be regulated by interaction with CK2. For example, CK2 phosphorylates the HIV-1 reverse transcriptase (Rev) in a CK2 β dependent mechanism (Marin et al., 2000). Viral proteins such as E7 from HPV (human papilloma virus), EB2 from EBV (Epstein-Barr virus), and many others from HSV(herpes simplx virus) are CK2 substrates (Chien et al., 2000; Massimi and Banks, 2000; Medina-Palazon et al., 2007; Bryant et al., 2000).

CK2 activity has also been linked to the pathogenesis of atherosclerotic disease as well as hypoxia signaling (Guerra and Issinger, 2008; Sarno and Pinna, 2008). Studies of MCP-1 (monocyte chemoattractant protein), a marker of macrophage activation, have shown that CK2 is in the pathway of interferon- γ signaling impacts macrophage function during atherosclerosis (Harvey et al., 2007). Studies reveal that CK2 activity is increased under hypoxic conditions, enhancing the activity of HIF-1 α (Hubert et al., 2006). Of note, several studies have shown that CK2 is involved in retinal neovascularization (Kramarov et al., 2006; Ljubimov et al., 2004), implicating this kinase in angiogenesis.

In addition the to its intracellular functions, CK2 can also be found on the external to cells where it can phosphorylates extracellular proteins or the external domains of proteins (Rodriguez et al., 2008). Substrates that have been identified for this ectokinase function of CK2 include vitronectin, a protein that regulates the cell adhesions (Stepanova et al., 2002; Seger et al., 2001); collagen XVII, a receptor whose

phosphorylation inhibits its degradation by metalloproteases (Zimina et al., 2007); and the C9 complement protein that controls cell lysis (Bohana-Kashtan et al., 2005). The major cellular functions of CK2 are summarized in Table 1.

1.3.3.3 Regulation of CK2 and CK2-Involved Pathways

While CK2 is constitutively active, *in vitro*, studies with intact cells have shown that CK2 activity can be modulated by treatment with serum (Orlandini et al., 1998; Carroll and Marshak, 1989), UV induced DNA damage (Kato, Jr. et al., 2003; Kapoor and Lozano, 1998; Keller et al., 2001), and growth factor or cytokine treatment (Ji et al., 2009; Ackerman et al., 1990; Litchfield et al., 1994; Sommercorn et al., 1987; Klarlund and Czech, 1988; Litchfield, 2003). In the EGF signaling pathway, CK2 α was shown to be a direct substrate of Erk2 at site T360/S362 (Ji et al., 2009). The CK2 β subunit has also been reported to be phosphorylated in response to EGF treatment, with concomitant activation of CK2 kinase activity (Ackerman et al., 1990). The factors that can regulate the activity or the stability of CK2 are summarized in Table 2.

The NF- κ B pathway is the most well studied pathway related to CK2. Within this pathway, CK2 can phosphorylate IKK2 and promote its activity and it has been proposed that this is a mechanism of aberrant NF- κ B activation in SCCHN (Yu et al., 2006). In addition, CK2 has been previously shown to phosphorylate multiple sites in the COOH-terminal PEST domain of I κ B α as well as S529 of the Rel/p65 subunit (McElhinny et al.,

1996; Kato, Jr. et al., 2003; Wang and Baldwin, Jr., 1998; Wang et al., 2000). TGF- β treatment of normal mouse hepatocytes leads to decreased CK2 expression and activity, with enhances stability of I κ B and decreased NF- κ B activity(Cavin et al., 2003).

CK2 is also believed to have a role in the regulation of the Wnt signaling pathway as it can phosphorylate β -catenin in the armadillo repeat protein interaction domain (T393) thereby stabilizing its expression, and counteracting GSK3 activity(Seldin et al., 2005; Song et al., 2000; Song et al., 2003). This region is also important for β -catenin's interaction with E-cadherin at cellular membrane and APC and axin in the cytoplasm and TCF/LEF transcription factors in the nucleus.

1.3.3.4 The role of CK2 in Cancer Development and Progression

There is increasing evidence that CK2 is overexpressed in multiple forms of cancer such as prostate, colon and lung cancers (Ahmed, 1994; Munstermann et al., 1990; Yamakin et al., 1994) and the level of expression is correlated with aggressive tumor behavior and poor prognosis in cancers (McCaffery et al., 1997; Landesman-Bollag et al., 2001a; Charoenrat et al., 2004; Gapany et al., 1995). In SCCHN, CK2 expression and activity are both elevated in tumors as well as cell lines (Gapany et al., 1995).When compared with normal oropharyngeal mucosa, the CK2 activity in SCCHN tumors was

increased, suggesting its role in tumorigenesis (Faust et al., 1996). The activity of CK2 in tumors correlates with aggressive behavior and poor prognosis of patients with SCCHN (Gapany et al., 1995; Faust et al., 1996).

Evidence from studies in genetically engineered mice that over-express CK2 in the lymphocytic compartment or mammary gland and go on to develop malignant transformation of lymphocytes and mammary glands emphasize the oncogenic role of CK2 (Seldin and Leder, 1995; Landesman-Bollag et al., 2001b). In studies in prostate cancer, over expression of CK2 in PC3 cells prevents etoposide induced apoptosis , and decreased expression of CK2 mediated by anti-sense DNA leads to reduced viability of PC3-LN4 *in vitro* and cell death *in vivo*(Unger et al., 2004; Slaton et al., 2004). In addition, antisense to CK2 α leads to the growth inhibition of squamous cell carcinoma cells, Ca9-22 (Faust et al., 2000). Anti-sense to both CK2 α and CK2 α' were required to enhance apoptosis in HeLa cells (Seeber et al., 2005). Interestingly, anti-sense CK2 α only had marginal pro-apoptotic effect on benign BPH-1 or normal PrEC prostate cells (Slaton et al., 2004), which indicates a minimal effect on normal or benign tissues supporting its potential utility as an anti-cancer target.

1.3.3.5 Conclusion

There appears to be a strong correlation between inflammation and the development and progression of head and neck cancer , as oral inflammation often precedes the development of oral cancer and the serum levels of inflammatory cytokines correlate with a tumor's response to treatment. This suggests a model in which carcinogen exposure together with inflammatory cytokines create an environment that initiates the mutation or deletion of tumor suppressor genes (such as 9p21 deletion), and activation of oncogenes and also promotes the growth of these genetically altered cells.

While the ability to restore the function of defective genes may hold therapeutic potential, we have also learned that, as they progress, most SCCHN cancers may become addicted to, and therefore dependent on, the aberrant activation of multiple signaling pathways including EGFR, NF- κ B, JAK/STAT, and Akt-mTOR. In spite of the multi-stage carcinogenesis and multiple signaling pathways involved, the development of cancers is not simply a summation of the effects. The functions of an oncoprotein are usually complex: while they may enhance proliferation, they may also activate senescence and anti-apoptotic pathways. As a consequence, cancer cells may be more dependent on the activity of a specific oncogene than normal cells, a process called “oncogene addiction” (Weinstein, 2002). The phenomenon provides a rationale for modern targeted therapy.

Table 1 Summary of the major cellular functions of CK2

Cellular Function of CK2
Proliferation
Anti-apoptosis
Inflammation
Viral infection
Cell migration
Angiogenesis
Motility and Invasiveness

Table 2 Summary of the known factors that regulate the activity of CK2 and proteins known to stabilize CK2 or destabilized by CK2

Factors that regulate CK2 activity	References
Serum	(Orlandini et al., 1998; Carroll and Marshak, 1989)
DNA Damage (e.g. UV)	(Kato, Jr. et al., 2003; Kapoor and Lozano, 1998; Keller et al., 2001),
Growth factors: EGF, IGF, insulin-like growth factor	(Ji et al., 2009; Ackerman et al., 1990; Litchfield et al., 1994; Sommercorn et al., 1987; Klarlund and Czech, 1988)
Cytokines: TGF- β , IL-6	(Litchfield, 2003)

Proteins that are stabilized by CK2 activity	References
c-myc	(Channavajhala and Seldin, 2002)
β -catenin	(Seldin et al., 2005; Song et al., 2000; Song et al., 2003).
Proteins that are destabilized by CK2 activity	References
I κ B α	(Kato, Jr. et al., 2003)
PML	(Scaglioni, et al. 2008)

1.4 The Role of Twist in Cancer Progression

1.4.1 Brief Review of Helix-loop-helix (HLH) Family of Transcription Factors

Twist is a highly conserved protein belonging to basic helix-loop-helix (bHLH) transcription factor family. Studies from *Xenopus laevis*, *Drosophila melanogaster* and mice have shown convincingly that HLH family proteins play important roles in many developmental processes such as lineage commitment, differentiation (e.g. myogenesis, neurogenesis and hematopoiesis), heart / pancreatic development and sex determination (Bain et al., 1994; Lee et al., 1995; Porcher et al., 1996). In addition, they have also been demonstrated to regulate some important metabolic pathways such as phospholipid biosynthesis and phosphate uptake (Hoshizaki et al., 1990; Ambroziak and Henry, 1994).

1.4.1.1 The bHLH Protein Structure

There are two important and high conserved regions in the bHLH protein family that enable these proteins to interact with DNA as well as other bHLH proteins: (1) a region containing basic residues that enhances binding to DNA and (2) an HLH domain, which facilitates protein-protein interaction with other bHLH family members to form homo- or heterodimers (Murre et al., 1994). HLH proteins that lack basic region lose the ability to bind DNA (Cabrera et al., 1994). The DNA binding site for bHLH proteins is known as the E box (CANNTG), which was originally identified as the immunoglobulin heavy chain gene Enhancer (Church et al., 1985). The E-box appears in promoter and enhancer

regions which regulate a large number of neuron-, muscle-, and pancreatic genes' expression. However, the E-box is not sufficient for binding with specific HLH proteins. Either the nucleotides flanking the E-box or other factors such as dimerization choice play a crucial role for the biological specificity of these proteins.

In addition to the basic region, the loop and C-terminal helix also make contact with the major groove of DNA (Harrison and Aggarwal, 1990). The helical structure is important for dimerizing with other HLH protein family member (heterodimer) or itself (homodimer). The interaction between HLH proteins occurs between four helices, which help stabilize the dimer proteins in the absence of DNA (Ferre-D'Amare et al., 1993). The activity of bHLH transcription factors is largely controlled by the dimerization of other partner proteins. For example, introducing the basic region of E12 to MyoD preserves the DNA binding ability but inhibits myogenesis (Davis et al., 1990). For heterodimers, usually one partner stimulates transcription (positive regulator) and the other inhibits it (negative regulator). Some bHLH family proteins such as Id (Inhibitor of differentiation) and emc, lack a basic region and they function as inhibitory regulator to other proteins since they still can dimerize with their dimer partner but forfeit their DNA binding ability and inactivate specific gene regulatory function (Williams and Ordahl, 2000).

1.4.1.2 Classification of bHLH Protein Family

As shown in Table 3, the bHLH family is currently divided into 7 classes according to their dimerization capacities, DNA-binding specificities and tissue distribution (Thompson et al., 1994). Class I bHLH proteins are ubiquitously expressed and known as E proteins, which include E12, E47, HEB, E2-2 and daughterless. They can form either homo- or hetero-dimers (Murre et al., 1989). For example, daughterless protein, which is a *Drosophila* HLH protein, can form heterodimer with achaete-scute T4 and control sex determination (Cline, 1989).

Class II bHLH proteins are tissue-specific transcription factors, including MyoD, myogenin, NeuroD/BETA2, achaete-scute-T4 and Twist. With very few exceptions, they can not form homodimers and preferentially form heterodimers with E proteins (Massari and Murre, 2000). For example, MyoD and myogenin are muscle-specific HLH proteins, known as myogenic regulatory factors (MRFs), which can convert mesodermal cell lines C3H10T1/2 to myoblasts (Olson and Massey, 1979). The heterodimers with E12 bind strongly with E2-box sites while the oligomers or homodimer of E12 or MyoD bind with low affinity (Murre et al., 1989).

Class III bHLH proteins are a group of proteins with an LZ (leucine zipper motif) adjacent to the HLH motif, including the Myc family, TFE3, and SREBP-1 (Zhao et al., 1993).

Class IV includes proteins such as Mad, Max, Mxi1 that can dimerize each other or with the Myc proteins. Class V bHLH proteins are negative regulators of Class I and II proteins. This group includes Id and emc, both of which lack a basic region and inhibit DNA binding ability through dimerization with Class I or II proteins (Benezra et al., 1990). Class VI bHLH proteins contain proline in their basic region and Class VII bHLH proteins have abHLH-PAS domain. This group includes the hypoxia-inducible factor 1 α (HIF-1 α).

The classification of bHLH proteins is summarized in Table 3.

Table 3 Classification of bHLH protein family

Class I	ubiquitously expressed	E12,E47, HEB, E2-2, daughterless
Class II	tissue-specific	Tal1, Hen1, eHAND, dHAND, neurogenin, NeuroD/BETA2, MATH1, MyoD, myogenin, achaete-scute-T4 , Twist
Class III	containing LZ (leucine zipper) motif	c-Myc, TFE3, Mi,TFEB, SREBP-1, SREBP-2
Class IV		Mad, Max, Mxi1
Class V	lack of basic region; transcription inhibitor	Id (Id1,Id2,Id3, & Id4) and emc
Class VI	Containing proline in basic region	HES-1, HES-3, hairy,
Class VII	Containing bHLH-PAS domain	Trh, Sim, AHR, HIF-1

1.4.1.3 The Potential Role of bHLH Proteins in Tumor Development

It is well established that E2A (or E) proteins (either E12 or its alternative splicing product, E47) are important regulators for B- and T- lymphocyte development (Zhuang et al., 1994; Bain et al., 1999). Knocking-out the expression of E2A in mice leads to abnormal T- cell development and rapid development of T-cell lymphomas (Bain et al., 1997), suggesting E2A proteins can act as tumor suppressors. However, E2A proteins are ubiquitously expressed in many tissues in addition to lymphocytes. In studies studying epithelial tissues or cell lines, E2A proteins were found to participate in the EMT process through repression of E-cadherin expression, thereby contributing to tumor progression (Perez-Moreno et al., 2001).

The bHLH protein, E47 has been found to induce α -smooth muscle actin (α -SMA) expression in multiple cell systems (Peinado et al., 2007) and E47 phosphorylation by p38MAPK regulates its oligomerization and DNA binding ability (Lluis et al., 2005). During B cells development, E12 and E47 can be phosphorylated by cyclin dependent kinases (CDKs) (Chu and Kohtz, 2001). In lymphocyte differentiation, the degradation of E47 is under control of Notch-ERK-mediated phosphorylation (Nie et al., 2003).

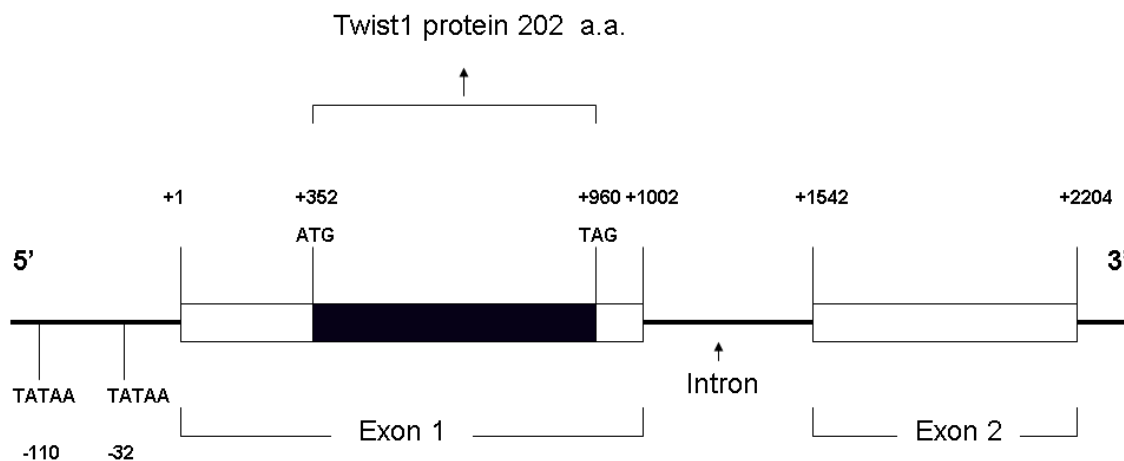
In spite of the active involvement of E47 in EMT, multi-drug resistance and tumor progression, the expression of E47 in human tumor samples has not been that well characterized. In contrast, studies in the inhibitors of the E2A protein such as Id have been shown to be overexpressed in over 20 types of cancers such as breast, colon

and prostate cancers (Wong et al., 2004) and correlate with metastasis and poor prognosis (Perk et al., 2005). Id-1 has been shown *in vitro*, to regulate cell senescence, growth and survival (Ohtani et al., 2001; Swarbrick et al., 2005; Ling et al., 2003), suggesting an oncogenic role for this bHLH protein in tumor progression. In pancreatic tumor specimens, Id-1 was associated with increased intra-tumor vascular density, indicating that Id-1 could play a role in promoting angiogenesis (Lee et al., 2004). A later study using highly specific Id1 antibody reassessing their previous work on prostate, breast and bladder cancer showed only 1/30 prostate cancer samples, 10/45 poorly differentiated breast cancer samples and most transitional bladder carcinomas were positive for Id1 (Perk et al., 2006). These results brought questions regarding the studies of Id1 expression in human tumors and emphasize the importance of specific Id1 antibody in such studies.

1.4.2 Twist Gene, mRNA and Protein structure

The human Twist1 gene (GenBank Accession No. NM_000474) (Howard et al., 1997) consists of two exons and one intron, encoding a 202 amino acid protein. (Figure 1.7). There are two TATA boxes at -32 and -110 upstream of the encoding region and two poly-A sites within exon 2.

Figure 1.7: Scheme of the human Twist1 gene structure



The coding strand of Twist consists of 606 nucleotides with GC % 71.6%. GC content in the third positions of codons (GC3%) is 91.6%. In mammalian cells, GC-rich genes lead to increased steady-state mRNA levels and were expressed several fold to >100 fold more efficiently than their GC-poor counterparts (Kudla et al., 2006).

Twist protein belongs to the Class II bHLH protein family that consists of a group of tissue-specific transcription factors. Comparisons of the Twist protein sequences from different species (ranging from zebrafish, frog, chicken, rat, mouse to human), has led to the identification of several high conserved domains.

In addition to the basic loop (position 108-120) and the two helix domains (at position 121-161) discussed above, four additional protein regions have been described in Twist (Singh and Gramolini, 2009):

- (1) NSEEE-motif (position 19-23): a highly conserved sequence domain at N-terminus, of unknown function.
- (2) NLS1 domain (RKRR; position 37-40): consensus motif for the nuclear localization signal
- (3) NLS2 domain (KRGKK, position 73-77)
- (4) WR-motif : another evolutionally conserved motif at the C-terminus(Castanon and Baylies, 2002). The WR motif is located 20 -55 amino acids C-terminal to the bHLH domain and its function is unclear. However, it might play role in

maintaining the stability of Twist mRNA or protein folding since a nonsense mutation before the WR motif can lead to mild presentation of Saethre–Chotzen syndrome, a human craniosynostosis disorder related to Twist mutation (Gripp et al., 2000).

1.4.3 The Regulation of Twist Function

It has been reported that the epidermal growth factor (EGF), fibroblast growth factor, and interleukin-6 (IL-6) can increase Twist expression and the Twist-dependent EMT (Lo et al., 2007; Zuniga et al., 2002; Sullivan et al., 2009). Twist has been reported to regulate the transcription of STAT3, HIF-1 and NF- κ B (Lo et al., 2007; Yang and Wu, 2008; Cheng et al., 2008b; Pham et al., 2007). Twist has also been shown to rapidly activate or repress other genes upon growth factor or cytokine treatment (Hamamori et al., 1997; Lee et al., 1997), suggesting the importance of regulation at a post-translational level. However, the mechanisms of the post-translational regulation of Twist have not been clearly defined, but potential mechanisms include: (1) Twist phosphorylation by protein kinase A (PKA) and dephosphorylation by protein phosphatase 2A (PP2A); (2) regulation of Twist's transcriptional activity through dimerization in which its binding partner determines whether there is transcriptional activation or repression ; and (3) Ubiquitin/proteasome mediated Twist degradation.

Studies of Twist mutations in patients with SCS have shown that Twist is phosphorylated by protein kinase PKA and dephosphorylated by PP2A) at T121/S123 (Firulli et al., 2005), which in turn regulates the dimerization choice and differential control. In cancer cells, a study has shown that Twist can be phosphorylated by Akt at Ser 42 (Vichalkovski et al., 2010), which leads to inhibition of p53 function in response to DNA damage. Similar to other proteins, Twist is degraded in a ubiquitin/proteasome-dependent pathway (Demontis et al., 2006)

1.4.4 The Role of Twist in Developing Tissues

Twist belongs to the bHLH class II protein family, which is actively involved in many developmental decisions such as osteogenesis, myogenesis, neurogenesis, and sex determination, and it was first described in *Drosophila* as a gene that regulates a series cell fate decisions in the mesodermal lineage. The genes identified in *Drosophila* to be regulated by Twist are listed in Table 4.

