METHYLATION IN HEAD AND NECK SQUAMOUS CELL CARCINOMA

DISSERTATION

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the Degree Doctor of Philosophy in the Graduate
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By

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ABSTRACT

In order to improve treatment and facilitate timely detection in Head and Neck Squamous Cell Carcinoma (HNSCC), elucidation of early detection markers is crucial. DNA methylation markers are advantageous, because DNA methylation is an early event. Following an overview on methylation in HNSCC in Chapter 1, we describe a genome-wide screen using Restriction Landmark Genomic Scanning (RLGS) in Chapter 2. This analysis found a set of potential tumor suppressor genes that are commonly methylated and downregulated in HNSCC.

Not all relevant candidates are detected by RLGS because of limitations of the assay. In Chapter 3, a candidate gene approach identified \textit{C/CAAT enhancer binding protein alpha (C/EBP\textalpha)}\textsuperscript{a}, a gene previously shown to exhibit tumor suppressor activity in acute myeloid leukemia (AML) and lung cancer, as a gene of interest. \textit{C/EBP\textalpha} tumor suppressor activity in lung cancer has previously been shown to be downregulated by epigenetic mechanisms. More recently, this gene has been found to be downregulated in HNSCC. This prompted investigation into the involvement of epigenetics in downregulating \textit{C/EBP\textalpha} in HNSCC. It was revealed that \textit{C/EBP\textalpha} is downregulated in HNSCC by loss of heterozygosity (LOH) and upstream DNA methylation. Also, \textit{C/EBP\textalpha}
overexpression in a HNSCC cell line (SCC22B) revealed its ability to provide tumor suppressor activity in HNSCC in vitro and in vivo.

Upstream methylation of $C/EBP\alpha$ correlates with decreased expression in HNSCC. In Chapter 4, investigation of previously unstudied AP2$\alpha$ binding sites within the upstream methylated region demonstrated that AP2$\alpha$ suppresses $C/EBP\alpha$ promoter activity and protein expression. Methylation analysis of the upstream $C/EBP\alpha$ sequence after AP2$\alpha$ downregulation revealed decreased methylation, suggesting that AP2$\alpha$ binding may precede and facilitate methylation and stable silencing of the gene. Finally, in Chapter 5 we discuss the relevance of the findings in the preceding chapters and the future direction of this body of work.
THIS IS DEDICATED TO MY PARENTS, SISTER, AND FIANCEE FOR THEIR UNCONDITIONAL LOVE, SUPPORT, ENCOURAGEMENT, AND CONFIDENCE IN ME WHICH HAS ENABLED ME TO OVERCOME CHALLENGES AND ACHIEVE THESE ACCOMPLISHMENTS.
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CHAPTER 1

PROMOTER DNA METHYLATION IN HEAD AND NECK SQUAMOUS CELL CARCINOMA (HNSCC)

1.1 HNSCC Risk Factors

Head and neck squamous cell carcinoma (HNSCC) is the 6th most common cancer in the world (1). According to the National Cancer Institute’s Surveillance Epidemiology and End Results (SEER) cancer statistics review for 2007, 34,360 individuals will be diagnosed with HNSCC, and 7,550 men and women will die of this cancer. Incidence rose significantly in the 20th century, particularly in people under 60 years of age (2). In the 1980’s, two US cancer centers reported an increase in oral tongue cancers (OTCs) in men less than 40 years old. Following this, there was a two-fold increase in OTC mortality rates for men less than 30 years of age, suggesting a strong effect of year of birth on incidence, such that men born after 1920 have increased HNSCC risk (2).

HNSCC, as its name suggests, affects different anatomical sites within the head and neck (Figure 1). Studies indicate that incidence is influenced by age, sex, race, primary site, date of birth after 1915, genetic factors, and geographic region. Furthermore, lifestyle choices (i.e. alcohol and tobacco use, oral hygiene, and sexual behavior) greatly influence risk for HNSCC.
HNSCC is primarily caused by exposure to alcohol and tobacco products, which influence major pathways of cell proliferation (3). The combination of both alcohol and tobacco use increases the risk for HNSCC 13-fold compared with either individual exposure alone (2). In the case of tobacco, increased risk may be due to genetic polymorphisms in enzymes that activate or detoxify tobacco carcinogens. p450 enzymes (CYP1A1, CYP2D6, and CYP2E1) convert tobacco-related carcinogens into reactivated intermediates which then form DNA adducts that need to be detoxified by a number of enzymes including glutathione S-transferase, UDP-glucuronosyltransferase, and N-acetyl
transferase. Therefore, increased HNSCC incidence may be related to the upset balance of activation, detoxification, and DNA repair because of impairment of the abovementioned enzymes. However, thus far there is inconsistent evidence for this hypothesis (2).