In the early stage of embryonal development, Twist is expressed in the invaginating ventral furrow cells. Homozygous Twist “knock-out” embryos die in the first few hours of *Drosophila* development due to failure to form mesoderm (gastrulation failure) with abnormal head involution and in the egg, the anterior end of the embryo is “twisted” (Simpson, 1983; Thisse et al., 1987). The name “Twist” is derived from the observation

of the “twisted” appearance” seen in the lethal mutants. At later stages, Twist is involved in mesodermal differentiation and muscle formation. In vertebrates such as mouse and human, Twist functions more actively and is involved in the “second gastrulation”, the highly conserved vertebrate process of neural crest migration. In Twist-null mice, the Twist-null embryo dies at day 11.5 with a failure of the cranial neural folds to close (Chen and Behringer, 1995), indicating that the most ancient and conserved function of Twist is to facilitate cell movement (Germanguz et al., 2007).

Table 4. Genes activated by Twist during *Drosophila* development

Gene	Function	References
<i>heartless (htl)</i> FGF receptor	Mesodermal migration	(Shishido et al., 1993; Beiman et al., 1996; Shishido et al., 1997; Gisselbrecht et al., 1996)
<i>NK-4</i> (also called <i>msh2</i> and <i>Tinman (tin)</i>)	Mesodermal development	(Lee et al., 1997)
	Heart/muscle development	(Azpiazu and Frasch, 1993; Bodmer, 1993)
<i>Mef2</i>	Muscle differentiation	(Lilly et al., 1995; Bour et al., 1995; Taylor et al., 1995)

In vertebrates, Twist is also involved in cell differentiation during osteogenesis (Murray et al., 1992), myogenesis (Hjiantoniou et al., 2008), neurogenesis (Verzi et al., 2002), hematopoiesis and cardiogenesis (Vincentz et al., 2008) in addition to mediating dorsao-ventral polarity as in *Drosophila*. In mice, Twist mediates growth and differentiation of the limb bud through SHH and FGF signal transduction pathways (O'Rourke et al., 2002).

In humans, mutations of the Twist1 lead to Saethre-Chotzen syndrome (SCS), an inherited autosomal–dominant craniosynostosis disease characterized by premature fusion of the calvarial bones, facial dysmorphisms, and limb abnormalities such as brachdactyly, duplication of distal phalanges, and cutaneous syndactyly (Reardon and Winter, 1994). After the syndrome was first described by Saethre and Chotsen in 1931-1932, it was later mapped to mutations in human chromosome 7p21 by linkage analysis in the affected families as well as sporadic cases in 1990s (Brueton et al., 1992; Rose et al., 1994) and Twist1 was identified as the responsible gene for the syndrome in 1997 (Howard et al., 1997).

In osteogenesis, the sutures of the skull bone are the primary sites of bone formation and differentiation during development. Overexpression of Twist can de-differentiate the osteoblasts and keeps them in a osteo-progenitor-like state and decreasing the expression of Twist can lead to cell differentiation, indicating its critical role during cell type

determination (Lee et al., 1999). In osteocyte/osteoblast cultures from SCS patients, Twist haploinsufficiency has been shown to promote apoptosis (Yousfi et al., 2002), linking Twist's function with cell survival.

Many studies reported that Twist usually plays a role as negative regulator in many cell types such as muscle and neurons. For example, in the direct interaction between MyoD and the basic domain of Twist, MyoD-mediated transactivation is inhibited (Spicer et al., 1996). Therefore, it is not surprising that Twist, a known myogenic differentiation inhibitor, is over-expressed in 50% of the tumors from rhabdomyosarcoma patients, in which tumor cells are derived from muscle precursor that fail to differentiate (Maestro et al., 1999).

1.4.5 The Role of Twist in Tumor Development

The cellular functions of Twist in cancer cells are listed in table 5.

Table 5 Functions of Twist in cancer cells

Functions	Cell lines used in the studies	References
Anti-apoptosis	MEF, Neuroblastoma cells,	(Yousfi et al., 2002; Maestro et al., 1999; Chen and Behringer, 1995; Valsesia-Wittmann et al., 2004; Sasic et al., 2003)
Drug resistance	Breast cancer, prostate cancer	(Li et al., 2009; Wang et al., 2004)
Angiogenesis	Hepatoma, Breast cancer	(Niu et al., 2007; Mironchik et al., 2005).
EMT	Breast cancer	(Yang et al., 2004)

1.4.5.1 Twist Inhibits Apoptosis and Promotes Drug Resistance

As mentioned above (section 1.4.2), in studies of rhabdomyosarcoma, Twist may play some roles in promoting tumorigenesis such as halting terminal differentiation and apoptosis inhibition (Maestro et al., 1999). Twist has been shown to have anti-apoptotic effects in multiple cell lines in vitro and animal models (Yousfi et al., 2002; Maestro et al., 1999; Chen and Behringer, 1995). The anti-apoptotic function can be mediated through targeting the Arf/p53 pathway induced by c-myc, which cooperates with N-myc to promote tumor proliferation in neuroblastoma (Valsesia-Wittmann et al., 2004), modulating TNF α expression (Yousfi et al., 2002), inhibiting mdm2, or through stimulation of the NF- κ B pathway (Sosic et al., 2003).

In addition to its involvement in differentiation and anti-apoptosis, Twist activity is also reported to contribute to multiple drug resistance in breast cancer and taxane resistance in prostate cancer (Li et al., 2009; Wang et al., 2004).

1.4.5.2 Twist Mediates Angiogenesis

Twist also has been reported to mediate angiogenesis, as overexpression of Twist in human hepatoma samples correlates with high tumor microvessel density and increased

intra-/extra-hepatic metastasis (Niu et al., 2007). In breast cancer cell lines and one xenograft mouse model, Twist overexpression is associated with increased vascular endothelial growth factor (VEGF) synthesis, suggesting the potential role in mediating angiogenesis through upregulation VEGF (Mironchik et al., 2005).

1.4.5.3 Twist Promotes EMT and Cell Motility

In addition to the functions mentioned above, Twist is well known to inhibit E-cadherin-mediated cell-cell adhesions and induce cell motility, in a process called the epithelial-mesenchymal transition (EMT) (Yang et al., 2004).

1.4.5.3.1 Definition and Classification of EMT

EMT is a biological process through which an epithelial cell undergoes multiple biochemical and morphological changes to gain a mesenchymal cell phenotype. In contrast to the epithelial phenotype which is characterized by tight cell-cell junctions and interactions with basement membrane through its basal surface, the mesenchymal phenotype consists of a fibroblast-type morphology, loosened cell-cell interactions, and enhanced migratory and invasive capacity. As a result, the cells are usually more resistant to apoptosis and chemotherapy.

EMT and the reverse process, the mesenchymal-epithelial transition (MET) are processes used during embryonic development to allow certain epithelial cells the capacity to transition back and forth between epithelial and mesenchymal status (Lee et al., 2006). Recently, EMT is classified into three subtypes (Kalluri and Weinberg, 2009):

- (1) Type 1 EMT: EMT during implantation, embryogenesis, and organ development
- (2) Type 2 EMT: EMT associated with tissue regeneration and organ fibrosis
- (3) Type 3 EMT: EMT associated with cancer progression and metastasis

The 3 subtypes of EMT are basically divided by the biological process, as there are no distinct differences between the involved signaling pathways. Type 1 is associated with development, Type 2 is involved in tissue regeneration, and wound healing both of which have strong associations with inflammation, trauma, and/or tissue injury. Once inflammatory signals attenuate, type 2 EMT tends to cease. Yet when type 2 EMT persists in the absence of inflammatory signals, it leads to disease state-organ fibrosis, which is commonly seen in kidney (renal tubular fibrosis), heart (cardiac fibrosis), liver (liver fibrosis), colon (some spots in patients with Crohn disease) and lung (idiopathic pulmonary fibrosis, Posttransplant bronchiolitis obliterans syndrome) (Zeisberg et al., 2007a; Zeisberg et al., 2007b; Kim et al., 2006; Pollack et al., 2007; Bataille et al., 2008; Hodge et al., 2009). Type 3 EMT is used by cancer cells to invade and metastasize during cancer progression.

Of note, cells involved in Type 2 EMT and type 3 EMT may be heterogeneous: some cells still continue expressing epithelial markers together with the acquired mesenchymal ones (also called “partial EMT”) , while others become fully mesenchymal phenotypes with complete loss of the epithelial markers (Kalluri and Weinberg, 2009).

1.4.5.3.2 Pro-EMT Cytokines

Many signal pathways are activated by cytokines to initiate EMT. During gastrulation, Wnt/ fibroblast growth factor (FGF) and transforming growth factor- β (TGF- β) pathways as well as transcription factors snail, Eomes and Mesps have been reported to be actively involved in promoting the EMT (Skromne and Stern, 2001; Liu et al., 1999; Chea et al., 2005; Skromne and Stern, 2002). During the EMT in neural crest formation, more signaling pathways such as BMP, c-Myb and msh homeobox 1 (Msx-1) are involved in addition to Wnt/FGF (Liem, Jr. et al., 2000; Karafiat et al., 2007).

In Type 2 EMT, participating growth factors or cytokines that have been reported include TGF- β 1, EGF, FGF-2 and PDGF(Kalluri and Neilson, 2003; Strutz et al., 2002)

In type 3 EMT, many studies have shown the EMT-inducing signals such as TGF- β 1, tumor necrotic factor α (TNF- α), interleukin -6 (IL-6), EGF, and PDGF derived tumor cells and/or the tumor-associated stroma can activate downstream transcription

factor molecules including Twist, Snail, Slug, zinc finger E-box binding hoeobox 1 (ZEB1), Goosecoid and FOXC2 (Thiery, 2002; Shi and Massague, 2003; Sullivan et al., 2009; Colomiere et al., 2009; Grund et al., 2008; Hardy et al., 2010; Ding et al., 2010; Logullo et al., 2010). The cytokine/growth factors identified in type 3 EMT are summarized in Table 6.

Table 6 Cytokines/growth factors involved in type 3 EMT

Cytokine/ Growth factors	Cell lines	References
HGF	Hepatoma , ovarian cancer	(Ding et al., 2010; Vergara et al., 2010; Gotzmann et al., 2006)
EGF	Breast cancer, ovarian cancer	(Colomiere et al., 2009; Hardy et al., 2010; Vergara et al., 2010)
IL-6	Breast cancer	(Sullivan et al., 2009; Colomiere et al., 2009)
PDGF	Malignant hepatocyte Prostate cancer Breast cancer	(van et al., 2009; Kong et al., 2009; Kong et al., 2008; Jechlinger et al., 2006)
TGF- β	Breast cancer , Ovarian cancer Hepatoma	(Shi and Massague, 2003; Logullo et al., 2010; Vergara et al., 2010; Gotzmann et al., 2006)

1.4.5.3.3. Twist and EMT

EMT in cancer cells may not only play a role in mediating a cell's migratory capacity and generating a high-grade, aggressive invasive cancer phenotype but also provide cancer cells with "stem cell like" properties. Twist was first to be reported in cancer-related EMT in a mouse breast cancer cell model (Yang et al., 2004). It was subsequently found to be overexpressed in a variety of cancer and associated EMT phenotypes. Further support for Twist in tumor progression came from studies employing an siRNA approach to inhibit Twist expression which, in turn decreases the metastatic potential of tumor cells in animal models (Yang et al., 2004).

1.4.5.4 Twist Overexpression in Human Cancers

To date, numerous clinical immunochemical studies have shown that Twist is overexpressed in multiple cancer cell types as listed in Table 7. The levels of Twist expression in cancers correlates with metastatic potential and invasiveness (Hoek et al., 2004; Kwok et al., 2005; Rosivatz et al., 2002; Li et al., 2009; Maestro et al., 1999; Matsuo et al., 2009; Mikheeva et al., 2010).

Table 7 List of human cancers that overexpress Twist

Cancers	References
Gastric cancer	(Rosivatz et al., 2002)
Rhabdomyosarcoma	(Maestro et al., 1999)
Breast cancer	(Yang et al., 2004; Li et al., 2009)
Neuroblastoma	(Mikheeva et al., 2010)
Hepatoma	(Matsuo et al., 2009)
Melanoma	(Hoek et al., 2004)
Prostate cancer	(Kwok et al., 2005)
Ovarian cancer	(Hosono et al., 2007)
Nasopharyngeal cancer	(Song et al., 2006)
Head and neck cancer	(Yang and Wu, 2008)
Esophageal cancer	(Sasaki et al., 2009)
Myelodysplastic syndrome	(Li et al., 2010)
Non-small cell lung cancer	(Hung et al., 2009)
Colon cancer	(Valdes-Mora et al., 2009)

1.4.5.5 Downstream Targets of Twist in Cancer Cells

Although Twist is known to be a transcription factor, relatively few Twist regulated genes have been identified. As listed in Table 8, Twist can trans-activate AKT2, YB-1 and Gli1, which are important for tumor metastasis and growth in cancers.

Table 8 Transcriptional target genes of Twist from literature search

Gene's name	Action	Functions	References
AKT2	Activation	migration	(Cheng et al., 2008a)
YB-1	Activation	Resistance to DNA damage Tumor growth	(Shiota et al., 2008)
Gli1	Activation	Tumor growth (rhabdomyosarcoma)	(Villavicencio et al., 2002)
E-cadherin	Repression	Cell-Cell adhesion	(Yang et al., 2004)

1.4.6 Conclusion

Currently, Twist is regarded as an oncogene (Maestro et al., 1999; Valsesia-Wittmann et al., 2004) since its over expression promotes colony formation of mouse embryonic fibroblasts in soft agar assay and participates in many important cellular function such as differentiation, anti-apoptosis and EMT, all of which are important for tumor establishment and progression. Many recent studies have shown that Twist can modulate cancer stem cells, and lead to drug resistance (Vesuna et al., 2009). Efforts to knock down Twist expression in cancer cell lines and animal models have shown promising therapeutic effects on inhibiting tumor growth and metastasis (Kwok et al., 2005). However, in spite of the importance and clinical associations of Twist expression in human tumors, the regulatory mechanisms of Twist expression as well as Twist's downstream targets remain poorly understood. Understanding the mechanism for Twist regulation could lead to the identification of novel prognostic factors and therapeutic strategies for cancer patients.

II. Statement of Objective

Oral cancers are frequently preceded with oral submucous fibrosis (OSF) or other inflammatory conditions associated with periodontal disease or poor dental hygiene months to years before cancer development. OSF is a chronic, progressive, precancerous lesion of the oral mucosa (Ranganathan et al., 2006) and can elicit an inflammatory reaction. The pathologic examination of such lesions usually reveals extensive fibrosis of the epithelium, suggesting a result from EMT similar to those in other organs (Iwano et al., 2002). The pro-inflammatory cytokine, IL-6, and Twist are not only important for the development of precancerous lesions, but their expression has also been shown to be correlated with the clinical outcomes clinical studies of head and neck cancer patients (Allen et al., 2007a; Yang and Wu, 2008). In our preliminary studies using cDNA microarray analysis to profile metastatic SCCHN cells and compare them to isogenic but non-metastatic cells, we identified a relative increase in expression of Twist1, in the more metastatic cell line, suggesting that Twist may be an important player in mediating motility and metastasis in SCCHN cells (Su, Pickering and Myers unpublished observations). Furthermore, several studies have shown that IL-6 treatment can increase Twist expression and Twist-dependent EMT in cancer cells. Although both IL-6 and Twist correlate with the tumorigenesis as well as tumor progression of SCCHN and both serve as important markers for predicting patients' prognosis, their relationship and the

mechanisms by which IL-6 can regulate Twist expression have not been clearly elucidated.

While Twist can be regulated by IL-6 at the transcriptional level through STAT3, HIF-1, and NF- κ B, we have found that Twist expression can also be regulated at the post-transcriptional level in SCCHN cells. While the sequence and secondary structure analysis of Twist predicts that its mRNA is quite stable, the Twist protein has a relatively short half-life between 1-2 hours. A comparative analysis of Twist in embryonic and adult tissues has shown a discrepancy between Twist mRNA expression and Twist protein expression, suggesting that Twist expression is regulated at the post-transcriptional level (Gitelman, 1997; O'Rourke and Tam, 2002). Therefore, we hypothesize that Twist expression is up-regulated by IL-6 stimulation of SCCHN cells at the post-transcriptional level and that this promotes SCCHN tumor progression. As studies have shown that decreasing Twist expression in cancer cells can reverse the metastatic phenotype both in vitro and in vivo (Yang et al., 2004; Yang and Wu, 2008), it follows that a greater understanding the mechanisms of TWIST regulation could hold therapeutic implications for SCCHN patients.

Our working model is shown in Figure 2.1.

Working model

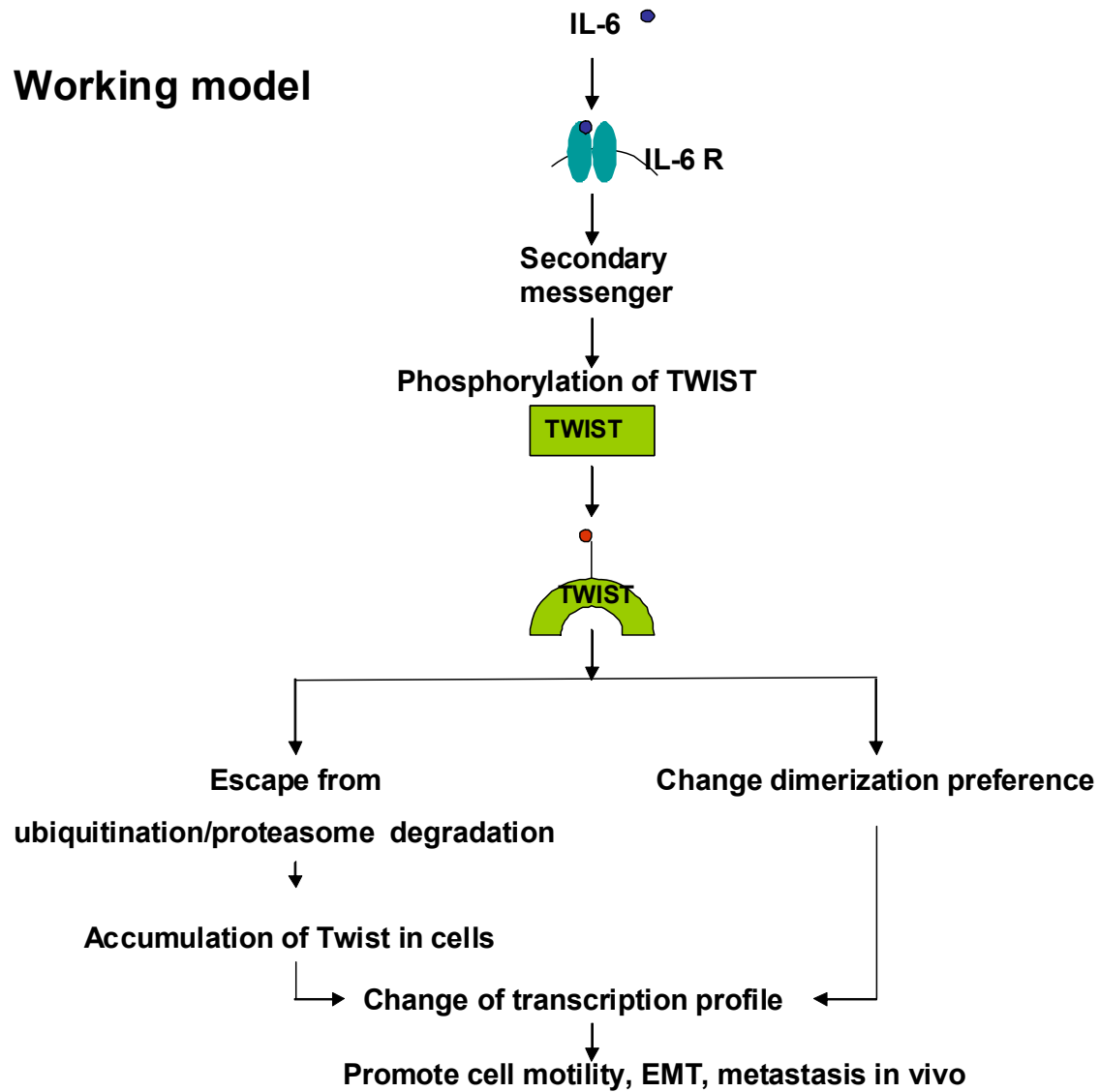


Figure 2.1 Working model for this project

The objectives of the project are:

Part 1: to characterize the relationship between IL-6 treatment and Twist protein expression in SCCHN cells. The levels of secreted IL-6, receptor for IL-6, Twist mRNA, and Twist protein in response to IL-6 are assayed, analyzed, and characterized and the functional importance of this axis is defined *in vitro*.

Part 2: to determine the mechanisms by which IL-6 stabilizes Twist, preventing its degradation. Since the expression level of Twist responds to IL-6 within minutes, phosphorylation modulation rather than transcription regulation is hypothesized as the mechanism. In this part, a search of kinase candidates that regulate Twist stability in this signaling pathway is conducted and verified by experimental approach.

Part 3: to identify the functional significance of the post-translational modification of Twist. Following part1, the most relevant functional consequence of Twist overexpression in SCCHN is increasing cell motility. In this part, phosphorylation site-modified Twist mutants are used to test the functional modulation by phosphorylation. Both *in vitro* migration as well as the lymph node metastatic rate in an orthotopic oral cancer mouse model are examined and discussed.

III. Materials and Methods

3.1 Drugs, Chemicals, Antibodies and Reagents

Cycloheximide (CHX), MG132, 2-Dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole (DMAT) or 4,5,6,7-tetrabromobenzotriazole (TBB), U0126, AG490, SB202130 and SP600125 were purchased from EMD Chemicals (Gibbstown, NJ). Human recombinant IL-6 and CK2 kinase were purchased from Cell Signaling (Danvers, MA). Phospho-threonin (42H4) mouse mAb, CK-2 α antibody, and Twist antibody were purchased from Cell Signaling Technology. Phosphoserine antibody was from Abcam (Cambridge, MA). Anti-HA antibody was from Roche Applied Science (Indianapolis, IN). Lipofectamine 2000 was purchased from Invitrogen. Nucleofector System was purchased from Lonza (Basel, Switzerland). CK2 kinase was purchased from Cell signaling. ATP, [γ - 32 P] was ordered PerkinElmer (Waltham, MA)

3.2. Cell Culture

The cell lines were used for experiments included, 293T, a human embryonic kidney cell line; A549, a human lung cancer cell line, a panel of SCCHN cell lines from the primary tumor of patients with squamous cell carcinoma of the head and neck (FaDu, HN30, and MDA686-TU) and SCCHN cell lines from the metastatic lymph nodes of patients with SCCHN (OSC-19, HN31, and MDA686-LN). All cells were authenticated by STR analysis and found to be unique, except for HN30 and HN31 which were confirmed to be isogenic as they were derived from the same patient.

All SCCHN cells and human 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin/streptomycin, L-glutamine, sodium pyruvate, nonessential amino acids, and a 2-fold vitamin solution. The A549 cell line is from human lung cancer and was cultured in RPMI 1640 supplemented with 10% FBS. All cells were grown in a humidified incubator at 37°C and 5% carbon dioxide. The cultures were free of Mycoplasma species and maintained for no longer than 8 weeks after recovery from frozen stocks. Neomycin-resistant stable cell lines were maintained in G418 (500 µg/ml).

3.3. Western Blot

3.3.1 Sample preparation

Cultured cells were washed with ice-cold PBS twice and then lysed with ice-cold RIPA lysis buffer (NP-40, 1%; deoxycholate, 0.5%; SDS, 0.1% , in PBS) supplemented with fresh 2 mM NaF, 0.5 mM Na₃VO₄, 1X protease cocktail and kept on ice or 4°C for 30 minutes. Then cell lysates were collected by a cell scraper and placed in eppendorf tubes for centrifugation. After removal of the insoluble fraction the protein concentration of the samples was determined by DC Protein Assay kit (Bio-Rad, Hercules, CA).

3.3.2 Immunoblotting

Each protein sample was diluted with 6X SDS sample buffer with freshly added β-

Mercaptoethanol, boiled at 95-100 °C for 10 min, and chilled on ice for 2-5 minutes. The samples were applied to each well of SDS-polyacrylamide gels (SDS-PAGE), electrophoresed, and electro-transferred to a PVDF (Polyvinylidene Fluoride) membranes. Then the membranes were blocked with either 5% non-fat dry milk, or 1% BSA, 10 mM Tris pH 7.5, 100 mM NaCl, 0.1% Tween 20) at room temperature for 30 min or 45 min respectively. Then the membranes were first incubated with the primary antibodies (1:1000 dilution in 1% BSA-TBS-T) at 4°C over night, washed ,then incubated with secondary antibodies (fluorescence– or HRP (horseraddish peroxidase) - conjugated) at room temperature for 0.5-2 hours. The bands were detected by Li-Cor imaging system or film development after ECL reagents added and quantified by software *Image J*.

3.4. Immunoprecipitation

Immunoprecipitation was conducted on cell lysates prepared as above and with concentrations of approximately 1000 µg/ml of protein samples with 0.5µg IgG control or 1-2 µg of antibody of interest according to the manufacturers' recommendations. After gentle rotation at 4°C overnight, 50 µl of pre-blocked agarose protein A or G beads were added for additional 1 h at 4 °C. Then the immunocomplex was washed for 4 times with complete IP lysis buffer 500 µl, vortexed, boiled at 95-100°C for 5 minutes, and applied to a SDS-PAGE gel for electrophoresis and western blotting.

3.5. Immunocomplex Kinase Assay

3.5.1 Generation of Hypo- & Hyper-phosphomimetic Twist mutants

To facilitate immunoprecipitation and protein purification, we first cloned 6 myc tags and 6 his (hexa-his) into pcDNA3.1+ between BamH1 and EcoR1 using a standard PCR cloning procedure with sequence verification. The entire human Twist cDNA coding sequence (NM_000474) was then cloned into EcoR1 site.

Point mutations were introduced by PCR using the QuikChange® Multi Site-Directed Mutagenesis Kit following the manufacturer's protocol (Stratagene, Cedar Creek, TX). The primer information for each condition is listed in Table 9. All of the inserted sequence was confirmed by using ABI PRISM 3730 DNA analyzer (Applied Biosystems, Foster, CA, USA) at the DNA core facility at UT MD Anderson Cancer Center.

Table 9 Primer sequences for Twist mutants using *in situ* mutagenesis

S18,20A-Twist	- tcgccggccgacgacagcctggcaaacgcagaggaagagccagaccggcagc-
S18,20D-Twist	- tcgccggccgacgacagcctggacaacgcagaggaagagccagaccggcagc-

3.5.2 Kinase Assay

The immunocomplex kinase assay was carried out in human 293T cells 48 hours after transfection. The Myc-Tagged Wild-type (WT) Twist and S18,20A –Myc- Twist were immunoprecipitated with Myc antibody then incubated with recombinant CK2 kinase (Cell Signaling) and 1 μ Ci γ -P³²-ATP in kinase buffer for 15 min at 30 °C. Then the reactions were terminated by adding 6X SDS sample buffer and boiling at 95-100 °C for 5 minutes. All the samples then were electrophoresed in SDS-PAGE, electro-transferred to PVDF membranes and subsequent autoradiography or western blotting.

3.6. Transfection

3.6.1 Lipofectamine Transfection

For 293T cell transfection, 4-6 x 10⁶ cells were plated onto 10cm dishes in order to reach 90-100 % confluency 24 hours before transfection. Lipofectamine 2000 20 μ l was diluted in 250 μ l PBS at room temperature for 5 min (Mix 1). Plasmids (5 μ g) were then diluted in 250 μ l PBS and mixed well with Mix1 and left at room temperature for 20 min. Before transfection, the cells were incubated in 5 ml of serum free medium. Then the plasmid / lipofectamine mixture was added drop by drop directly into the culture dish and the cells were kept at 37°C in the incubator for 4-6 hours. After replacing the serum free media with 10% serum containing media, the cells were left to grow for 48 hours before harvest for western blot or immunoprecipitation.

3.6.2 Electroporation

For difficult-to-transfect SCCHN cells, electroporation was used. The experiment was carried out in Nucleofector System (Lonza, Basel, Switzerland) with solution V. For this method, 2×10^6 cells were suspended well in 100 μ l of electroporation buffer and mixed with plasmid DNA (4 μ g). Then electroporation was carried out on the mixture in an electroporation cuvette using different programs according to cell lines. (V-020 for OSC-19, T-001 for HN30/HN31, and X-001 for A549, use). The electroporated cells were then recovered in pre-warmed (37°C) serum-free RPMI 500 μ l for 5 min and transferred to 6-well plates and placed in the incubator.

3.6.3 Selection of Stable Clones

For establishment of stable lines (HN31-control, HN31-WT_Myc-Twist, HN31-S18,20A-Myc-Twist, and HN31-S18,20D-Myc-Twist), G418 (Neomycin) 500 μ g/ml was added to the complete medium 48 hours after transfection together with a control plate without transfection. Starting at the 4th week after selection, all (100%) of the cells in the control plate died while a portion of the transfected cells still grew. These cells were then expanded and cryo-preserved for future experiments.

3.7 CK2 Kinase Activity Assay

Cell lysates were collected in hypo-osmotic buffer after treatment with PBS (control) or IL-6 (20ng/ml) for 30 min, and the cell lysates (5 μ g), synthetic peptide CK2

substrate (RRRADDSDDDDD; 0.1 mM)(Genescript, Piscataway, NJ), and γ -P³²-ATP were incubated in the assay dilution buffer for 10 min at 30 °C. The phosphorylated substrate was then separated on P81 phospho-cellulose paper and quantified with a scintillation counter.

3.8. Real Time RT-PCR

The RNA was extracted from cells with RNeasy ® Mini Kit (Qiagen, Valencia, CA), and real time RT-PCR was carried out with Verso 2-step QRT-PCR kit (Thermo Fischer Scientific, Worcester, MA) that uses SYBR fluorescent dye to detect the amplified PCR product. The relative gene expression was normalized to endogenous GAPDH expression. The primer information was listed in the below table:

Table 10 Primer information for real time PCR

Gene	Primer's name	Product size	Primer sequence
Twist	Forward	150 bp	Ccttaccaggtcctccaga
	Reverse		Cagaatgcagaggtgtgagg
IL-6R	Forward	86 bp	Tgagctcagatatcgggctga
	Reverse		Cgtcgtggatgacacaagtga
GAPDH	Forward	188 bp	Atcatccctgcctctactgg
	Reverse		Ccctccgacgcctgcttcac

3.9 IL-6 Enzyme-Linked Immunosorbant Assay (ELISA)

The secreted IL-6 in the supernatants of cultured cells was determined with a Human IL6 immunoassay (ELISA) kit (R&D systems, Minneapolis, MN). Briefly, the cells were cultured to subconfluency and serum starved overnight before replacement with 2% serum containing medium for 48 hours. The supernatants were then centrifuged at 2000 rpm for 6 minutes to remove the floating cells before assaying for IL-6 expression. Meanwhile, the cultured cells were trypsinized and the total numbers of cells were counted. The data was expressed as pg/million cells.

3.10 Transwell Migration Assay

The transwell migration assay was performed in 24-well BioCat control inserts (BD Biosciences, San Jose, CA) with an 8µm pore size polycarbonate filter. The lower chamber contained 700 µl of 10% medium as a general chemo-attractants . Cells were washed once in serum free medium and plated onto the upper chamber in 500 µl of serum free medium to create a concentration gradient toward the lower chamber. The experiment was carried out within 20-22 h after cell plating and incubation in a 37 °C incubator. The non-migratory cells on the membranes were removed by cotton rod swiping and the migrated cells were stained for cell counting.

3.11 Orthotopic Xenograft Tongue Cancer Mouse Model

The care, use, and treatment of all of the animals in this study were conducted on an Institutional Animal Care and Use Committee (IACUC) approved protocol. For the orthotopic xenograft nude mouse model of tongue cancer, 5×10^4 cells from each stable cell line established from the HN31 cell line were suspended in 30 μ L of serum-free Dulbecco modified Eagle medium (DMEM) and injected into tongues of athymic nude mice. The mice were examined twice a week for weight loss and tumor measurement with calipers. If a mouse lost more than 20% of their preinjection body weight, euthanasia was performed. Fourteen days after tumor injection, all mice were sacrificed and necropsy was performed with removal of tongue tumors and cervical lymph nodes. All tumors and cervical lymph nodes were fixed in formalin and embedded in paraffin for hematoxylin-eosin (H&E) and pan-cytokeratin (Lu-5) staining for evaluation of metastasis.

3.12 Statistical Analysis

Statistic analyses were carried out in the SPSS 16.0. For comparing the means from two independent groups, two-sided Student's *t* test was used in all statistical analyses.

H_0 : is null hypothesis , supposing the means from the two groups are equal ;

H_1 : is alternative hypothesis , supposing the means from the two groups are not equal.

Statistical significance was accepted when the P value was less than 0.05, which means if the means are equal as null hypothesis, the chance to have the result is less than 5 in 100. The smaller the P value, the higher the chance to reject H_0 . Since the H_0 is rejected, we conclude there is significant evidence to support H_1 .

IV. Results

4.1. IL-6 production and biological functions in Squamous Cell Carcinoma of Head and Neck Cells *in vitro*

4.1.1. The Expression of the Receptor for IL-6 (IL-6R) and IL-6 in SCCHN Cells

It has been reported that most non-small cell lung cancer and squamous cell carcinoma of head and neck (SCCHN) cell lines express the receptor for IL-6 (Shinriki et al., 2009). To further characterize the level of the receptor for IL-6 (IL-6R) in SCCHN lines, mRNA for IL-6R was quantified by real-time RT-PCR. As shown in Figure 4.1, there is no expression of IL-6R in the human immortalized keratinocyte epithelial cell lines HOK16B and NOM (normal oral mucosa) 9. In contrast, all of the SCCHN cell lines examined had elevated IL-6R relative to HOK16B or NOM 9 control lines (all $p < 0.05$ by student t 's test) .

The secreted IL-6 in the supernatants of cultured SCCHN cells was determined using a human IL-6 ELISA kit as described in section 3.9. As shown in Table 11, all of the SCCHN lines excreted increased levels of IL-6 relative to conditioned culture media from immortalized human keratinocyte cell lines.

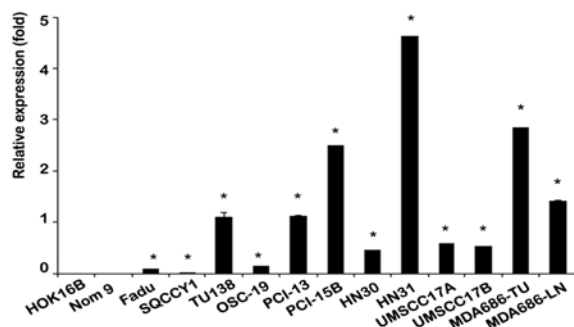


Figure 4.1 Relative expression of the receptor for IL-6 (IL-6R) in SCCHN cells

The levels of IL-6R mRNA expression were normalized to the expression levels of the housekeeping gene GAPDH and were expressed as the mean fold change from basal \pm s.e.m. Because there is no detectable mRNA expression in HOK16B and Nom 9 lines, all expression levels were normalized to SQCCY1. All experiments were done in triplicate for each cell line. (*: $p < 0.05$)

Table 11 Levels of secreted IL-6 in SCCHN cell lines

	conc (pg/million cells)	STDEV
10% DMEM	0	0.00
Nom 9	0	0.20
Sqccy1	4.79	0.68
UMSSC17B	41.16	3.82
TU138	41.96	2.14
UMSSC10B	111.88	2.41
Fadu	113.29	3.19
HN30	214.85	2.71
PCI-15B	465.05	44.87
PCT-13	659.99	32.20
OSC-19	840.33	21.12
JHU011	1423.83	47.77
UMSSC11B	5609.98	8.72

4.1.2 Proliferation Rate of SCCHN Cells in Response to Exogenous IL-6 Treatment

Since all the SCCHN lines express various levels of IL-6R and IL-6, next we determined the effect of exogenous IL-6 on proliferation of these cells. Cell proliferation was measured by MTT assay after 48 hours treatment of the cells in IL-6-containing 5% DMEM. As shown in Figure 4.2, there is a dose-dependent inhibition of cell proliferation with IL-6 treatment.

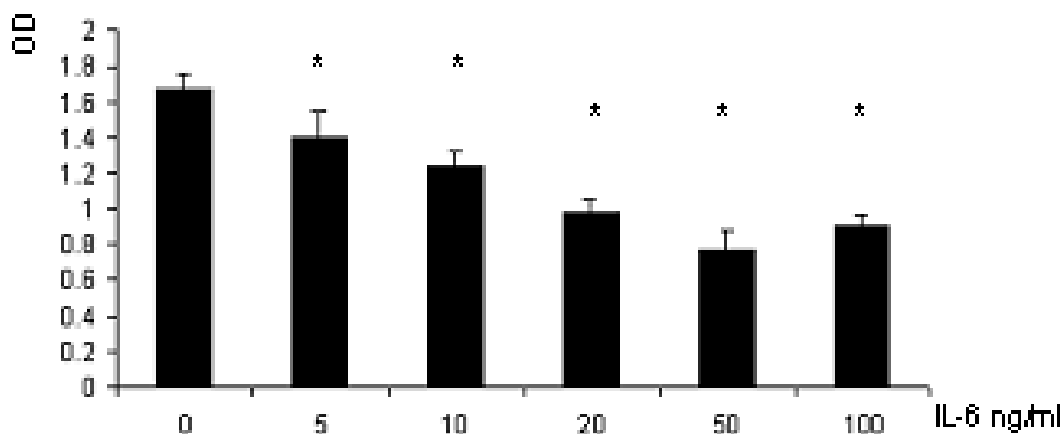


Figure 4.2 Proliferation rate of OSC-19 SCCHN cells in the presence of IL-6

A total of 100,000 cells were plated into each well in 1ml volume in a 24-well plate. After the cells had attached they were serum starved overnight, and the medium was replaced with 5% DMEM containing IL-6 at the indicated concentration for 48 hours. On the day of harvest, 200 μ l of MTT solution (2 μ g/ml) was added to each 1-ml medium for 2 hours. Then the cells were lysed and prepared for optic density (OD) measurement by adding 1ml of DMSO each well after all the remaining medium was aspirated. The y-axis in the figure shows the OD at 570 μ m wave length in a plate reader. (*: compared with no treatment group (concentration =0), $p < 0.05$ by student t 's test)

4.1.3 IL-6 as a Chemoattractant in Transwell Migration Assay

To determine if IL-6 is a chemoattractant for SCCHN cells, the transwell migration assay was performed with various concentration of IL-6 added to the 0.1% serum containing DMEM in the lower chamber while 200,000 cells of OSC-19 were plated onto the upper chamber in 0.5 ml of serum free medium. The migratory cells were determined after 22 h of incubation.

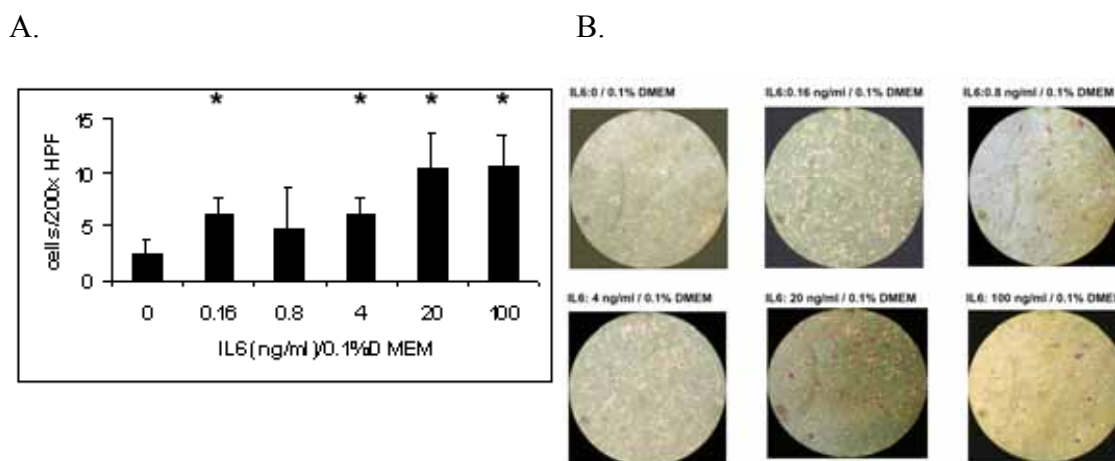


Figure 4.3 IL- 6 as a chemoattractant in trans-well migration assay

A. The average number of migratory SCCHN cells in each condition is expressed as average number of cells/200 per high powered field \pm s.e.m. *: represents $P < 0.05$ by student t 's test comparing treated and control (IL6 =0) groups. B. Representative pictures for each condition are shown in the lower panel. The motility of SCCHN cells is increased as the concentration of IL-6 is increased.