Alcohol may act as a solvent to enhance carcinogen exposure. Acetaldehyde (a metabolite of alcohol) can form adducts with DNA, interfering with DNA synthesis and repair (4, 5). Polymorphisms in alcohol dehydrogenase (ADH) (which metabolizes alcohol into acetaldehyde) have been found to affect its enzymatic activity and change local levels of acetaldehyde. For example, individuals with “fast-metabolizing” ADHs have higher concentrations of salivary acetaldehyde (6). However, different case studies have provided conflicting results on association of “fast-metabolizing” ADHs and increased cancer risk. Therefore, it may ultimately be alcohol’s solvent effect that provides the increased risk for HNSCC.

In addition to alcohol and tobacco exposure, Human Papillomavirus (HPV) infection is also a principle component in HNSCC risk. HPV is sexually transmitted and is associated with sexual behavior. HPV oral cancers generally arise from lingual and palatine tonsils within the oropharynx (7). HPV16 seropositivity has been found to pose a 14-fold increase in oropharyngeal cancer progression. HPV infection in combination with alcohol and tobacco use may act synergistically to increase HNSCC risk (8, 9).

The relationship between diet and risk of oral cancer has been found to be among the strongest for any malignancy. Oral cancer prevalence in alcohol and tobacco users decreases with increased consumption of vegetables and fruits (10-14). In contrast, high intake of eggs, butter, meat, and dairy provides an elevated risk for HNSCC (10).
As one may suspect, oral hygiene is also associated with risk of oral cancer. Increased tooth loss has the greatest correlation with increased risk, and frequency of tooth brushing and dental visits provide a decrease in HNSCC risk (10). Oral hygiene correlates and acts in synergy with alcohol and tobacco use (15).

Genetic predisposition also plays a role in development of HNSCC. Fanconi anemia (FA) increases the chance for HNSCC by more than 500-fold (16), and high-risk HPV is detected in 84% of oral cancers from FA patients. Oral cancers also arise in patients with xeroderma pigmentosum, dyskeratosis congenital, and Blooms syndrome (17). HNSCC can develop at a young age in individuals with functionally inactivated p16 (18). Also, there is a 2-4 fold increase in risk for people who have positive family history for the disease, since this increases their sensitivity to mutagens (19). In regard to this, an in vitro mutagen sensitivity assay has been developed to detect the amount of chromatid breaks in metaphase spreads as a biomarker of possible complex genetic defects in DNA repair (20).

1.2 Genetic alterations in HNSCC

Extensive research has led to the identification of several molecular alterations relevant in HNSCC. Increased aberrant expression of cytokines, cyclooxygenase-2, nuclear B-catenin, MMP2-MMP9, and plasminogen activation inhibitor-1 (PAI-1) have suggested potential as prognostic factors for HNSCC patients (21-23). Although the clinical relevance still remains unknown, hypoxic markers within the tumor microenvironment (such as hypoxia inducible factor 1 alpha) may hold predictive value (24).
DNA copy number gains commonly occur in HNSCC, allowing oncogene amplification (25). For example, aberrant NFκB expression has been shown to lead to oncogene overexpression (26), and ErbB receptor has been found overexpressed in HNSCC (27). Furthermore, cyclin L1 amplification has been found to associate with lymph node metastases (28), and co-amplification of cyclin D2 and bcl-1 has been evidenced in HNSCC (29). Also, MYC and p53 are very well known HNSCC oncogenes (30, 31).