4.1.4 IL-6 Promotes the Motility of SCCHN cells

In order to further assess the impact of IL-6 treatment on cell motility, OSC-19 SCCHN cells were pre-treated with IL-6 at 20ng/ml for 24 h then re-plated onto the inner chamber for studying the cell migration in a trans-well assay. As shown in Figure 4.4, the IL-6 pre-treated SCCHN cells have greater motility relative to the control group with $P < 0.05$. The lower panel showed the representative pictures under microscopic exams in the corresponding conditions.

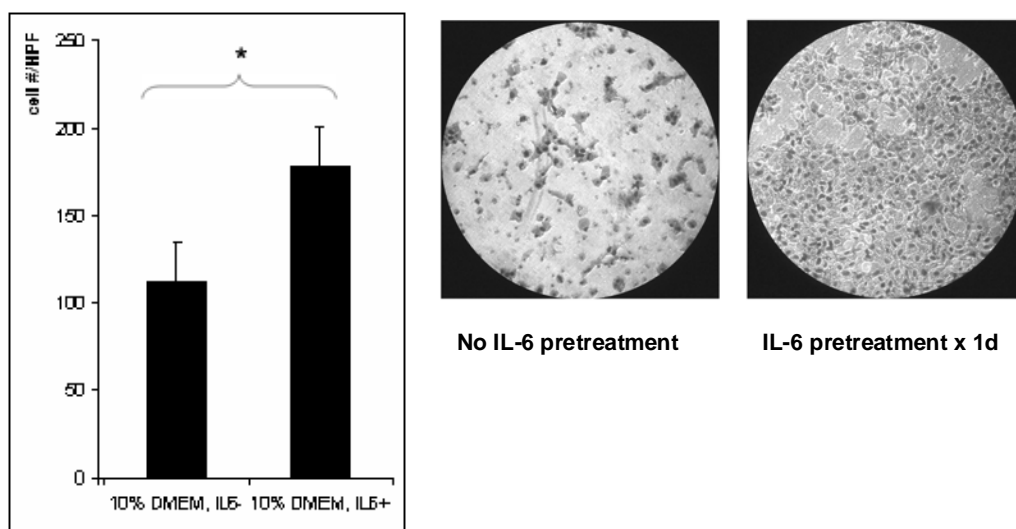


Figure 4.4 IL-6 pretreatment of OSC-19 SCCHN cells increases the cell motility in a trans-well migration assay. *: $P < 0.05$ by student t 's test

4.1.5 Changes of EMT Markers under IL-6 Stimulation in SCCHN

Because EMT is frequently associated with increased cell motility as reviewed in Section 1.4, we next examined the impact of IL-6 treatment on the expression of EMT markers in OSC-19 SCCHN. The cells were serum starved overnight then treated with IL-6 20ng/ml and harvested for western blotting at the indicated times. As shown in Figure 4.5, the protein expression levels of Twist increased over time while the epithelial marker E-cadherin and Snail, a mesenchymal marker, remained at similar levels. α -SMA (smooth muscle actin), another mesenchymal marker for EMT, appeared briefly after 3-6 h of IL-6 treatment and went back to the basal level. These data show differential effects on the expression of EMT markers on IL-6 stimulation.

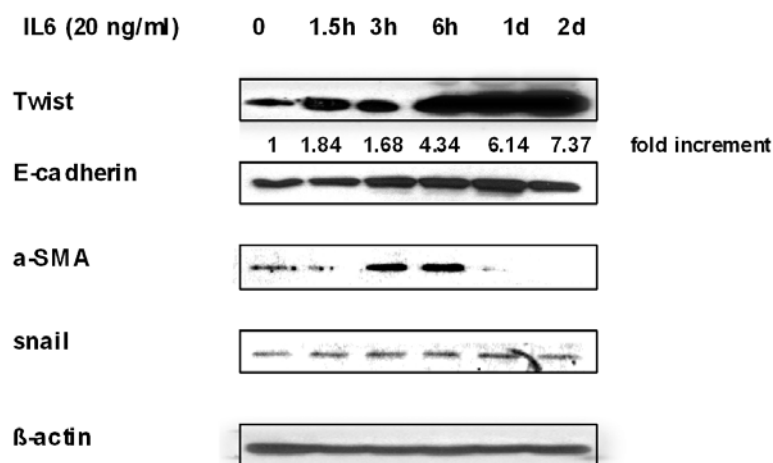


Figure 4.5 Changes of EMT markers following IL-6 treatment in SCCHN cells.

4.1.6 Conclusions and Discussion

In our preliminary data, almost all SCCHN cells express receptors for IL-6 and secrete varying levels of IL-6 into the culture medium. Despite the presence of an apparent autocrine loop, some of the cells still can respond to exogenous IL-6 stimulation, suggesting that the receptors for IL-6 were not saturated. The hypothetical model of how IL-6 works on SCCHN tumor cells through autocrine/paracrine and systemic pathways is illustrated in Figure 4.6. We found that IL-6 promotes cell motility as well as induction of several mesenchymal markers of EMT. Although morphological EMT was not observed following short-term stimulation of IL-6 (data not shown), the appearance of mesenchymal markers in the epithelial cancer cell lines suggest at least partial EMT induction by IL-6. Of all the mesenchymal markers tested, the levels of Twist expression continue to increase with the length of exposure to IL-6, suggesting that Twist is a downstream target of the IL-6 signaling pathway. This observation is examined further in the following sections.

Several research and clinical studies have shown that IL-6 promotes cancer cell proliferation and tumorigenesis (Mueller et al., 2010; Lederle et al., 2010; Pitulis et al., 2009; Grivennikov et al., 2009), however, there are discrepancies between *in vivo* and *in vitro* studies (Regis et al., 2009; Knupfer and Preiss, 2007). Consistent with the published results of other investigators, we observed an IL-6 dose-dependent inhibition of SCCHN

cell growth *in vitro* cultures (Figure 4.2). Whether or not this is cell- line specific or whether it is only specific for *in vitro* condition needs to be evaluated in further experiments. As reviewed in section 1.3.2, the status of chromosome 9p21 (INK4/ARF) may also contribute to the inhibitory effects of IL-6. Since 9p21 deletion is commonly seen in oral premalignant lesions as well as SCCHN in 39-67% of these patients (Gollin, 2001), it will be worthwhile to incorporate genetic information regarding this locus along with a cell's growth response to IL-6 in future experiments.

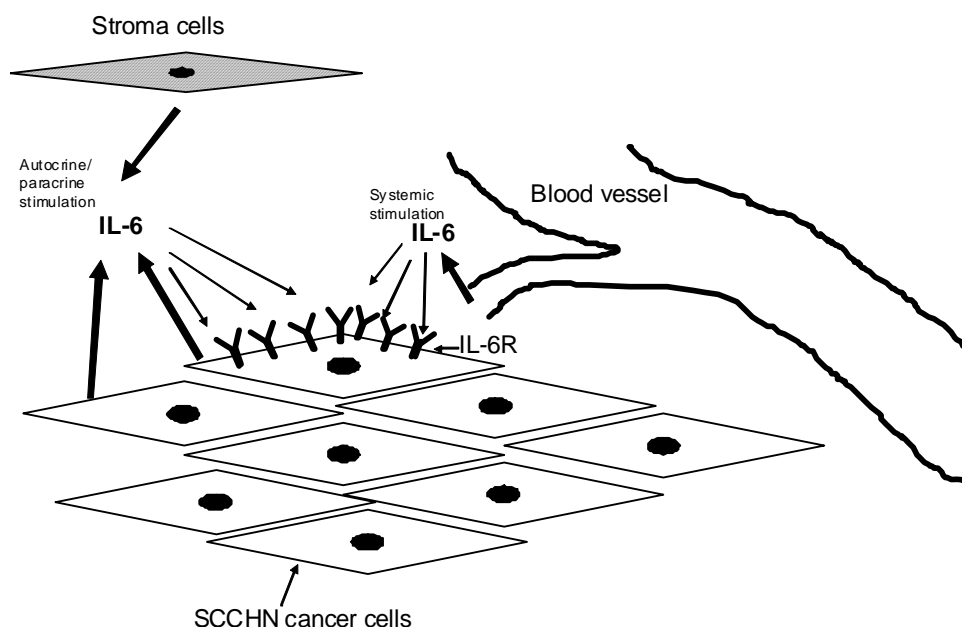
4.2. Twist Regulation by IL-6 in SCCHN Cells

4.2.1 Time–Course of Twist Regulation by IL-6

Since we have shown that the up-regulation of Twist by IL-6 is the most striking changes among the EMT markers, we next examined the impact of IL-6 treatment on Twist expression in a shorter period (0-4h). All cell lines used in Figure 4.6 are from SCCHN except A549 and H1703, which are lung cancer cells. As shown in Figure 4.7, Twist expression is up-regulated shortly after IL-6 treatment in not only OSC-19- but also in HN31-, FaDu-, & MDA 686-LN- SCCHN. Of note, Twist up-regulation after IL-6 treatment is also observed in the lung cancer cell line A549, indicating that the

phenomenon is not cell-type specific or tissue-specific. In this figure, there are three “paired” or “isogenic” cell lines, e.g. HN30 & HN31, MDA686-TU & MDA686-LN, UMSSC17A & UMSSC17B, which were established from primary tumors (HN30, MDA686-TU, UMSSC17A) or metastatic lymph nodes (HN31, MDA 686-LN, UMSSC17B) of the same patients. Two out of these paired cell lines showed the Twist up-regulation is increased in the metastatic lines as compared to the lines of the primary sites, suggesting an underlying different biological response to IL-6 in metastatic lines. This is also observed in other lines established from metastatic LNs such as OSC-19.

Figure 4.6 Hypothetical model for how IL-6 works on SCCHN tumors



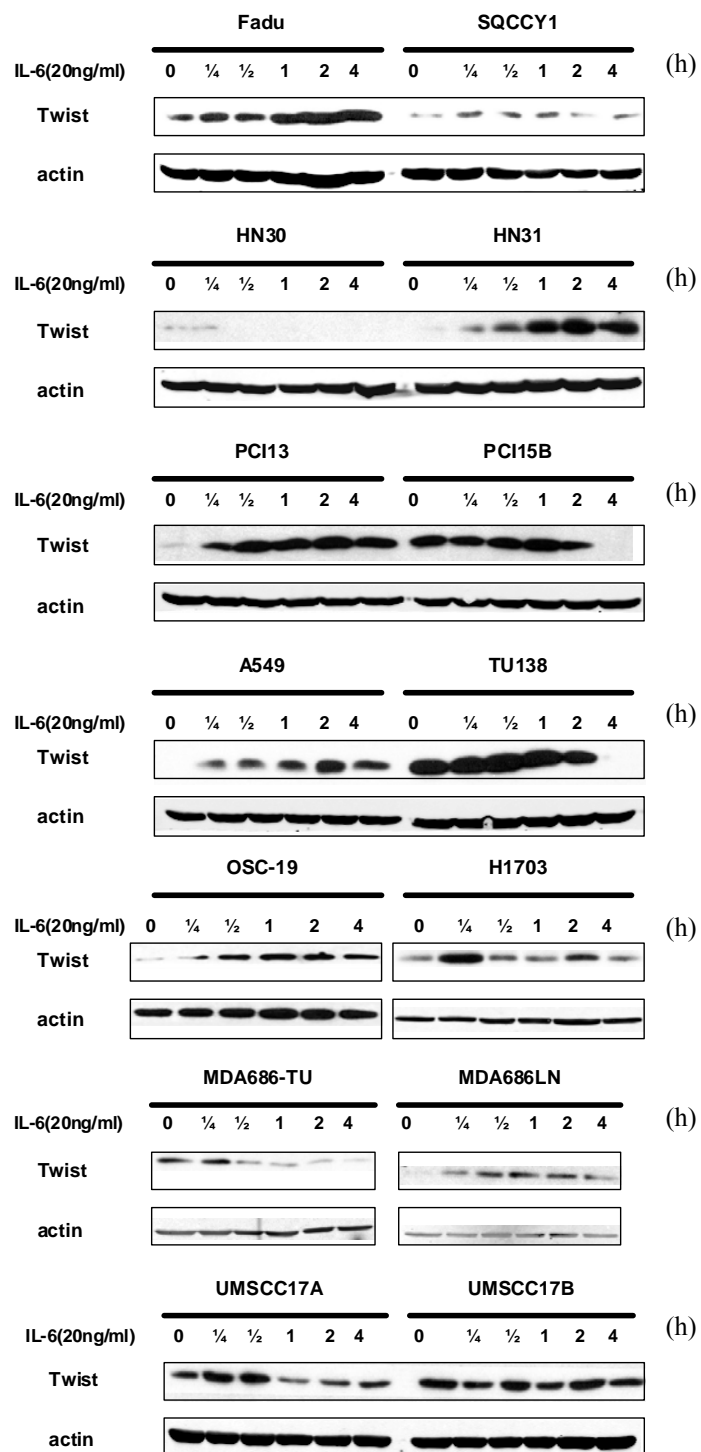


Figure 4.7 Twist expression in response to IL-6 stimulation in SCCHN cells

4.2.2 Up-regulation of Twist by IL-6 is Transcription-independent

Since Twist has been reported to be a transcriptional target of STAT3 (Lo et al., 2007), which is also a major downstream target of IL-6 signaling, we evaluated whether IL-6 regulates Twist expression at the transcriptional level. Using RT-PCR for Twist, the level of Twist mRNA was examined in IL-6 treated HNSCC cells.

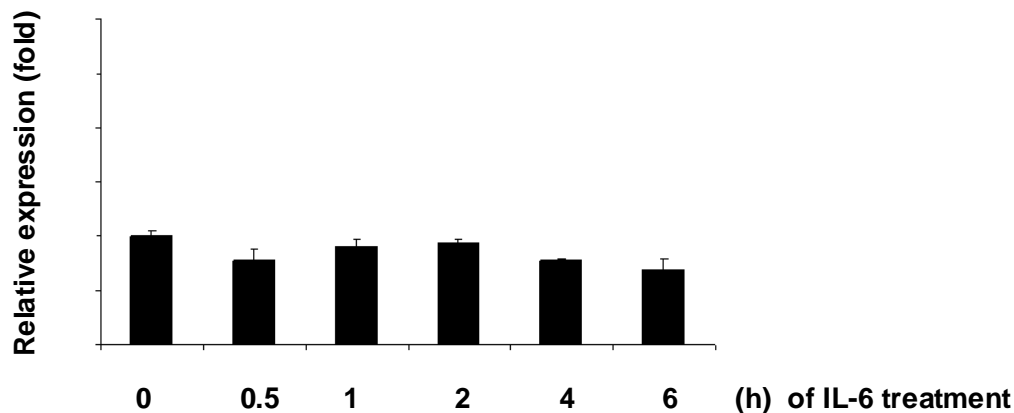


Figure 4.8 Twist mRNA expression in response to IL-6 stimulation in OSC-19 SCCHN cells

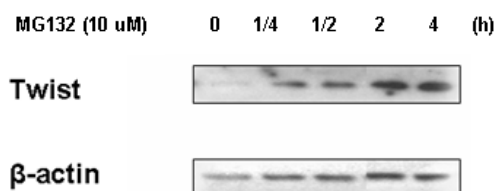
The experiments have been performed in duplicate in the OSC-19 and HN-31 SCCHN lines. Figure 4.8 shows a representative result from OSC-19 with the corresponding expression levels of Twist mRNA quantified by real-time PCR. Values were normalized to the expression levels of the housekeeping gene GAPDH, and are expressed as the mean fold change from basal \pm s.e.m.

In contrast to the dramatic increase of the Twist protein after IL-6 treatment, Twist mRNA levels remained relatively unchanged under the same experimental conditions, indicating an underlying transcription-independent mechanism controlling the increase in Twist expression that is induced by IL-6 treatment.

4.2.3 IL-6 Upregulates Twist Expression by Inhibiting Its Degradation

The observation that IL-6 up-regulates Twist expression post-transcriptionally led us to investigate whether or not the degradation of Twist was in turn modulated after IL-6 treatment.

A.



B.

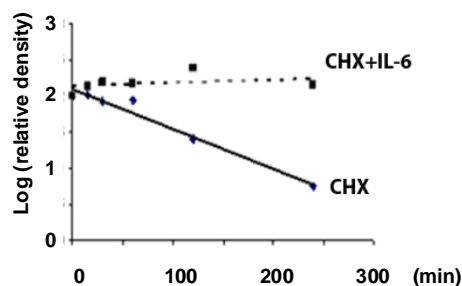
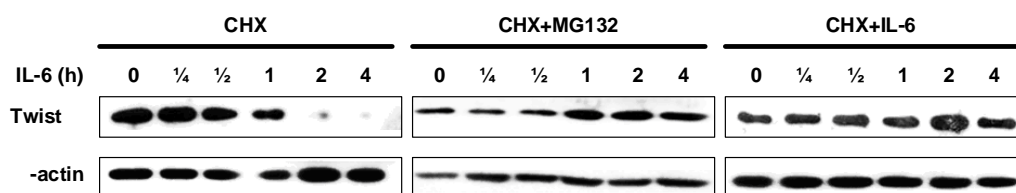


Figure 4.9 IL-6 upregulates Twist expression by inhibiting its degradation

As shown in Figure 4.9 (A) the expression of Twist protein in OSC-19 SCCHN cells is induced rapidly after treatment with the proteasome inhibitor MG132, suggesting Twist is degraded through a proteasomal degradation pathway.

To determine the half-life of Twist (Figure 4.9(B)), cells were treated with CHX, a protein synthesis inhibitor and cell lysates were collected at 0, 15, 30 min and 1, 2, 4 h and the amount of protein at each time point was determined by western blotting and quantified by densitometry. In Figure 4.8, the steady state of Twist has a half-life of 1.2h. In the presence of proteasome inhibitor MG132 or IL-6, less Twist is degraded and the half-life is not achieved in this experiment, indicating that IL-6 up-regulates Twist expression through inhibiting its degradation.

4.2.4 Conclusions and Discussion

Twist can be upregulated by IL-6 treatment of SCCHN cell lines through inhibition of Twist degradation. Though, it has been reported that Twist is a transcriptionally up-regulated by STAT3, a major downstream of IL-6 (Lo et al., 2007). Twist protein levels elevate at a faster rate than mRNA levels. Consistent with previous studies (Demontis et al., 2006), our data support that Twist is degraded through a ubiquitin/proteasome degradation pathway since levels of the short-lived Twist can be stabilized by the proteasome inhibitor MG132. Given the concept that the high GC content of Twist mRNA may contribute to its stability as discussed in Section 1.4.2, the expression of the

Twist protein may be largely controlled at the post-transcriptional level in this system. Thus, Twist is maintained at a low basal level with very short half-life; and when external factors such as EGF or IL-6 are available, Twist is stabilized and up-regulated within a short time to meet the needs for cell survival, migration, or differentiation.