In contrast to amplification, loss of heterozygosity (LOH) also accounts for a portion of HNSCC patients. In fact, LOH has been found to be more common in HNSCC progression than microsatellite instability (32). Some examples include loss of RAD17, which leads to decreased DNA repair (33), and LOH for KLF6 (Kruppel-like transcription factor), which is associated with tumor recurrence and decreased HNSCC survival (34). Tumor suppressor genes MTUS1, RHOBTB1, and RASSF1A also demonstrate LOH at their loci (8p21.3, 10q21, and 3p21.3 respectively) (35-37). Although a study revealed no predictive value for LOH at 3p, 7q, 8p, and 9p (38), loss of 18q has been shown to be associated with tumor progression (39).

Apoptotic cell death and the balance between pro and anti-apoptotic proteins has revealed significance to radiotherapy response (i.e. more caspase 3 = radiosensitivity, more survivin = resistant to irradiation) (40, 41). In addition to the disturbed apoptosis pathway, increased tyrosine kinases can cause aberrant kinase signaling in HNSCC (42). Patients with HPV-positive HNSCC have better prognosis with increased chance of survival (43), whereas overexpression of HER-2/neu, KI67, and p53 indicates poor prognosis (44).
Selective inhibition of metalloproteins (MMPs) (endopeptidases which digest the extracellular matrix) and their pathways has been investigated for its potential in clinical management of HNSCC. MMP expression correlates with metastasis, invasiveness, and prognosis. Indeed, a putative MMP, metalopanstimulin, has been discovered to have potential in HNSCC treatment (45). Also, salivary glutathione levels (which indicate oxidative stress in upper respiratory airways) have provided promise for chemoprevention, such that metabolic alterations of saliva could serve as an epidemiological marker for HNSCC (46). However, there is still much room for improvement in HNSCC detection and treatment.

1.3 HNSCC detection and treatment

HNSCC is generally non-symptomatic until advanced, metastatic stages of cancer, making therapy difficult. Surgery is performed only in cases of locally advanced HNSCC, and following surgical treatment many HNSCC patients are prone to relapse and ultimately systemic dissemination (47). However, recent development of hybrid accelerated radiotherapy for locally advanced HNSCC revealed 55% locoregional control and manageable toxicity, suggesting promise for improved treatment if used in combination with chemotherapy (48). Neck dissection followed by chemotherapy allows substantial regional control, but very few patients achieve long-term remission (3). Also, isolated neck recurrence following definitive radiotherapy for lymph nodes and HNSCC salvage is unfortunately fairly common (49).

HNSCC surgery can be invasive, and neck dissection in particular greatly diminishes the patients’ quality of life (50). Vocal expression is impeded for laryngectomized patients. This has led to the development of a double membrane-based
voice producing element that appears promising for female patients (51). Also, patients who undergo parotidectomy often form Frey syndrome because of aberrant regeneration of cut parasympathetic fibers (52). Therefore, new research is constantly generating new radiation tactics. For example, external beam radiation combined with interstitial brachytherapy suggests improvement for oropharyngeal SCC treatment (53). Yamane et al. discusses noninvasive intraoral ultrasonography and its newfound success in HNSCC diagnosis and metastasis prediction when used in combination with a computer-aided diagnosis system (54). Also, intensity-modulated radiation therapy in HNSCC has recently introduced innovative 3-D conformal radiotherapy. This utilizes computer-driven treatment planning and dose-distribution and delivery, providing more accurate treatment delivery and minimizing the affected adjacent normal tissue (55).

Because surgery and radiation may be invasive and/or ineffective in preventing recurrence, several drugs have been developed in an attempt to improve HNSCC treatment. Cidofovir (targets HPV type 16) was tested against HPV6 (type involved in recurrent respiratory papillomatosis), but it was found unsuccessful (56). However, artemisinin (involved in apoptosis induction) has recently shown promise for improved HNSCC chemotherapy, because it has greater antitumor effect than cisplatin and paclitaxel (57). Cetuximab (a chimeric antibody against EGFR) has been tested for its efficacy in targeting the aberrant kinase signaling that occurs in HNSCC (42). Combined treatment with 13-cis-retinoic acid, interferon-α2α, and α-tocopherol has been shown to induce cell cycle arrest and apoptosis in HNSCC (58).
However, despite these great efforts in the scientific community to improve management and treatment of the disease, HNSCC persists as an aggressive cancer with 50% of the patients dying within 5 years post-diagnosis (50). Therefore, early detection and better disease prediction becomes crucial. Currently, there is not a well-accepted method for preoperative determination of the presence of cancer in nodal diseases. Fine-needle aspiration cytology (FNAC) has been suggested to provide 75% sensitivity and specificity, with positive and negative predictive values of 94 and 38%, respectively; whereas, CT-scans allow only 79% positive and 20% negative predictive value (59). Therefore, FNAC may provide improved diagnostic modality for recurrent nodal disease, but the possible caveat of negative neck dissection persists and must still be conveyed to the patients (59).

Probably the most novel diagnostic tool involves the recent interest in the stem cell hypothesis. Recent work demonstrates the presence of cancer stem cells (CSCs) in HNSCC, suggesting promise for the development of HNSCC treatment aimed at cellular targets unique to stem cells (60). Although there has been advancement in HNSCC treatment, additional biomarkers are still crucial for improving early detection of this disease.

In general, biomarkers represent any measurable biological index associated with HNSCC onset (61). Gene expression profiling has been applied for biomarker discovery and development (62), and immunohistochemistry is utilized to measure protein expression of biomarkers (i.e. mismatch repair protein expression, which correlates with microsatellite instability) (63) (64). The amount of autoantibodies in the serum can indicate autoimmune disease, and repair of DNA damage serves as a measure for risk of
lung cancer (65, 66). Alterations in mitochondrial DNA and microsatellite length have been found to serve as markers for different disorders as well as the amount of telomerase activity (67, 68). Also, tumor hypoxia markers and EGFR expression are established for predicting therapy response (69). Mutation and epigenetic inactivation of several genes has been investigated for their potential in cancer diagnosis (64).

In HNSCC, global proteomic analysis has revealed certain genes that can classify HNSCC patients by biological differences (70). For example, E-cadherin, claudin 4 and claudin 7 have been suggested to be useful in predicting response to HNSCC treatment (71), and p53 expression serves as a biomarker for HNSCC metastasis (72). Also, epigenetic inactivation of several genes by DNA methylation has been found to associate with HNSCC progression (73-78). However, even though such markers provide great promise for early detection, epigenetic biomarkers have not yet been clinically implemented (79, 80).

1.4 DNA methylation

1.4.1 DNA methyltransferases (DNMTs) and methylation

DNMTs are a family of enzymes involved in epigenetic modifications. These modifications are capable of altering the expression status of a gene without changing the actual genetic sequence. This is accomplished via the addition of a methyl group to the 5’ position of the nucleotide cytosine by de novo DNMT (Figure 2a) (Figure 4a) (81). DNMT3A/B and 3L act as the de novo methyltransferases to establish the initial methylation pattern within the genetic sequence (82), and DNMT1 maintains methylation after replication (83).
Figure 2. DNA methylation and its cycle.  

a. DNA methylation occurs via addition of a methyl group to the 5’ position of cytosine via DNMT.  

b. DNA methylation cycle: Methionine reacts with ATP, resulting in S-adenosylmethionine (AdoMet), which serves as the methyl donor for DNMTs. S-adenosylhomocysteine (AdoHcy) is the demethylated product that can inhibit DNMTs. AdoHcy is quickly hydrolysed to homocysteine, which can be remethylated into methionine using folate, vitamin B12, methionine synthase, and the methyl donor methyltetrahydrofolate (CH₃THF).
During early embryogenesis, a wave of demethylation is followed by establishment of distinct patterns of DNA methylation. This provides for proper tissue and organ formation by tissue-specific gene methylation (84). Within mammalian genomes, imprinted genes contain differential methylation (i.e. maternal allele is methylated to ensure paternal-specific expression of that gene) (84), X-chromosome inactivation occurs through DNA methylation (85), and repetitive sequences, LINE elements, and transposons are normally methylated to prevent chromosomal instability (86, 87). Furthermore, increased methylation within the mammalian genome occurs naturally with aging (88).