4.3 Determination of the Potential Downstream Target to IL-6 to Stabilize Twist

4.3.1 Twist is Phosphorylated in Response to IL-6

Post-transcriptional modification of transcription factors, especially through phosphorylation, has been shown to be important for regulating their function as this provides a mechanism for cells to rapidly initiate transcriptional programs in response to external stimuli (Holmberg et al., 2002). Therefore, we examined whether or not Twist can be phosphorylated in response to IL-6 treatment by immunoprecipitating the treated Twist protein from IL-6 treated cells and performed western blotting with antibodies to phospho-tyrosine or phospho-serine/phospho-threonine. Limited by the poor efficiency of current commercially available antibodies to immunoprecipitate endogenous Twist, SCCHN cells transiently transfected with HA-tagged Twist were used for this HA pull-down assay. Although there was no band detected by the anti-phospho-tyrosine antibody (data not shown), there was a clear increase in phospho-serine detection following IL-6 treatment. (Figure 4.10)

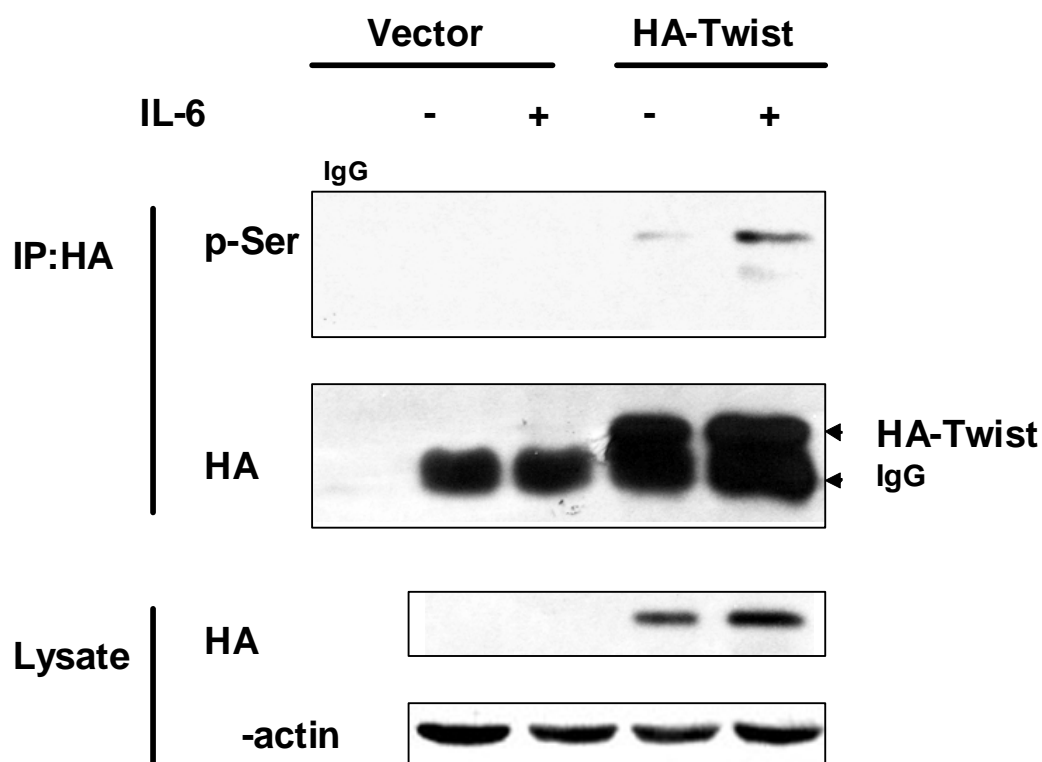


Figure 4.10 Twist is serine-phosphorylated by IL-6 treatment

4.3.2 Signaling Pathway Inhibitor Screening Assay

To determine the potential downstream signaling pathway responsible for Twist phosphorylation after IL-6 stimulation, we used an inhibitor screening strategy to test whether or not any known canonical signaling pathway is participating in the stabilization of the Twist protein through its phosphorylation.

As shown in Figure 4.11, Twist can be up-regulated by IL-6 in spite of the usage of inhibitors blocking JAK (AG490), PI-3 kinase (Wortmannin), Erk (U0126), p38 MAPK (SB202130), and JNK (SP600125) pathway, indicating the Twist up-regulation is independent of these pathways.

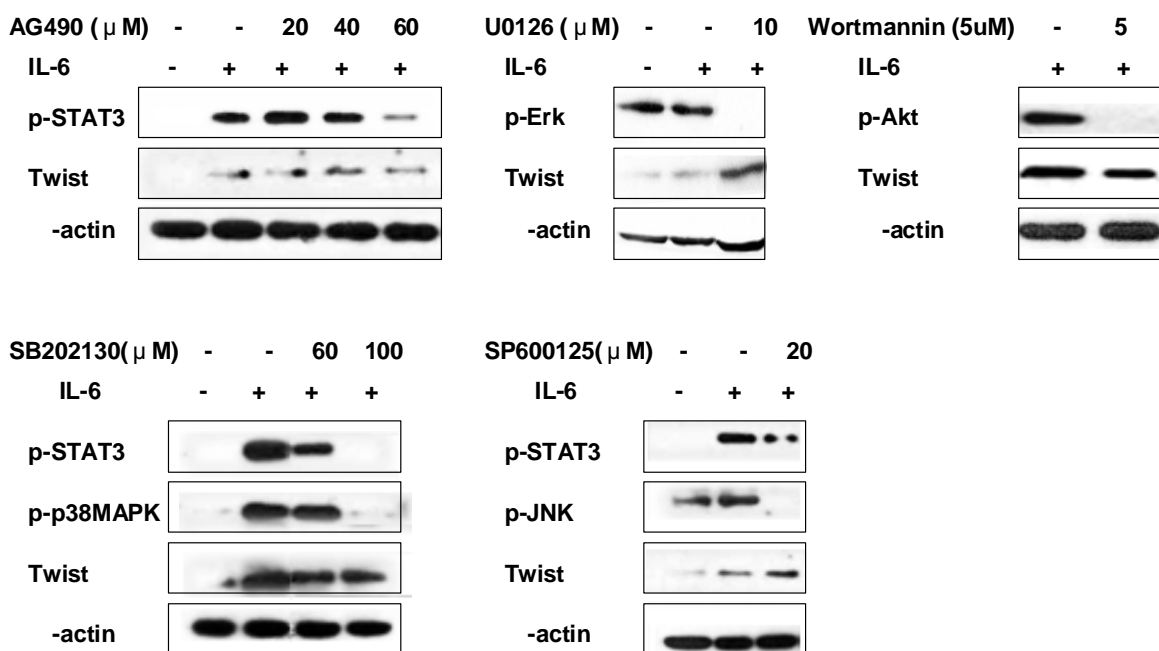


Figure 4.11 Inhibitor screening assay shows Twist up-regulation by IL-6 is independent of the known canonical signaling pathways.

4.3.3 CK2 Phosphorylation Motif on Twist is Conserved Across Species

Since none of the known pathways that we tested appeared to be involved in mediating the IL-6 - induced phosphorylation of Twist, we next scanned the amino acid sequence in the webserver <http://hits.isb-sib.ch/cgi-bin/PFSCAN>, and we identified a CK2 substrate consensus motif (SNSE) within Twist at residues 18 through 21 that is conserved across species (Figure 4.12).

```

Homo sapiens: .....11spaddsl21snseepdrqqpp.....
Mus Musculus : .....11spaddsl21snseepdrqqpa.....
Frog Xenopus laevis: .....11spvds21snseeldkqqskr.....

```

Figure 4.12 Predicted CK2 phosphorylation motif is conserved across species.

4.3.4 CK2 Inhibitors Destabilize Twist in Response to IL-6

To determine whether CK2 could be mediating the IL-6 induced stabilization of the Twist protein, we evaluated the effect of small molecule inhibitor of CK2, DMAT and TBB on Twist expression after IL-6 treatment. As shown in Figure 4.13 both DMAT and TBB were able to block the up-regulation of Twist by IL-6 at very low concentrations in more than one cell line.

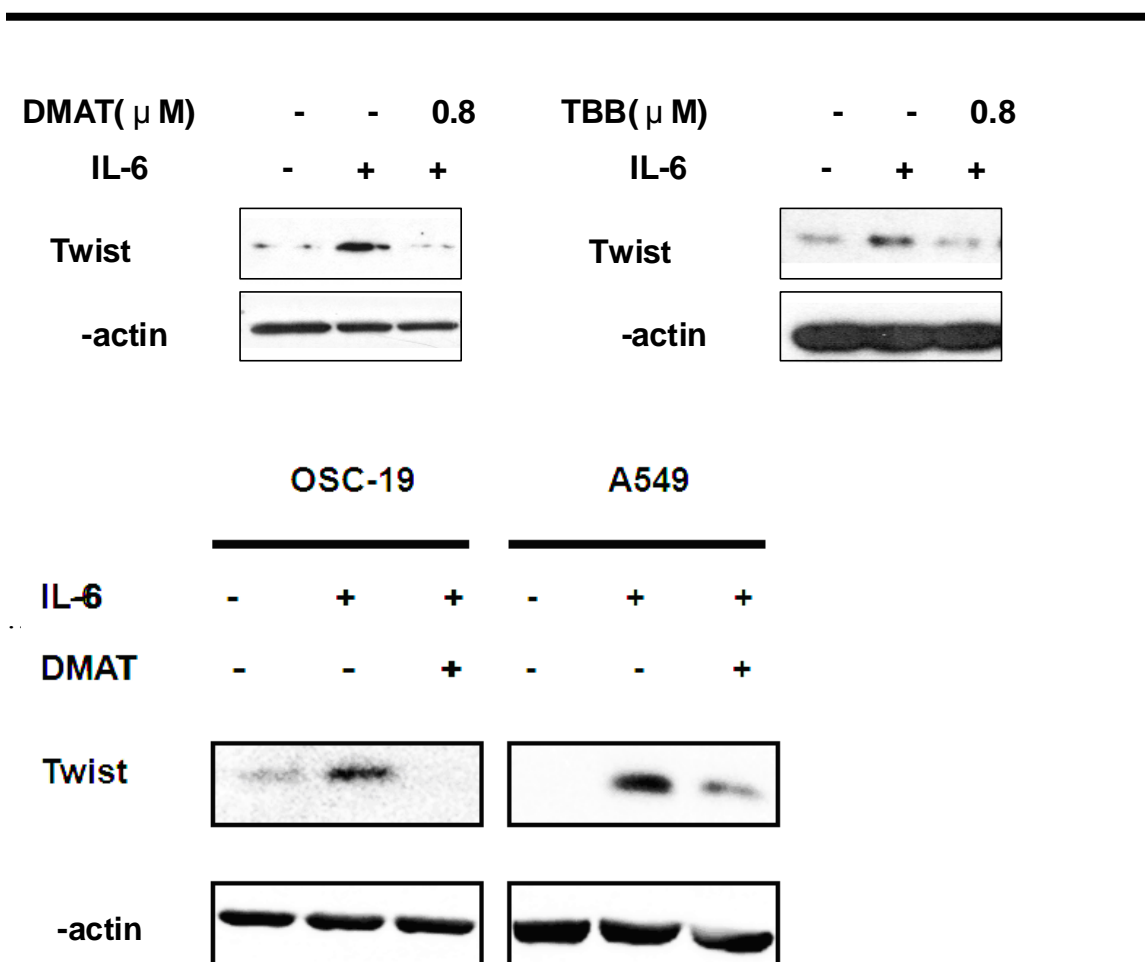


Figure 4.13 Twist expression is blocked by CK2 inhibitors

4.3.5 CK2 shRNA Decreases Twist Levels in IL-6-treated SCCHN Cells

To confirm the findings seen with the CK2 inhibitors, we next examined the effect of short hairpin (sh) RNAs specific for the catalytic subunit of CK2 (CK2 α) on the IL-6 induced elevation of Twist levels in SCCHN cells.

To initiate these studies, OSC-19 SCCHN cells were electroporated with either control vectors or shRNA to CK2 α for 24 hours. The cells were then treated with either IL-6 20 ng/ml or PBS control for 30 minutes and lysed for subsequent western blotting for Twist protein. As shown in Figure 4.14, IL-6 failed to up-regulate Twist protein level when the CK2 expression was diminished with two different shRNA constructs.

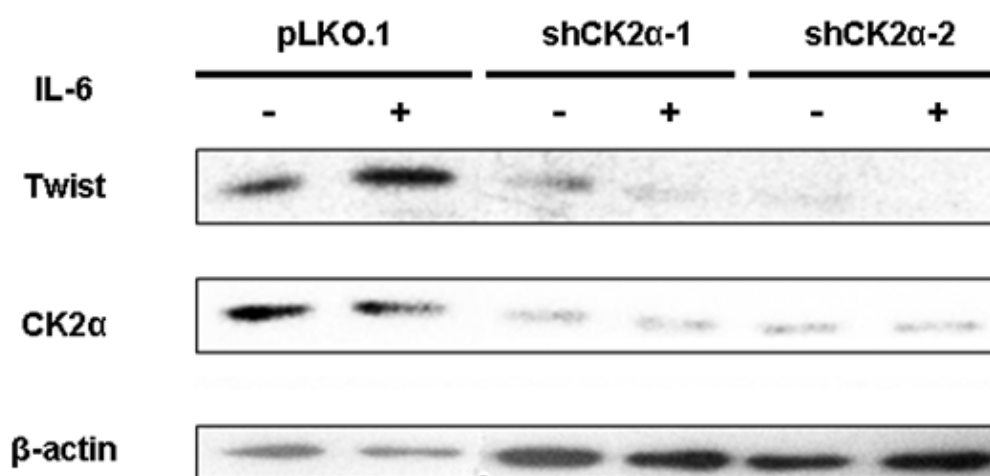


Figure 4.14 CK2 α shRNAs block Twist upregulation by IL-6

4.3.6 CK2 Kinase is Activated by IL-6

CK2 is a ubiquitous serine/threonine kinase regulating many relevant biologic processes, including inflammation (Cozza et al., 2010) that used to be regarded as a constitutively active intracellular kinase. However, several recent studies showed CK2

can be activated in response to external stimuli although though underlying mechanisms remain unclear (Litchfield, 2003).

We next evaluated the effect of treating SCCHN cells with IL-6 and found that the CK2 kinase is activated by IL-6 as assessed by a specific CK2 kinase assay (Figure 4.15).

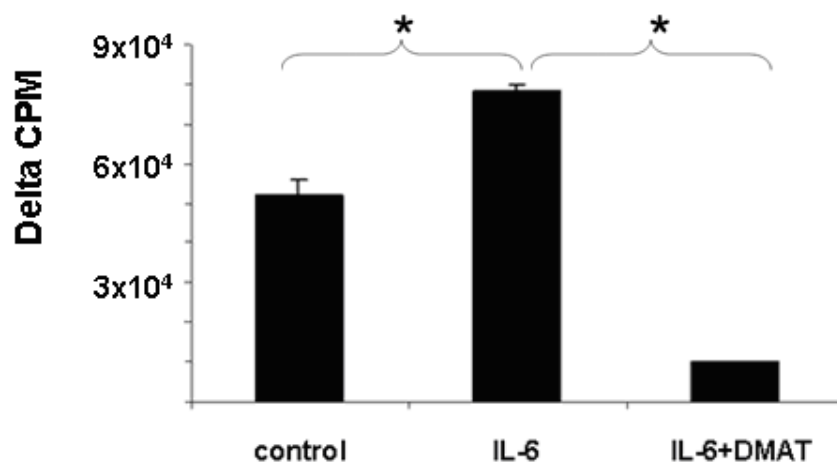


Figure 4.15 CK2 kinase activity assay shows CK2 kinase activity is increased by IL-6 in HN31-SCCHN cells.

To initiate this study, HN31 SCCHN cells were serum starved overnight, pretreated with DMSO or DMAT (10 μ M) for 1h, and treated with either phosphate-buffered saline (PBS) (control) or IL-6 (20 ng/ml) for 30 minutes. The endogenous CK2 activity was then examined in the cell lysates (5 μ g) by using a synthetic peptide CK2 substrate (RRRADDSDDDDD; 0.1mM) and γ^{32} P-ATP and counting the radioactive uptake

(Pagano et al., 2004). The y-axis represents the count per minute (CPM) of the radioactivity after normalizing to the control (no substrate).

4.3.7 Conclusions and Discussion

In this section, we determined that CK2 is in the pathway between IL-6 and Twist up-regulation by using a CK2 inhibitors, shRNA, and a CK2 kinase activity assay.

Although CK2 has been shown to be activated by many factors and conditions including serum, DNA damage, growth factors and cytokines (as reviewed in section 1.3.3), the mechanisms of CK2 activation are still not known. Using a web-based computational prediction of the phosphorylation motifs within the CK2 α and CK2 β amino acid sequences, only several Erk phosphorylation consensus motifs have been found. Although it has been reported experimentally that Erk2, not Erk5 is responsible for CK2 α activation by EGF stimulation in neuroblastoma cells (Ji et al., 2009), in our SCCHN lines, as shown in Figure 4.11, phosphor-Erk is not increased by IL-6 and blocking Erk activation with U0126 did not blocks the effect of Twist upregulation by IL-6. These findings suggest an Erk-independent pathway is responsible for CK2 activation by IL-6. Since the computational prediction could not provide us more information of the unknown kinase responsible for CK2 activation, alternative approaches such as Mass Spectrometry to identify the potential phosphorylation site(s) on CK2, or phosphoprotein

array comparing untreated and treated samples may help us to identify the potential targets that mediate signaling between IL-6 and CK2. .

4.4 Protein Interaction Between Twist and CK2

4.4.1 In 293T Transient Transfectant Lysates

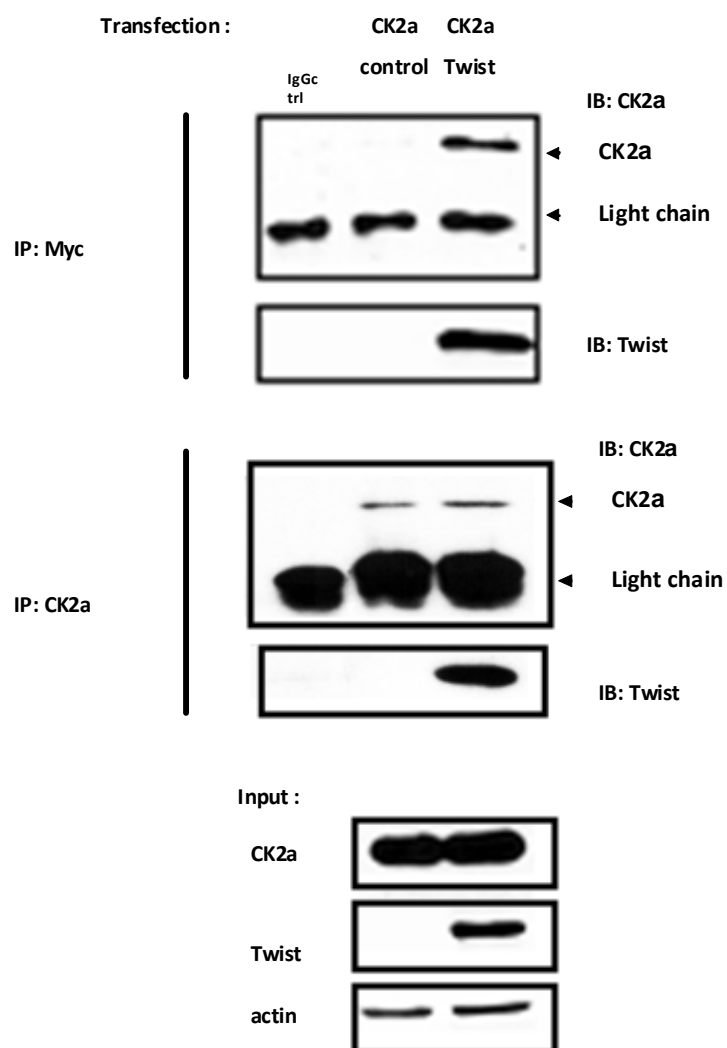


Figure 4.16 CK2 α interacts with Myc-Twist in 293T transient transfectant lysates

In Section 4.3, we demonstrated that CK2 is in the pathway between IL-6 and Twist up-regulation and that the Twist transcription factor is phosphorylated by IL-6 treatment. The observation prompted us to investigate whether or not there is a direct interaction between CK2 and Twist.

To address the question, 293T cells were transfected with both wild-type Myc-Twist and the catalytic subunit of CK2 (CK2 α) for 48 hours. The lysates then were subjected to immunoprecipitation with either anti-CK2 α antibody or anti-Myc antibody, followed by western blotting.

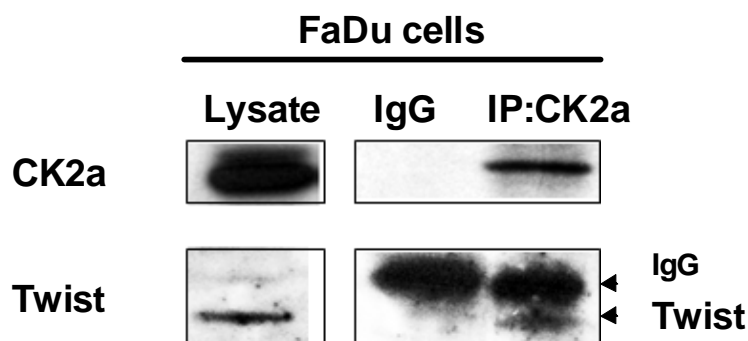
As shown in figure 4.16, CK2 α was co-immunoprecipitated with Myc antibody and vice versa. The bidirectional co-immunoprecipitation experiments suggest that CK2 α and Myc-Twist are found within a protein complex.

4.4.2 Co-immunoprecipitation in FaDu Cell Lysates

Because of the concern that non-specific protein interaction can occur in co-immunoprecipitation studies using transient protein over expression systems in which the proteins are over-expressed, we next examined if the protein interaction can be identified with the endogenous CK2 and Twist proteins in SCCHN cells.

The FaDu cell line was chosen for this study because it has a high basal levels of CK2 protein. In Figure 4.17, endogenous Twist (MW: 26 kDA) was co-precipitated with CK2 α pull-down, indicating there is interaction between CK2 and Twist in SCCHN cells.

Figure 4.17 Endogenous CK2 α interacts with Twist in Fadu SCCHN cells



4.4.3. Co-immunoprecipitation in Myc-Twist Stably Expressing HN31 Cell Lysates

In order to further study the interaction between Twist and CK2 α by, we established a Myc-Twist stable expression HN31-SCCHN cell line by stable transfection through G418 selection, as all currently available Twist antibodies from Cell Signaling, Sigma-Aldrich, and Santa-Cruz were found to have very poor efficiency in immunoprecipitating endogenous Twist.