Dietary factors (i.e. folic acid and vitamin B12) determine the levels of homocysteine, which is the amino acid involved in the DNA methylation pathway (89). Enzymes assist in processing methylation substrates, yielding products that can be complementary or inhibitory to the ability of DNMT to function (Figure 2b) (90). The balance between S-adenosylmethionine and S-adenosylhomocysteine dictate the degree of DNMT activity and methylation(90). Therefore, folate deficient diets and pregnancy (which decreases a woman’s homocysteine levels) can upset this process (89). Ultimately, this cycle provides methyl groups that allow DNA methylation and regulation of gene expression.

1.4.2 CpG islands, histone modifications, and gene regulation

Although most CpGs within the genome are methylated, 1-2% reside within regions dense in CpG dinucleotides (otherwise known as CpG islands) (Figure 3a). CpG islands are generally classified as sequences with greater than 50% GC content in a stretch of at least 200bp. There are ~30,000 CpG islands associated with 50-60% of known genes. They can range from 200 bp to several kb in length and generally are associated with
promoter or exonic regions. CpG islands are normally unmethylated in order to encourage transcriptional activation (91) (Figure 3b). Modifications (i.e. methylation, acetylation, ubiquitylation, phosphorylation, and SUMOylation) to specific amino acid residues on histones (H2A, H2B, H3, and H4) can change the “open” or “closed” configuration of the associated chromatin. Generally, acetylation is “activating”, and methylation, SUMOylation, and ubiquitylation are “repressing”. However, dependent on the methylation moiety (i.e. mono, di, or tri) and the affected residue, histone methylation may be either “active” or “repressive” (which is also the case for ubiquitylation) (92).

Certain enzymes are responsible for adding histone marks, such as histone methyltransferases (HMTs) which add methyl groups and acetyltransferases (HATs) which add acetyl groups. Other enzymes are involved in removing histone marks, such as deiminases (remove arginine methylation), histone deacetylases (HDACs) (remove acetylation), and ubiquitin proteases (remove mono-ubiquitin) (93-95). The typical model suggests that cytoplasmic signaling to transcription factors helps establish “active” histone tail marks within promoter sequences for gene activation. Furthermore, “repressive” marks are established via DNA-bound repressors during gene suppression. However, more recent observations suggest the histone code may not be so straightforward. This is due to the findings that certain marks may be termed “repressive” or “active” dependent on the transcriptional setting (i.e. initiation, elongation, and attenuation of transcription) (92). One explanation of this phenomenon has been that these types of marks may act first in establishing an active chromatin state and then later in reinstating gene repression (92).
Figure 3. DNA methylation distribution and the relationship to transcription.

a. **Diagram of normal DNA methylation distribution in the genome.** The lollipops represent CpGs dinucleotides (open=unmethylated, closed=methylated); arrowheads=repetitive elements; arrows=transcription start sites. Unmethylated CpGs are in promoter regions, and methylated CpGs reside within intronic and repetitive sequences.

b. **Diagram of a normal, unmethylated, CpG-island-associated gene.** The upstream sequence is unmethylated, associated nucleosomes (blue circles) are open, and histone tails (green beads) contain active marks (pink circles=acetylation) to allow transcription.

c. **Diagram of a methylated CpG-island-associated gene.** The upstream sequence is methylated, associated nucleosomes (blue circles) are tight, and histone tails (green beads) contain repressive marks (orange circles=methylation), preventing transcription.
this manner, the marks have the ability to recruit multiple effector proteins for different transcriptional outcomes (96). Both DNA methylation and histone modifications are dynamic, so the presence of certain marks should not always be taken to indicate a strict “on” or “off” status (92). Furthermore, even though much advancement has been made in the field of chromatin and histone research, there is still considerable work to be done to provide a clearer appreciation for the complexity of histone modifications.

1.4.3 Aberrant methylation in cancer

In cancer, changes to the methylation status of promoter CpG islands can cause aberrant up or downregulation of oncogenes or tumor suppressors, respectively (Figure 3c). Such epigenetic changes are understood to occur very early in life. In fact, aging organs retain a “memory” that spans through their adaptive responses to environmental stimuli all the way back to their fetal history. Changes in the epigenotype are thereby crucial, primary events in disease progression, and are of great clinical importance (88).

An abundance of recent epigenetic research has revealed the crucial role that both DNA methylation and histone chromatin changes play in cancer development (88). Consequently, techniques that allow accurate methylation assessment across the genome (i.e. bisulfite sequencing or MassARRAY) have been developed (97). Now, instead of focusing on individual genes, large chromosomal regions can be effectively studied. This improved technology may allow researchers to determine the hierarchy of events, addressing the controversy of whether or not DNA methylation preceeds histone modifications. Also, information from such broad analysis would advance detection of biomarkers, which benefits disease diagnosis, prognosis, and treatment (98, 99).
Capitalizing on the reversibility of epigenetic alterations provides great opportunity in treatment of malignancies, allowing prevention and/or cure from the disease. Small molecule inhibitors (cytidine analogues) against DNMTs (i.e. 5-azacytidine and decitabine) (Figure 4b, modified from previous publication) have shown great success in treatment of leukemias but much less efficacy in solid tumor malignancies (81, 100). However, recent work suggests promise for solid tumor treatment using drugs that target DNMT protein degradation without incorporation into the DNA (which decreases toxicity to the cells) (86). Reversible chromatin modifications have also been the target of drug development [i.e. Trichostatin A (TSA)] (101).

1.5 Methylation in HNSCC

Epigenetic modifications, especially in the form of DNA methylation, have emerged as a relevant factor in the disease progression of HNSCC (102). Despite the fact that global DNA methylation screens demonstrate relatively lower levels of promoter methylation in HNSCC as compared to other malignancies (Table 1), several epigenetically silenced cancer-related genes have been described in HNSCC. These include SFRP family genes, LHX6, p16 (103), TCF21 (74), p53 (104), and members of the Fanconi anemia/BRCA1 pathway (105). Also, there has been evidence for a positive correlation between methylation of Tissue Inhibitor of Metalloproteinase 3 (TIMP3) and Death Associated Protein Kinase (DAPK) (76). Soluble CD44 hypermethylation has very recently indicated promise as an early detection marker (77). A recent study has especially emphasized the importance of methylation through investigation of the potential for a CpG island methylator phenotype (CIMP) in HNSCC (75).
This group found a correlation between promoter hypermethylation of several tumor suppressor genes and methylation of 3 Methylated in Tumor (MINT) loci, which serve as CIMP classifiers (75). Although this study does not elucidate the etiology of the disease, it does suggest promise for using methylation as a screening tool in HNSCC (75).

Figure 4. DNA methylation, DMT, and demethylation by 5-aza-2’deoxycytidine. a. DNA methylation by DNA methyltransferase (DMT). Cytosine undergoes nucleophilic attack by DMT, allowing for the addition of the methyl from S-adenosylmethionine (S) to the 5’ carbon. The intermediate undergoes Hydrogen removal via B-elimination. b. Demethylation via 5-aza-2’deoxyeytidine. This cytosine analogue is incorporated into the DNA, methyl transfer occurs, and DMT becomes covalently bound to the DNA, preventing it from continuing to methylate DNA as DNA replication occurs.
<table>
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<tr>
<th>Cancer</th>
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<tr>
<td>ABT</td>
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<td>MBT</td>
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Table 1. Summary of overall methylation incidence in a variety of malignancies using RLGS analysis. ABT = adult brain tumor, AML = acute myeloid leukemia, BRE = breast cancer, CER = cervical cancer, CLL = chronic lymphocytic leukemia, CLN = colon cancer, HNM = metastatic head and neck tumor, HNP = primary head and neck tumor, LNG = lung cancer, MBT = metastatic brain tumor. The number of samples out of 95 showing methylation in more than 1% of the genomic loci represented in RLGS analysis (i.e. decreased or lost fragment intensity = methylation) are shown.
Figure 5. RLGS analysis. a. The DNA is digested by NotI followed by radioactive end labeling (P$^{32}$), such that only unmethylated DNA is digested and labeled. This is followed by an EcoRV digestion, and the products are run out in “tube gels” (1D). A HinfI in-gel digestion is then performed, and the fragments within this gel are then run through another gel in a second dimension (b.), yielding a large profile containing thousands of fragments that represent genomic loci. b. The numbers indicate kb, because the molecular weight can be determined based on the distance the fragment can traverse through the gel.