In Figure 4.18, CK2 α is co-immunoprecipitated in the HN31- anti-myc immunoprecipitants, further supporting the interaction between the two proteins in SCCHN cells.

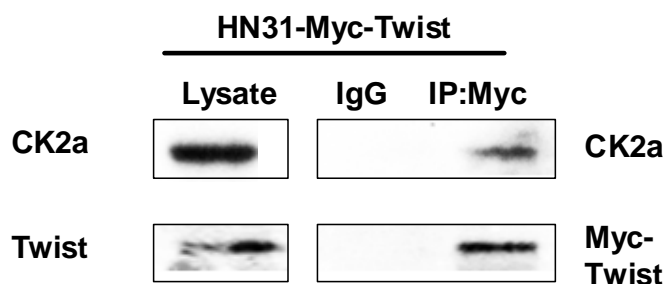


Figure 4.18 Endogenous CK2 α interacts with Myc-Twist in HN31 SCCHN cells

4.4.4 Conclusions and Discussion

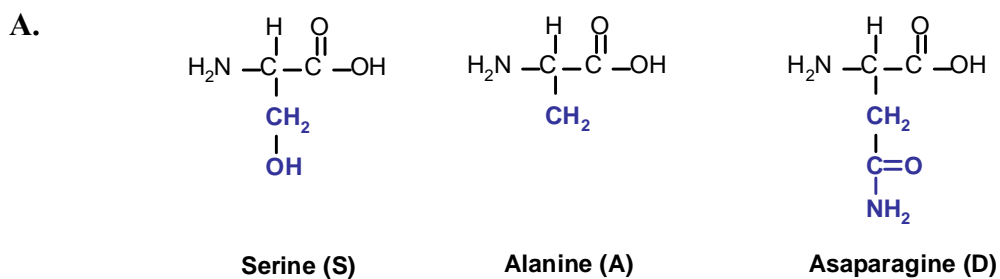
Immunoprecipitation is one of the commonly used approaches to demonstrate protein-protein interactions. Because the commercially available Twist antibodies do not work well for immunoprecipitation in our hands, a small epitope Myc tag was added in front of Twist as a fusion protein in an expression plasmid in for immunoprecipitation purposes. From the first experiment using 293T transfectant lysates, the interaction between CK2 α and Myc-Twist was demonstrated using bi-directional immunoprecipitation. The two proteins were found to interact endogenously within SCCHN cells and this was further demonstrated in the immunoprecipitation experiment in FaDu and HN31-Myc-Twist over expression stable lines. Thus, we provide evidence that there is interaction between the two proteins in different approaches and cell lines. One of the disadvantages of this type experiment is that the proteins co-immunoprecipitated may not necessarily interact directly but rather through a third docking protein. To further

answer the question, an *in vitro* GST protein pull-down assay using proteins isolated from a bacterial protein expression system should provide complementary information.

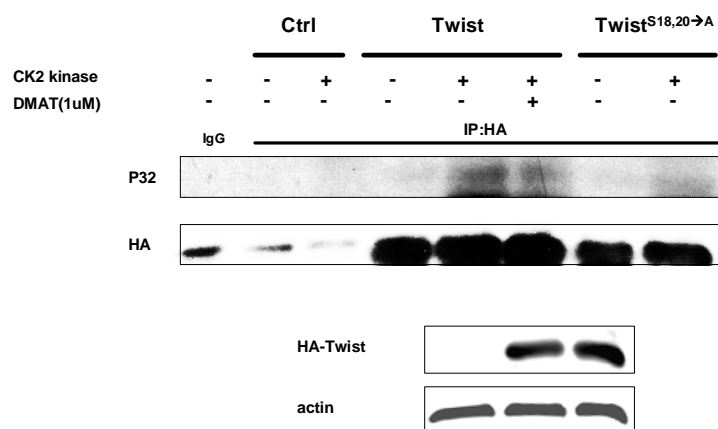
4.5 Twist Phosphorylation by CK2

4.5.1 Mapping the Phosphorylated Twist Residue by Immunocomplex Kinase Assay

As mentioned in Section 4.3.3, there is a putative CK2 phosphorylation site (SNSD) between the 18th and 21st amino acids of Twist as determined by sequence analysis for the CK2 phosphorylation motif. To determine whether or not CK2 directly phosphorylates Twist at this site, we performed immunocomplex kinase assay in 293T cells over expressing either wild-type (WT) Myc-Twist or mutant Myc-Twist in which the S18 and S20 are substituted with alanine by site-directed mutagenesis (S18,20A). As shown in Figure 4.19A, the amino acid alanine (A) is structurally similar to serine (S) except the –OH residue on the second carbon, therefore alanine can not be phosphorylated by a kinase and therefore functionally mimics the unphosphorylated serine. As shown in Figure 4.19 B, the purified CK2 kinase can phosphorylate WT Twist, but this phosphorylation is strongly reduced in Twist S18,20A. Furthermore, this phosphorylation is also abolished in the presence of the CK2 inhibitor, DMAT. The experiment was done using different tagged forms of Twist (HA-Twist in figure 4.18B and Myc-Twist in Figure 4.19C), with both experiments showing consistent results.



B.



C.

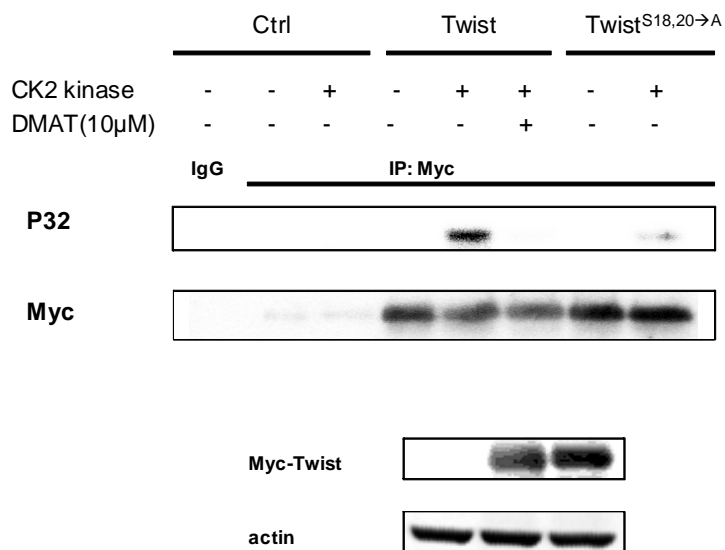


Figure 4.19 Immunocomplex CK2 kinase assay

4.5.2 Mass Spectrometry

To more definitively demonstrate the site where the phosphorylation of CK2 occurs in IL-6 treated SCCHN cells, mass spectrometry was attempted several times. To increase transfection efficiency, electroporation was optimized and stable cell lines were established. However, even when using the Twist stable expression cell lysates, the protein detected by western blotting was not enough for subsequent mass spectrometry phospho-site identification.

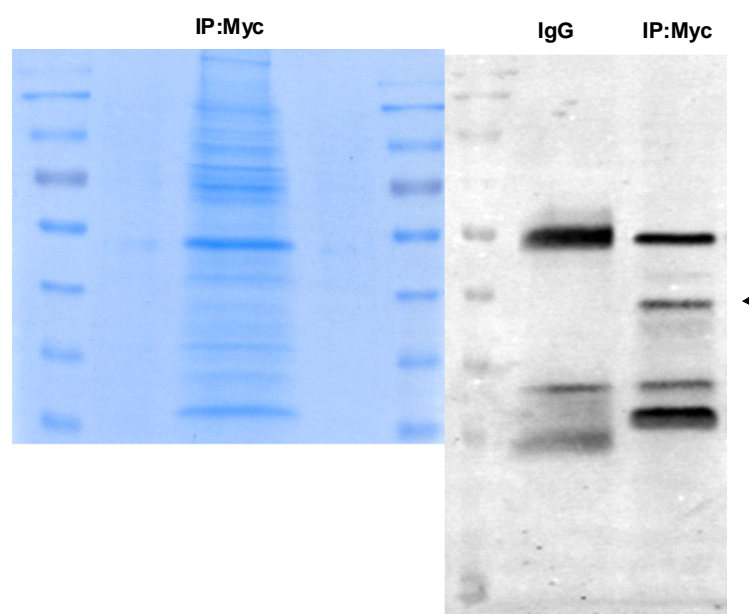


Figure 4.20 Coomassie gel staining for mass spectroscopy and corresponding western blotting

As shown in Figure 4.20, the corresponding protein band in Coomassie gel staining (left) is weak in contrast with the clear band by IP western blotting (right, arrow, IP: myc , IB: Myc)

4.5.3 Conclusions and Discussion

In the section, we show the evidence that Twist is a substrate by CK2 as the γ -P³²-ATP incorporation is increased after CK2 kinase is added to the Twist/ γ -P³²-ATP/kinase buffer mixture. By either adding CK2 inhibitor or substitution of the potential phosphorylated serines with alanines, the phosphorylation is diminished, supporting our hypothesis. To demonstrate that the phosphorylation can occur in cancer cells, mass spectrometry was tried several times, however, was not successful due to insufficient Myc-Twist protein was immunoprecipitated by Myc antibody. To further improve the assay, we may repeat MS by using GST-Twist pull-down assay which can enrich the protein in the gel or try to raise mice that produce monoclonal antibody against the phospho-Twist protein and test the antibody in vitro and in vivo.

Of note, the identified CK2 phosphorylation site is with the NSEE motif (as reviewed in section 1.4.2). NSEE is a highly conserved sequence domain at N-terminus and the function is unknown to date (Singh and Gramolini, 2009). Our data may contribute to the current knowledge of the function of this motif.

4.6 Functional Significance of Twist Phosphorylation by CK2

4.6.1. Twist Phosphorylation by CK2 α Changes the Stability of Twist

Because our earlier data show that IL-6 stimulation leads to stabilization of Twist and CK2, mediates the signal, by associating with and phosphorylating Twist, we next examined whether or not the stability of Twist is affected by site-directed mutagenesis of the putative CK2 phosphorylation sites of Twist. As discussed in section 4.5, when the amino acid serine (S) is substituted by alanine (A), which has a similar structure except the –OH residue on the second carbon is replaced with a methyl group, phosphorylation can not occur and therefore functionally mimics the unphosphorylated serine. When serine is substituted with aspartic acid (D), a negatively charged amino acid side, it functionally mimicks a phosphorylated serine. Following these principles, hypo- and hyper-phosphorylated mimicking mutants of Myc-Twist constructs were generated as S18 and S20 were substituted with alanine (S18,20A-Myc-Twist) or aspartic acid (S18,20D-Myc-Twist) by site-directed mutagenesis. The protein degradation rate of WT Twist or its phosphorylation site mutants were examined after the treatment with translation inhibitor CHX and the half life of Twist and its isoforms was determined. The results from experiments performed in triplicate are shown in Figure 4.21A. The numbers below each western blot are the relative intensity of the band by densitometric

measurement as determined from Image J. Figure 4.21B represents the protein amount (in log axis) versus time of CHX treatment. Figure 4.21C is the summary of the half-lives of WT –Myc-Twist and Twist mutants from the three experiments. The average half-lives for Twist S18,20D, Twist 18,20A and WT-Twist are 32.3 ± 5.94 h, 9.3 ± 0.64 h and 13.4 ± 0.92 h respectively. All P values are less than 0.05 by comparing means from any two groups with student *t* test.

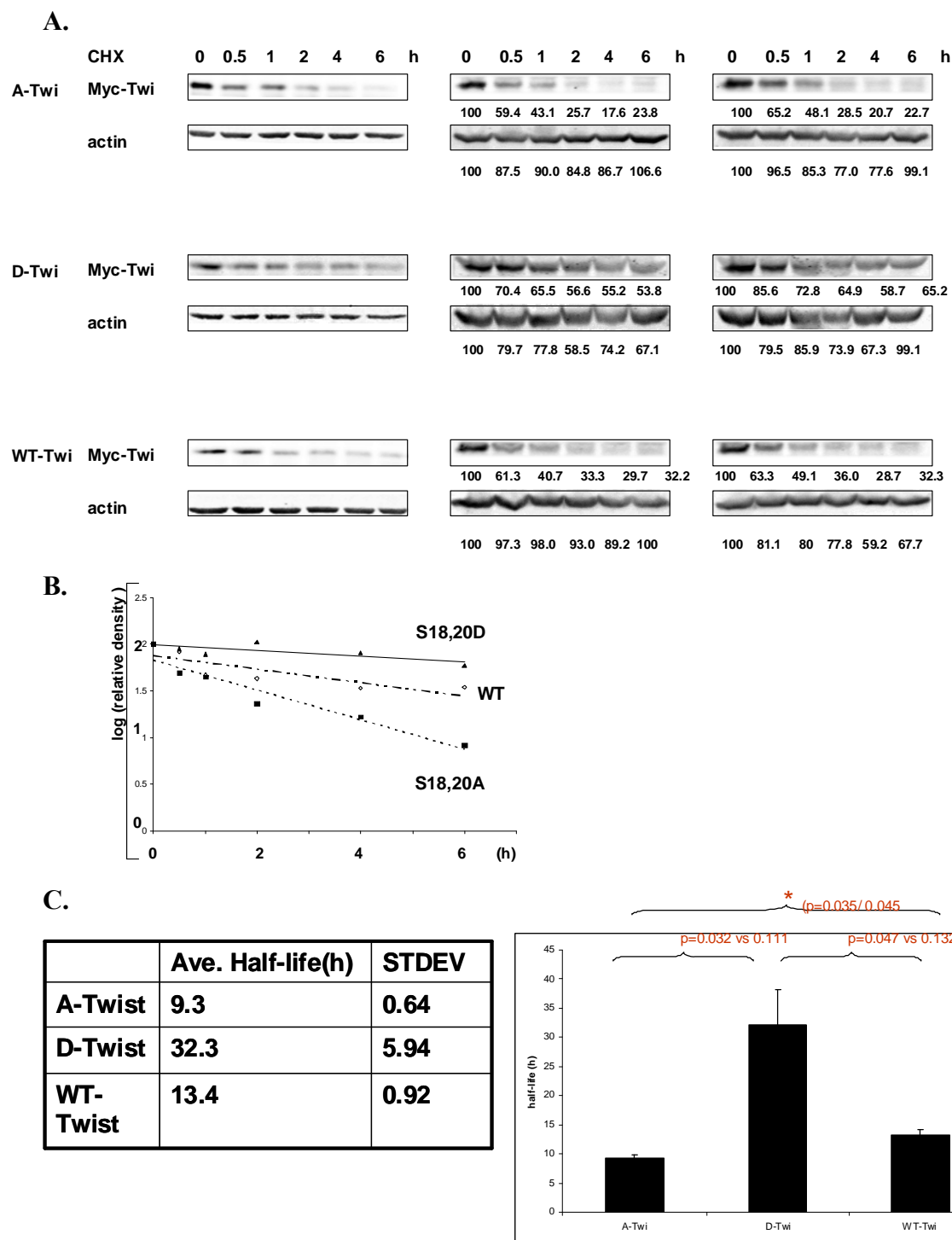


Figure 4.21 Twist stability is affected by CK2 phospho-modification

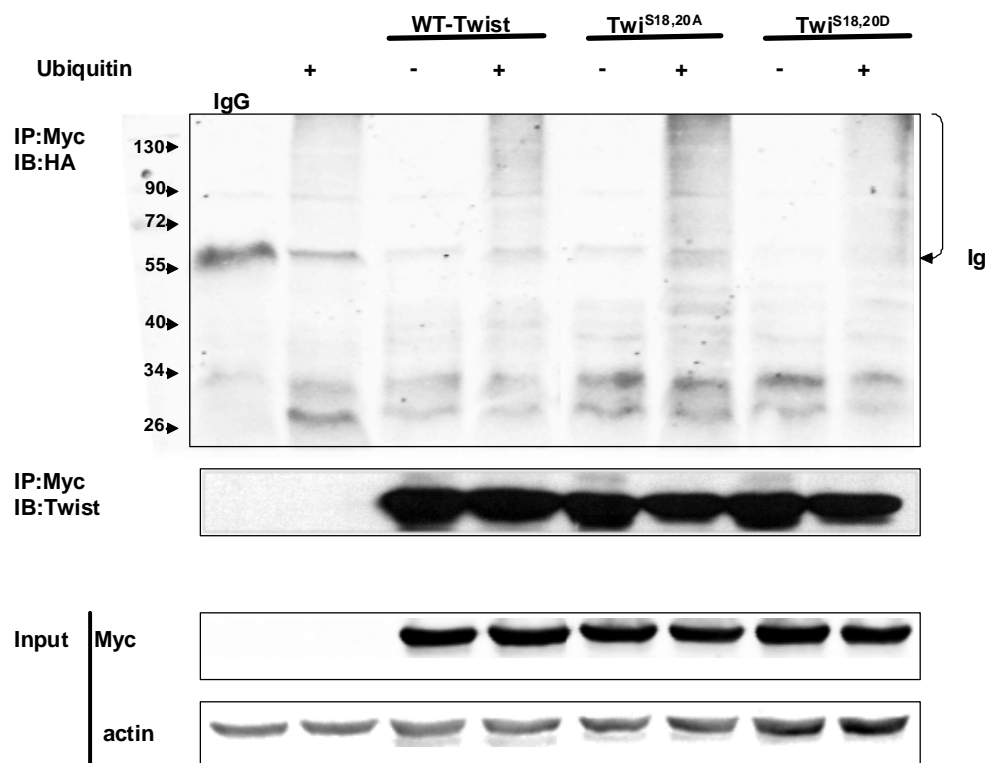


Figure 4.22 The *in vivo* Ubiquitination Assay in 293T transient transfectant lysates

Since Twist has been reported to be degraded through ubiquitin/proteasome pathway, we next examined whether or not there is increased ubiquitination in the hypo-phosphorylated Twist mutant. In this *in vivo* ubiquitination assay, 293T cells were transfected with WT- or mutant- Myc-Twist as well as HA-tagged ubiquitin expression plasmids for 48 hours. Then the cell lysates were collected and immunoprecipitated with Myc tag, electrophoresed, transferred, and immunoblotted with HA or Myc antibodies. As shown in Figure 4.22, there is hyperubiquitination of the S18,20A Twist in 293T cells when co-transfected with HA-tagged ubiquitin relative to WT-Twist and

S18,20D –Twist. The result between WT and S18,20A –Twist groups was consistently observed in three independent experiments, supporting that the phospho-modification may affect the stability through affecting its ubiquitination status.

4.6.2. The Interaction Between Twist and E12 Protein is Increased after IL-6 Treatment

Previous studies have shown that bHLH proteins form active dimers that can bind to the regulatory elements of downstream genes (Firulli and Conway, 2008). Therefore, we examined whether there is an association between Twist and its dimer partner, the E2A protein (E12) in the presence of IL-6. As shown in Figure 4.23, the total amount of endogenous E12 that was co-immunoprecipitated with WT-Twist was increased in the Myc-Twist over-expressing HN31 cell lysates after a short treatment (30 min) with IL-6.

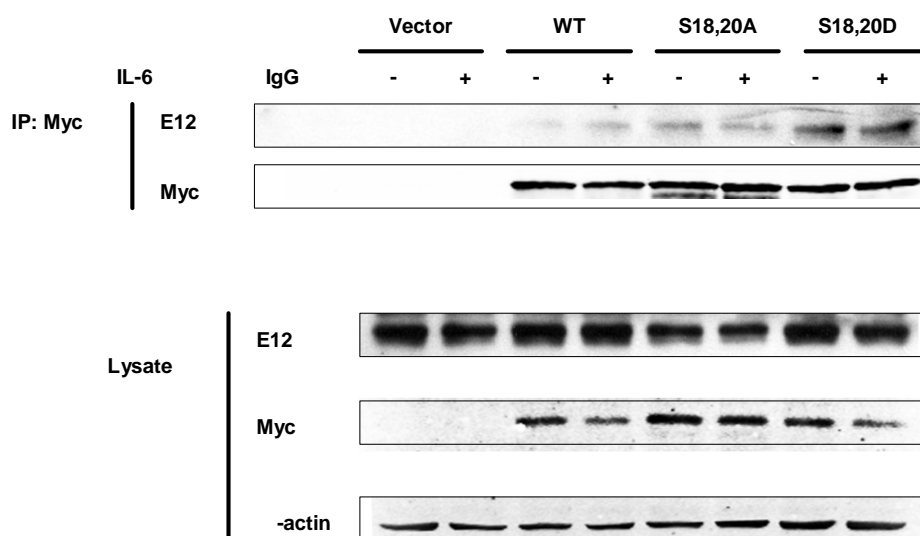


Figure 4.23 The interaction between Twist and E12 is increased after IL-6 treatment

From this blot, comparison between groups can not be made since the basal levels of E12 in different lines are not equal. However, the total amount of E proteins associated with S18,20D is higher than those with wild type or S18,20A. The evidence in this figure suggests that IL-6 may play some role in mediating E12 and Twist interaction.