In addition to specific promoter hypermethylation, global DNA hypomethylation also occurs in HNSCC (106). Hypomethylation, which can result from low folate intake, is associated with increased risk of disease (107). Consequently, global DNA methylation detected through whole blood has been suggested for screening purposes (107).
However, one may realize the potential caveats associated with this technique, given the involvement of both global hypomethylation of repetitive sequences and specific promoter hypermethylation. Therefore, elucidation of epigenetically regulated genes that correlate with poor prognosis would aid in screening and greatly benefit HNSCC early detection (108).

Our laboratory utilizes a technique called Restriction Landmark Genomic Scanning (RLGS) for genomewide promoter methylation analysis (109) (Figure 5). Previous RLGS analysis on several HNSCC patient DNAs revealed more methylation in metastatic HNSCC samples compared to primary tumors, and the methylated loci in metastatic vs. primary tumors within the same patient were found to be dissimilar (102). Also, RLGS analysis has been utilized to identify amplification of potential oncogenes in HNSCC (25). In the following study, we demonstrate how we used these RLGS profiles to identify potential tumor suppressor genes that are frequently methylated in HNSCC.

1.6 C/EBP\(\alpha\) tumor suppressor protein

C/EBP\(\alpha\) is part of a family of basic region/leucine zipper transcription factors involved in cell cycle regulation and differentiation (110). It promotes anti-proliferation through inhibition of CDK2, 4, 6 and repression of S-phase gene transcription (Figure 6a). It is alternatively translated via leaky translation, creating 42 and 30 kDa proteins. Although both isoforms are able to bind to C/CAAT elements in target promoters, only the 42 kDa isoform contains the anti-mitotic activity (111) (Figure 6b). In this way, the truncated isoform behaves as a dominant negative.
Figure 6. C/EBPα cell cycle inhibition and alternative translation. a. C/EBPα interacts with CDK proteins, preventing phosphorylation of RB and release of E2F. Therefore, E2F cannot bind to and activate S-phase genes, thereby blocking cell cycle progression. b. Leaky translation creates two C/EBPα isoforms: full length 42 kDa, and truncated 30 kDa, which lacks the first 119 amino acids which contain a large portion of the transactivation domain (TDI-III) that confers the anti-mitotic activity. However, both isoforms contain the DNA binding domain (DBD) and Leucine zipper (LZ).

Because of its role in cell cycle regulation and cellular differentiation, C/EBPα became a very attractive tumor suppressor candidate. Its involvement in granulocyte and neutrophil differentiation led to investigating C/EBPα in AML (112).
This study revealed for the first time that C/EBP\(\alpha\) N-terminal mutations allow translation of only the dominant negative isoform (112). Abolishing the anti-mitotic 42 kDa protein causes the differentiation block evidenced in AML (112).

More recently, C/EBP\(\alpha\) has been investigated for its relevance in lung cancer, because it is expressed in bronchial cells (113). This study not only exposed C/EBP\(\alpha\) as a novel tumor suppressor in lung cancer, but also revealed significantly greater downregulation in poorly differentiated and advanced stages of the disease (113). Subsequent to these findings, the possibility for epigenetic downregulation of C/EBP\(\alpha\) in lung cancer was investigated (114). This analysis demonstrated a correlation with upstream DNA methylation and decreased C/EBP\(\alpha\) expression (114).

C/EBP\(\alpha\) is also expressed in the epidermal keratinocytes and is essential for squamous cell differentiation (115). Therefore, it is not surprising that it was found to be downregulated in squamous cell carcinomas (115). Its decreased expression in skin carcinomas was shown to be associated with negative regulation from oncogenic Ras, and reexpression of C/EBP\(\alpha\) provided decreased proliferation (116). Almost simultaneously, another study revealed significant downregulation of C/EBP\(\alpha\) in HNSCC that correlated with poor prognosis (i.e. lymph node metastasis) (117). However, how this downregulation occurs remained to be shown.