4.6.3 The Motility of SCCHN Cells is Increased by Expression of Forced T/E

Hetero-dimer

Given that the both E12 and Twist are involved in the EMT process, trans-well migration assays were performed in HN31-SCCHN cells transiently expressing either WT-Twist, E12, or both, the forced T/T homodimer, or the T/E heterodimer (The T/T and T/E dimer constructs are a gift from Dr. Spicer, Maine Medical Center) (Connerney et al., 2006). The scheme of the construct design of the forced dimers is shown in Figure 4.24A. The western blot after transfection for each condition is shown in Figure 4.24B. The counted migrated cells in Boyden chamber migration assay is shown in Figure 4.24C.

As shown in Figure 4.24C, over expression Twist or E12 increased the amount migrated cells relative to the control vector. However, increased expression level of Twist

does not further increase cell migration, indicating the existence of another rate-limiting factor rather than Twist. Although co-expression of both Twist and E12 did not show further increases in motility, there is a statistically significant difference between the forced T/T and T/E groups, suggesting a different transcription consequence between the two groups.

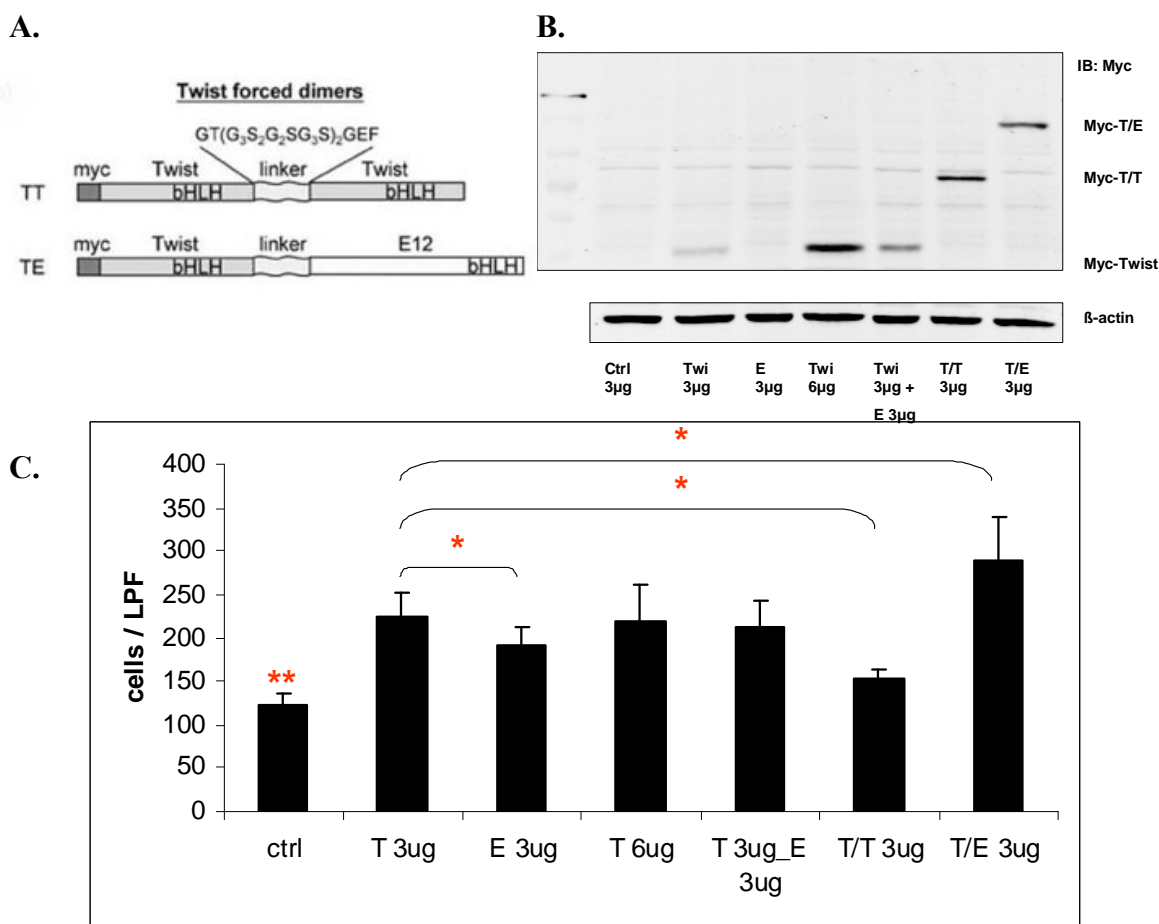


Figure 4.24 The motility of HN31 SCCN cells is increased by expression of forced T/E heterodimer. *: represents P value < 0.05

4.6.4 Twist Phosphorylation Promotes Cell Motility *in vitro*

We next examined the effects of IL-6 treatment on OSC-19 SCCHN cell motility as measured by wound healing assay. Figure 4.25A shows the representative pictures before (0h) and 12h after indicated treatment and the migratory distance during the 12h treatment was quantified by Image J and expressed as relative values (set control= 100) in Figure 4.25B. The experiment was done in duplicate and at least 12 measurements in each well were analysed.

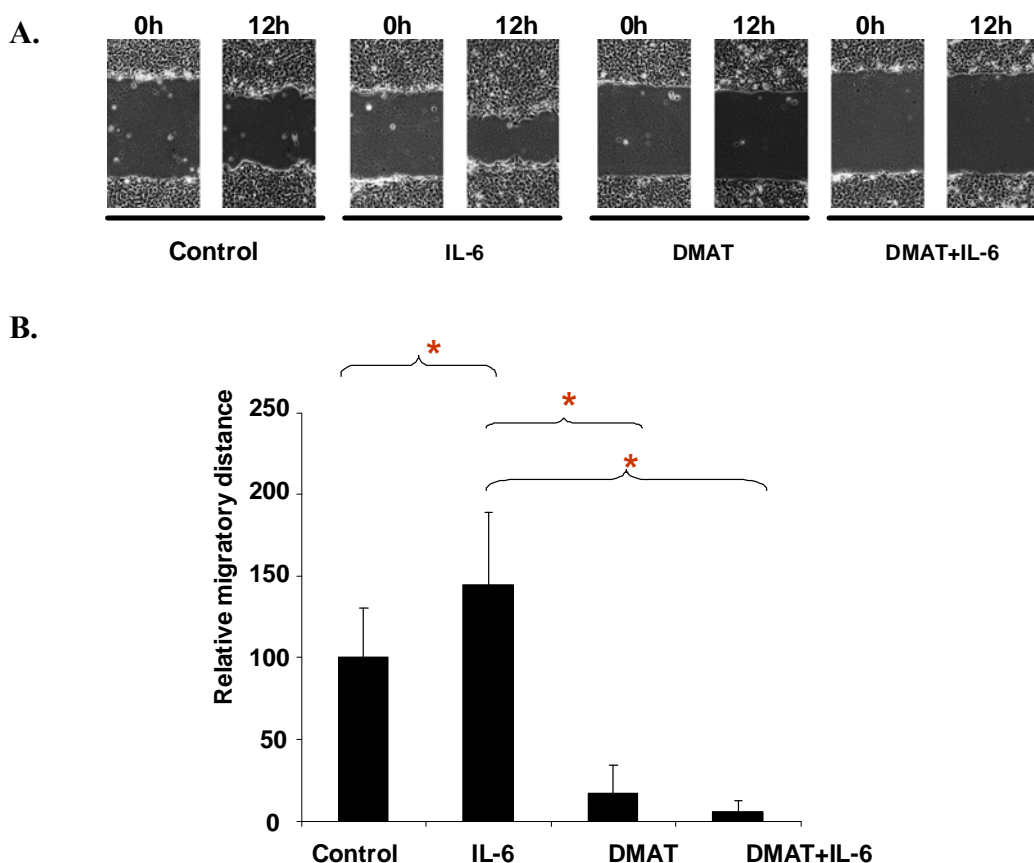


Figure 4.25 The motility of OSC-19 SCCHN cells is promoted by IL-6 and blocked by the CK2 inhibitor, DMAT.

We next examined the effects on cell motility of shTwist in the presence of IL-6. As shown in Figure 4.26, IL-6 increases the cell motility in the trans-well assay, which is consistently seen in the wound healing assay. In this 22h trans-well assay, blocking the expression of Twist decreases the cell migratory ability in the absence or presence of IL-6, suggesting that the IL-6-mediated cell migration is, only in part, through Twist since in the absence of Twist, the cells still could respond to exogenous IL-6 stimulation.

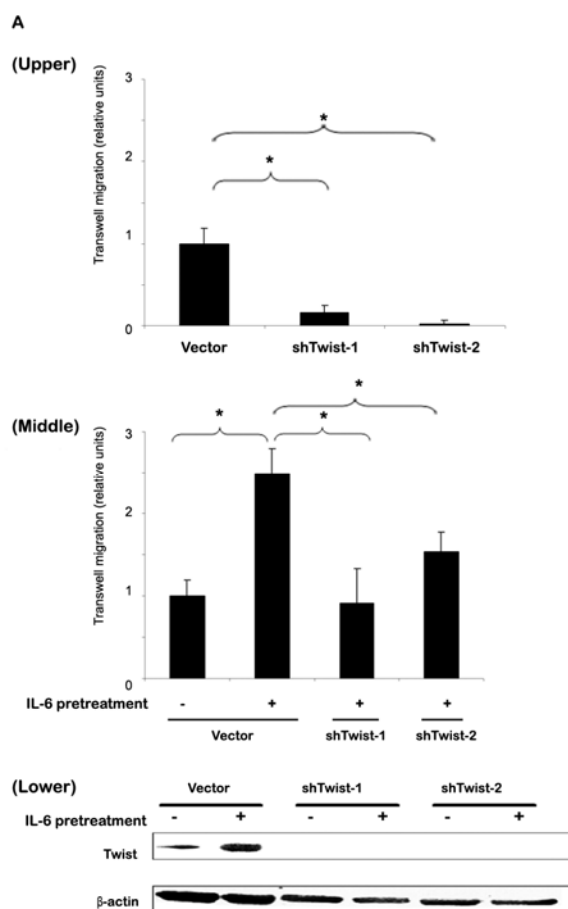


Figure 4.26 IL-6 promotes OSC-19 SCCHN cell motility, in part, through Twist

Then we examined the motility of HN31 SCCHN stabling expressing WT—Myc-Twist, and Twist mutants. As shown in figure 4.27, over-expression of WT Twist but not S18,20A Twist in both OSC-19 and HN31- SCCHN cell lines promotes cell migration relative to the control. Furthermore, over expression of S18,20D Twist further increases the motility relative to WT or S18,20 A Twist, suggesting that this mutation further promotes cell motility.

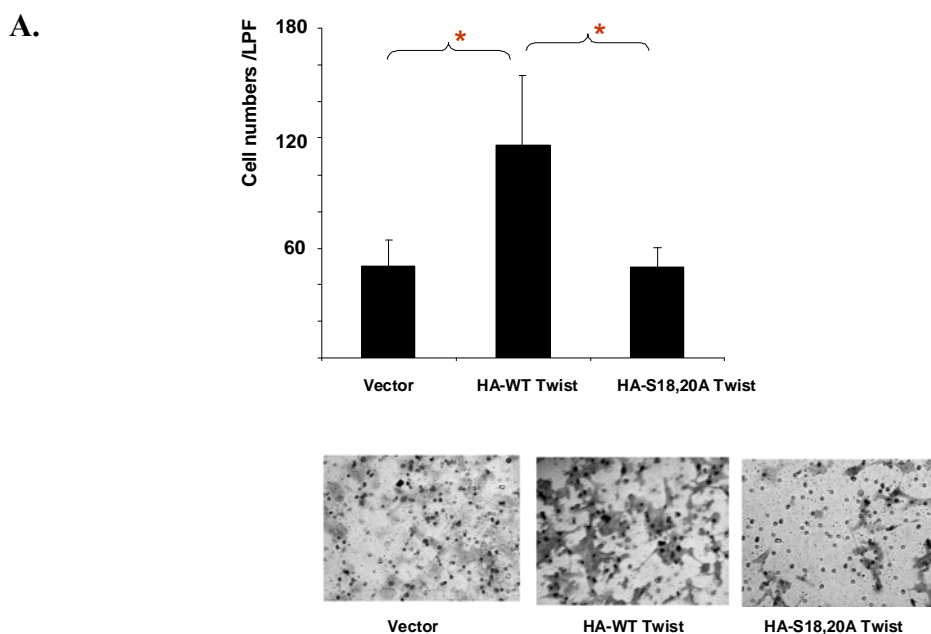
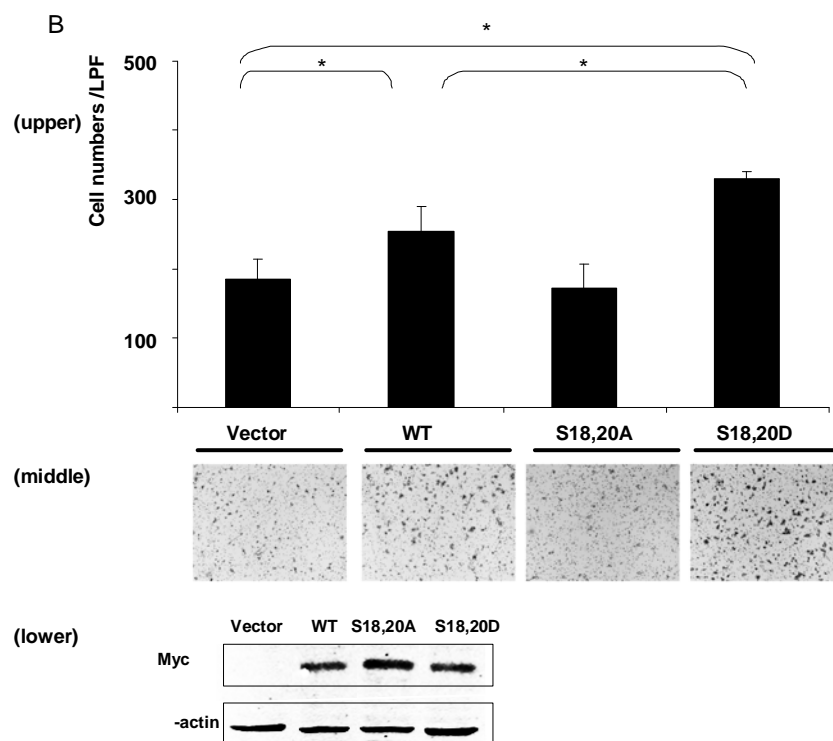


Figure 4.27 Overexpression of Myc-WT- Twist, not Myc-S18,20A –Twist promotes the motility of SCCHN cells



(continued) Figure 4.27 Over expression of Myc-WT- Twist , not Myc-S18,20A –

Twist promotes the motility of SCCHN cells

In Figure 4.27A, the experiment was performed with OSC-19 SCCHN cells after transient transfection by electroporation for 48 h. Then the cells were trypsinized and re-plated for subsequent trans-well migration assay. In Figure 4.27B, the experiment was performed using HN31-SCCHN cells. The representative images of the migrated cells from the corresponding trans-well membranes are shown in the lower and middle panels of Figure 4.27 A and B respectively. The quantitative data are shown in the upper panels and expressed as mean \pm SEM of the cell counts from each group in lower-powered field microscopic exams. * $P < 0.05$.

4.6.5 The effect of Twist phosphorylation by CK2 on lymph node metastasis *in vivo*

To investigate the potential impact of the over expression of Twist and its phospho-modificated isoforms on the incidence of lymph node metastasis in a murine orthotopic model of tongue cancer, we inoculated a small number of cells from the HN31 stable-Twist transfectant lines (10,000 cells / mouse) into the lateral tongues of nude mice and observed them for tumor growth and lymph node metastasis for 8 weeks.

During the 8-week period of observation, the tumorigenicity was found to be 8/10 and 7/10 mice in the control and S18,20A –Twist groups respectively and is markedly decreased in the WT-Twist and S18,20D –Twist group (Both are 4/10). The tumors in the S18,20A Twist group grew the biggest among the 4 groups ($P < 0.05$) as summarized in Table 12.

	Control	Twist	A-Twist	D-Twist
Tongue Tumor formation	8/10	4/10	7/10	4/10
Ave tumor vol at sac (mm³)	32.6 ± 25.6	18.3 ± 9.4	90.1 ± 90.9	41.5 ± 29.1
Microscopic tumor	8/10	6/10	7/10	6/10

Table 12 The volumes and rates of tumor formation in mice 8 weeks after inoculated orthotopically in the tongue with 10,000 HN31 cells stably expressing wild- type Twist and its mutants .

Results

However, at sacrifice, there are more grossly detectable neck lymph nodes in WT-Twist and S18,20-Twist group as shown in Figure 4.28. A total of 30 and 25 lymph nodes were found in WT-Twist and S18,20 D Twist group respectively; Only 14 and 10 lymph nodes were found in the control and S18,20A –Twist group respectively despite the higher rates of tumorigenicity and bigger average tumor volumes in these two groups. In contrast, in some cases in WT-Twist and S18,20D Twist groups, they had unusually enlarged lymph nodes despite the lack of detectable tumor formation in the tongues.

Microscopically, only non-specific inflammation was noted in the enlarged lymph nodes, as no cancer metastasis were found in these enlarged lymph nodes. As result, the total lymph node metastasis rate, as determined by microscopic examination, is 1/10, 1/10, 2/10, and 1/10 in control, WT-Twist, S18,20A Twist, and S18,20D Twist group respectively. No statistical significance was found.

Although there is no statistical significant difference between groups in the lymph node metastatic rate, the tumor size in each group did differ.

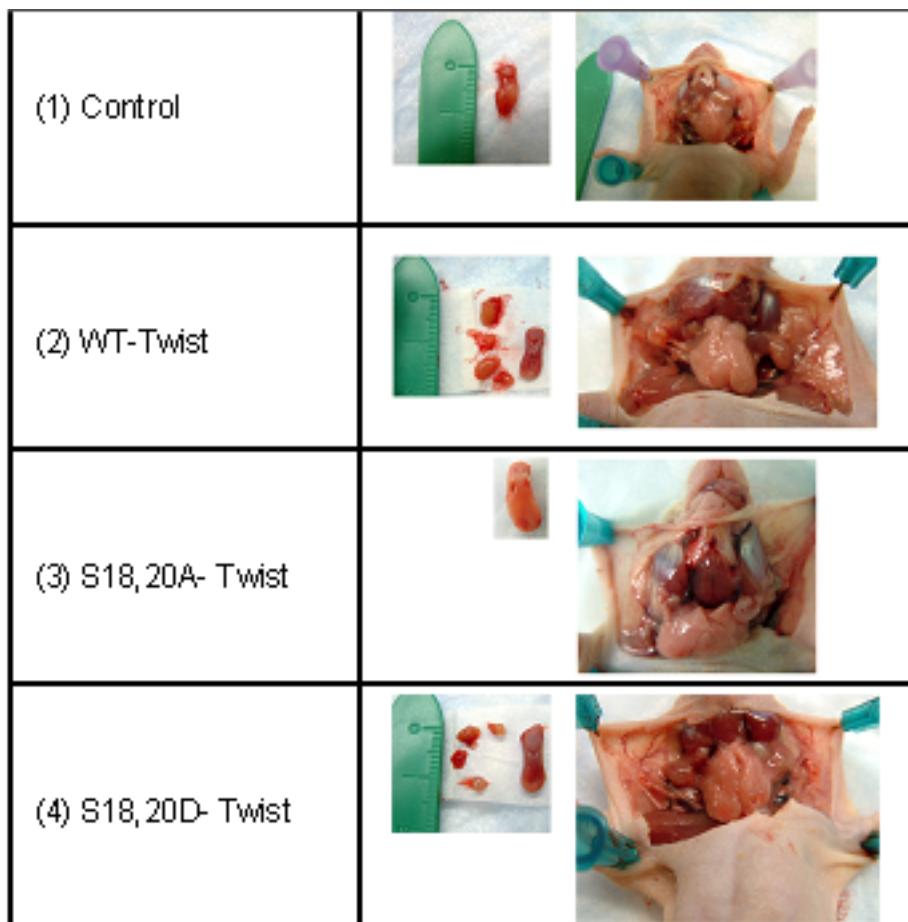


Figure 4.28 Representative pictures of the cervical lymph nodes at sacrifice (Right) .
The dissected nodes and tongues with tumors are listed at left panel for comparison.
Mouse inoculated with WT-Twist, not S18,20A, over expression HN31 stable lines
had more grossly detectable neck lymph nodes.

We next examined the incidence of lymph node metastasis 14 days after inoculation of a larger number of cancer cells (50,000 cells/ mouse) into the tongues of nude. In this experiment, tumorigenesis was 100% in all mice group as listed in Table 13. There is no statistical difference in the tumor sizes between except the S18, 20D Twist group, which is less than the other groups (Figure 4.29).

	Control	Twist	A-Twist	D-Twist
Tongue tumor formation	10/10	10/10	10/10	10/10
Ave. tumor Vol. at Sac (mm³)	28.8 ±10.6	30.3 ±8.9	23.8 ±7.7	21.0 ±5.5
Microscopic tumor	10/10	10/10	10/10	10/10

Table 13 The volumes and rates of tumor formation in mice 2 weeks after inoculated orthotopically in the tongue with 50,000 HN31 cells stably expressing wild- type Twist and its mutants

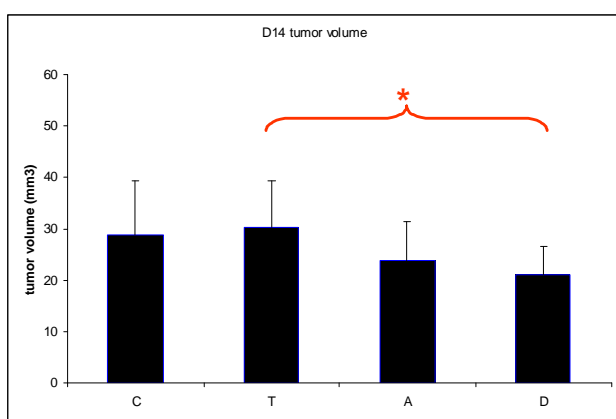


Figure 4.29 *In vivo* tongue tumor volume of each HN31 stable lines 2 weeks after the inoculation with 50,000 cells inoculation.

For this set of experiments, the microscopic lymph node metastasis per group (10mice) is listed in Table 14. There is a tendency towards increased lymph node metastasis in the WT-Twist and S18,20D Twist groups (3/10 and 5/10 relative to 2/10 in the control and 1/10 in the S18,20A group) . However, there is no statistical significance. The representative microscopic lymph node metastasis in H&E staining and cytochrome (Lu-5) staining of each group is shown in Figure 4.30.

	LN metastatic rate
control	2/10
Twist ^{wild-type}	3/10
Twist ^{S18,20→A}	1/10
Twist ^{S18,20→D}	5/10

Table 14 Incidence of lymph node metastasis 2 weeks after inoculation with a higher cell number (50,000 cells)

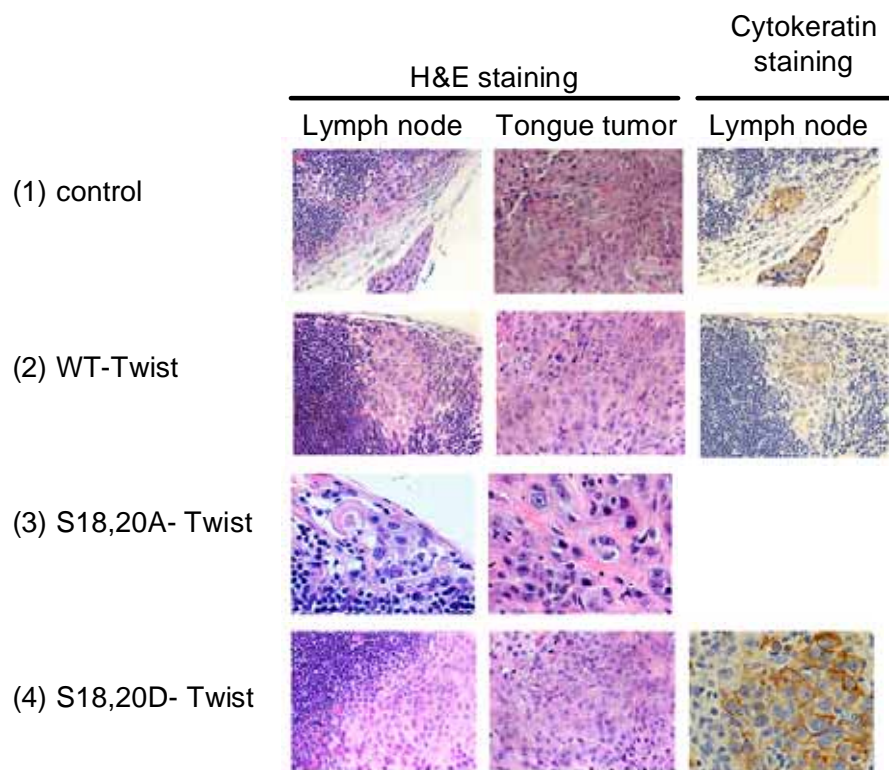


Figure 4.30 Representative pictures of immunohistochemical staining (H&E and cytokeratin Lu-5) of tumors and metastatic lymph nodes in each group of mice that was inoculated with 50,000 cells

In addition to lymph node metastasis, there was increased histiocyte infiltration observed in the lymph nodes of the S18,20D-Twist group, which was not observed in other three groups. Usually, histiocytosis in the reactive lymph adenitis suggests a foreign body reaction, such as in infection like tuberculosis or early cancer cell infiltration.

Since histiocytosis only observed in the S18,20D-Twist group and the mice are generally healthy and maintained in a pathogen free environment, early cancer cell infiltration is highly suspected.

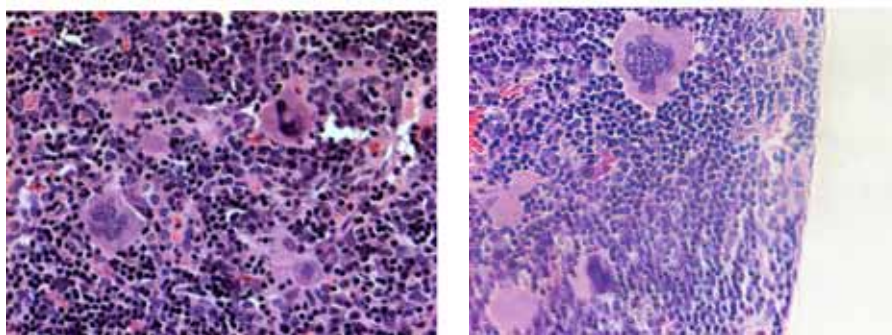


Figure 4.31 Representative H&E staining of 2 lymph nodes from the S18,20D Twist group shows increased histiocyte infiltration.

4.6.6 Conclusions and Discussion

In this section, we confirmed that phospho-modification of Twist by CK2 may play a role in mediating its stability as the S18,20D phosphorylated-mimetic Twist mutant has the highest half-life as compared to S18,20A mutant and wild-type Twist. There is increased HA-tagged ubiquitin conjugation with the S18,20A Twist mutant compared to wild-type and S18,20D mutant, suggesting increased ubiquitination in the less stable and unphosphorylated-mimetic mutant Twist. We also confirmed that IL-6 promotes the

cell motility in SCCHN cells *in vitro*. The IL-6 mediated cell motility is blocked by either the use of CK2 inhibitor or shTwist, indicating both CK2 and Twist are important in mediating this phenomenon. Further, the CK2 hyperphosphoric mimic mutant Twist (S18,20D), and not S18,20A-Twist, can increase the cell motility, supporting that IL-6 mediating cell motility is through Twist by CK2 phosphorylation.

The mechanisms of how Twist phosphorylation promotes cell motility remain unclear. It has been reported that Twist promotes the motility of breast cancer cells through transcriptionally upregulating the expression of AKT2 (Cheng, GZ et al., 2007). However, in either 293T cells or SCCHN cells, we were not able to demonstrate the upregulation of AKT2 promoters by different Twist plasmids in a luciferase promoter assay. Another potential target, E-cadherin suppression, which has been reported to result from Twist over expression in breast cancer cell lines (Ynag J et al., 2004), was not observed in the cell lysates from the Myc-Twist HN31 stable lines or transiently over expressing HA-Twist in the OSC-19 cell line. These negative results may be due to the different cell line systems used. To solve the problem, we could use comparative transcriptional profiling such as cDNA microarrays to identify and validate the potential downstream targets of Twist phosphorylation.

On the other hand, our preliminary data showed there might be increased interaction between Twist and E12 after brief treatment of IL-6 in SCCHN cells. Given the

background that E proteins have been found to participate in EMT (Thiery, JP, 2005), which is associated with regulation of cell (Lluis FE, et al., 2005), our data, although preliminary, suggests cooperative Twist and E protein transcriptional regulation in response to cytokine signals. From computational analysis of the E12 protein sequence, there are several CK2 phosphorylation consensus motifs. Further studies need to be performed to determine whether or not E12 is also phosphorylated by CK2 and how the phosphorylation of E12 and Twist might work in concert to regulate EMT could enhance our current understanding of the mechanisms of EMT regulation by Twist.

Although the *in vivo* experiments using different number of injected cells (10,000 and 50,000 respectively) in the orthotopic xenographic tongue cancer model did not show statistically significant differences in the incidence of lymph node metastasis, these studies might be confounded by endogenous Twist, the tumorigenicity or residual immune reaction in the lymph node of athymic nude mice.

Our mice experiments reveal the limitations of *in vivo* studies to demonstrate the small difference observed *in vitro*. For a 30-40% difference in cell migration, we may need 200 mice per group to have enough statistical power, which is not feasible for experimental animal studies. Another approach to studying the role of stable wild type and phospho mutant Twist expression and is to utilize different SCCHN cell lines. Experimentally, HN31 SCCHN, which was used for establishing stable cell lines due to the high transfection efficiency by electroporation, is a very resistant and aggressive cell

line. Also, these cells express higher levels of endogenous Twist than other SCCHN cells which may interfere with the observations of specific phenotypes for mutant Twist. At this aggressive background, over-expression Twist in such cell line may only minimally increase cellular invasiveness, which may result in insignificant findings *in vivo*.

V. DISCUSSION AND FUTURE DIRECTIONS

Over expression of Twist, which functions as a master regulator of tumor metastasis, has been correlated with advanced tumor metastasis, advanced tumor stage and poor prognosis in many types of cancer, including SCCHN (Yang and Wu, 2008; Kwok et al., 2005). In addition to facilitating cell motility both *in vitro* and *in vivo*, Twist has also been shown to enhance apoptosis inhibition, angiogenesis and chemoresistance *in vitro* (Cheng et al., 2007). Although a number of downstream transcriptional targets of Twist have been found, the control of Twist expression at post-transcriptional level has been less well studied. The discrepancy between Twist mRNA expression and Twist protein expression in a comparative analysis in both embryo and adult tissues indicates that Twist expression is regulated at the post-transcriptional level.(Gitelman, 1997; O'Rourke and Tam, 2002). In this project, we have shown that IL-6 treatment increases Twist protein expression through CK2 phosphorylation of Twist. This post-transcriptional modification can stabilize the Twist protein, allowing Twist to regulate cell motility.

5.1. The Importance of Twist Phosphoregulation

Although Twist phosphoregulation has been described in studies for Twist mutations in patients with Saethre-Chotzen syndrome (SCS)(Firulli et al., 2005), an autosomal dominant disorders of craniosynostosis , the role of phosphoregulation of Twist in cancer

cells, to our knowledge, has only been discussed in one study that addresses the p53-related anti-apoptotic function in the presence of Akt phosphorylation of Twist (Vichalkovski et al., 2010). In SCS, Twist dimerizes differently depending on whether it is phosphorylated by protein kinase PKA or dephosphorylated by the trimeric protein phosphatase 2A, at site T121/S123. Since Twist has been shown to play both positive and negative roles in mesenchymal differentiation, it is believed that heterodimerization with different partner proteins plays an important role in modulating its transcriptional function during cranial bone development (Connerney et al., 2008; Connerney et al., 2006).

Interestingly, not only Twist, but also its dimer partner, E12, are involved in the regulation of EMT (Connerney et al., 2006; Perez-Moreno et al., 2001). In this project, we have shown that the increased E12 protein was co-immunoprecipitated with WT-Twist in the presence of IL-6 in SCCHN cells (Figure 4.22). The data, although still preliminary, suggests that the phospho-modification of Twist may affect its dimerization with E12. A similar search for the potential CK2 phosphorylation site on the E12 protein with computational prediction, reveal that there are several CK2 sites found on this protein. Since E protein has been known as a phospho-target of p38 MAPK (Lluis et al., 2005), it would be interesting to study if E12 is also a target of CK2 and how the CK2 modification effects the interaction between Twist and E12. In our transwell migration study, cell motility is marked increased in the forced T/E dimerization relative to wild

type Twist group, however it is not seen in the co-transfection with Twist and E12. Whether phosphorylation plays a role in mediating the dimerization and transcription activity needs further investigation. Whether the transcriptional activity of Twist is modified by CK2 phosphorylation remains to be explored but could provide useful information for many labs studying EMT since the inconsistent observation across cell lines, which have raised controversies, may be reconciled thorough the phosphorylation modification of EMT proteins.

5.2. The Role of CK2 in Response to Cytokine Treatment

In this project, we chose to focus on the cytokine IL-6, which was originally regarded as a regulator of immune and inflammatory responses and its expression is detectable in many epithelial tumors and correlates with unfavorable clinical outcomes (Nishimura et al., 2000; Bachelot et al., 2003; Schafer and Brugge, 2007; Colomiere et al., 2009; Allen et al., 2007). There is also evidence that IL-6 plays a role in tumor progression of EGFR mutated lung cancer (Gao et al., 2007). However, since SCCHN tumorigenesis and progression has long been known to be influenced by multiple growth factors and cytokine signaling factors other than IL-6, we analyzed the post-transcriptional regulation of Twist in response to other SCCHN-relevant cytokines/growth factors and found that the stabilization of Twist is not IL-6 restricted (data not shown). Twist can also be upregulated within minutes after EGF and VEGF-C treatment in SCCHN lines. Since CK2 is also reported as a phosphorylation target in the

EGFR signaling pathway in neuroblastoma lines (Ji H et al., 2009), it would be interesting to validate whether or not CK2 is a downstream to these pathways in SCCHN lines because CK2 plays an important role in mediating the activity of NF- κ B, an important mediator in cytokine pathways and of prognostic value in SCCHN.

5.3. Connecting Inflammation, Twist, and Cancer progression

Our study, which links Twist, an important mediator for tumor progression, with inflammatory cytokine IL-6 and CK2, may contribute to the current knowledge regarding inflammation and the tumor progression. SCCHN tumorigenesis often arises in the inflammation within the pre-malignant lesions, leukoplakia, erythroplakia, or oral submucous fibrosis. CK2 has been reported to be activated by multiple inflammatory cytokines and growth factors related to inflammation and can regulate the activity of NF- κ B (as reviewed in section 1.3). Activated CK2 is correlated with chronic inflammatory disease such as glomerulonephritis and SLE, indicating the biological importance of CK2 in inflammation. In this study, we add Twist to the list of CK2 phosphorylation targets. Since Twist can mediate the migration of both embryonal cells and cancer cells, the link between IL-6 and CK2 may underscore the importance of inflammation to cancer development and progression.

Our experiments show that despite high levels of cancer-secreted IL-6 in the cultured supernatants, these cancer cells still can respond to exogenous IL-6 and undergo

partial EMT, indicating that cytokines from the tumor microenvironment can promote EMT and tumor progression. Recent studies showed the cytokines such as IL-8 secreted by tumor cells could further attract IL-6 secreting neutrophils to tumors resulting in a mutually beneficial microenvironment for both immune cells and cancer.

Although some of the process may be irreversible (i.e. mutation), however, some of them, such as the Twist expression by IL-6 stimulation, may be reversible if CK2 kinase activity is blocked. Although EMT in cancer may be induced in the dysregulated inflammatory response, our understanding of molecular mechanisms involved in this process may provide insight for new strategies to reverse the process.

5.4. Targeting CK2 as an Anti-cancer Therapy

In this report, we also showed that the level of Twist expression can be manipulated using a pharmacological approach with an inhibitor to CK2, suggesting that CK2 could be a useful therapeutic target in patients with SCCHN. CK2 is a highly conserved and ubiquitously expressed serine/threonine kinase, which consists of catalytic ($\alpha\alpha$, $\alpha'\alpha'$, or $\alpha\alpha'$) and two β regulatory subunits (section 1.3.3). CK2 was recently described as a “master kinase” because it controls the activity of many other kinases and regulates many important cellular process such as cell cycle, transcription, survival and viral infection. CK2 is overexpressed in multiple forms of cancer and its expression level has been correlated with prognosis in cancers (Lallemant-Breitenbach and de, 2006; Charoenrat et

al., 2004; Gapany et al., 1995). In addition, in a mouse model, silencing the expression of CK2 in SCCHN suppresses tumor growth and metastases (Ahmad et al., 2005; Ahmed et al., 2002). Because many important regulatory cellular proteins are targets of CK2 (Lallemand-Breitenbach and de, 2006) and CK2 is oncogenic in transgenic mice (Litchfield, 2003), it is not surprising that CK2 has previously attracted attention as a potential target for therapy. Currently, an oral CK2 inhibitor is available and is being investigated clinically (Perea et al., 2008; Solares et al., 2009); further preclinical and molecular studies are needed to define the therapeutic potential of CK2 in treating patients with SCCHN.

5.5 Conclusions and Future Directions

Twist, a bHLH family transcription factor, plays important roles in the control of cell survival, differentiation and motility. Despite the clinical correlation of Twist over-expression with poor prognosis in patients with a variety of cancers, the mechanisms by which Twist levels are regulated post-transcriptionally are less well understood. We provided evidence that the expression level of Twist is stabilized by IL-6 stimulation through CK2 phosphorylation on Twist at residue S18 and S20. The phosphorylation not only increases its stability but also promotes cancer cell motility.

Our preliminary data also show that there is increased heterodimerization between Twist and E12 following IL-6 treatment, which may change the transcriptional profile to

promote aggressive cellular function. Since there have been reports showing that E12 is a phospho-target of p38 MAPK and there are several putative CK2 phosphorylation sites within the E12 protein sequence, it is conceivable that phospho-modification of E12 could play an important role in EMT and dimerization with Twist. Further investigation is clearly needed to discern the roles of E12 and Twist in SCCHN tumor progression.

Our findings support previous studies that cytokines can regulate the EMT of cancer cells. The finding of CK2 in mediating these effects in SCCHN cells suggest that this kinase could be an interesting target for therapy. This is important since the progress of molecular targeting treatment for SCCHN lags behind other cancers and there is an urgent need for innovative therapy for SCCHN patients.

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VII VITA

Personals

Name : Ying-Wen Su
Date of Birth: March 17, 1971
Sex : Female
Marital Status : Married
Nationality : Taiwan

Education:

2005-2010, Ph. D. Program of Cancer Biology, The University of Texas at MD Anderson Cancer Center, Houston, Texas, US

Dissertation for Ph.D. thesis: “The post-translational regulation of Twist in squamous cell carcinoma of head and neck”

- Mentor: Dr. Jeffrey Myers M.D., Ph. D., (professor, Head and Neck Surgery)

1989-1996 M.D., Taipei Medical University

Profession and occupational experience:

2002-2005 Attending physician, Hematology/Oncology department in Mackay Memorial Hospital, Taipei, Taiwan

1999-2002 Clinical Fellow, Subspecialty Hematology/Oncology, Mackay Memorial Hospital, Taipei, Taiwan

1996-1998 Medical Resident, National Taiwan University Hospital (sponsored by Koo Foundation Dr. Sun Yat-Sen Cancer Center), Taipei, Taiwan

1995-1996 Medical Internship, National Taiwan University Hospital, Taipei, Taiwan

Certifications and Licensures :

1997 Medical Licence in Taiwan (No. 027447)

1999 Diplome, Board of Internal Medicine in Taiwan (No. 5336)

2001 Subspecialty Board of Medical Oncology in Taiwan (No. 90006)

2002 Subspecialty Board of Hematology in Taiwan (No.0359)

Poster Presentation :

“IL-6 stimulates cell motility in squamous cell carcinoma of head and neck (SCCHN) cells through CK2 phosphorylation and stabilization of TWIST “ **Ying-Wen Su**, Tongxin Xie, Daisuke Sano, Mitchell J. Frederick and Jeffrey Myers. Abstract #: 3958, the 101th Annual Meeting of the American Association of Cancer Research (AACR)

Publications:

- (1) “IL-6 stabilizes Twist and enhances tumor cell motility in head and neck cancer cells through activation of casein kinase 2” **Ying-Wen Su**, Tongxin Xie, Mitchell Frederick , Daisuke Sano , Ge Zhou, Jeffrey Myers (in submission)
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