1.6.1 Regulation of C/EBP\(\alpha\)

Upstream Stimulatory Factor (USF) and SP1 proteins have been shown to be primary transcriptional activators of C/EBP\(\alpha\). USFs and AP4 regulate C/EBP\(\alpha\) expression in adipocyte differentiation (118). USF1 and USF2 bind as heterodimers on a required E-
box within \( C/EBP \alpha \)'s promoter, increasing transcriptional activation; whereas AP4 can bind the same E-box and repress transcription (118). Therefore, USF1/2 and AP4 are inversely expressed during adipocyte development to allow for proper \( C/EBP \alpha \) expression and adipocyte differentiation (118). Sp1 is also a factor required for \( C/EBP \alpha \) activation in adipocytes (119), brain, and lung cells (114). However, the activation offered by SP1 is specific to human \( C/EBP \alpha \), because it is repressive to the murine promoter and has no effect on the Xenopus promoter (120). Instead, the murine promoter utilizes auto-regulation via \( C/EBP \alpha \) binding to C/CAAT elements within its own promoter (110). Gfi1 transcriptional repression and Stat3 activation of \( C/EBP \alpha \) after G-CSF stimulation have been shown to be required for proper myeloid differentiation (121, 122).

Activator Protein 2 alpha (AP2\( \alpha \)) has been shown to act as a \( C/EBP \alpha \) suppressor in adipocytes, hepatocytes, and keratinocytes (119, 123, 124) by inhibiting adjacent SP1 binding and \( C/EBP \alpha \) activation. Interestingly, decreased AP2\( \alpha \) expression and increased \( C/EBP \alpha \) expression is required for adipocyte differentiation. AP2\( \alpha \) is one of five members within a family of AP2 transcription factors. These proteins are developmentally regulated, retinoic acid-inducible genes that are involved in apoptosis, cell growth, and differentiation. These genes regulate many genes in differentiated tissues and tumor cells. For example, AP2 binds with Rb to regulate E-cadherin in epithelial cells. It has also been shown to bind to p53 and p21 and induce cell cycle arrest (125). During retinoic-acid-induced cell differentiation, AP2\( \alpha \) recruits nucleophosmin (NPM) to promoters of retinoic-acid-responsive genes where they act as co-repressors via HDAC recruitment (126).
1.6.2 C/EBPα in HNSCC

In a recent study, microarray expression profiling was performed on 40 HNSCC tumor samples to identify gene expression differences correlating with poor prognosis (117). C/EBPα was one of the genes analyzed, and the authors found significant C/EBPα downregulation in the HNSCC tumor samples (117). Furthermore, a significant correlation was found between C/EBPα downregulation and patients with extensive lymph node metastasis (117).

Decreased C/EBPα expression could contribute to the tumor cells’ characteristics of uncontrolled proliferation and loss of differentiation. This has already been demonstrated in acute myeloid leukemia (AML) and lung cancer (113), the latter of which has more recently demonstrated downregulation by epigenetic mechanisms (114). The studies described in the proceeding chapters will demonstrate epigenetic downregulation of potential tumor suppressor genes, including C/EBPα, in HNSCC.
CHAPTER 2
FREQUENTLY METHYLATED GENES IN HNSCC

2.1 Background

HNSCC, a cancer primarily caused by alcohol and tobacco exposure (127), is the 6th most common cancer worldwide (1). According to the National Cancer Institute’s Surveillance Epidemiology and End Results (SEER) cancer statistics review, 34,360 individuals will be diagnosed with HNSCC in 2007 (24,180 of those being men and 10,180 being women). Furthermore, 7,550 men and women will die of this cancer in 2007. Treatment of HNSCC is complex, utilizing radiation therapy or surgery for early stages and a combination of surgery with chemoradiation for locoregionally advanced disease. Unfortunately, the latter is limited to a complete response of 50% (47), and patients often require long-term rehabilitation. Early detection markers for better prediction and outcome would greatly benefit this aggressive disease.

DNA methylation within the promoter region of a gene can result in chromatin compaction and inhibition or downregulation of gene transcription (128). Aberrant promoter methylation is often responsible for gene silencing in a variety of malignancies (129). Although overall CpG island methylation incidence in HNSCC is lower compared to other cancer types (102), several genes have been shown to be epigenetically downregulated in HNSCC (73, 78, 130). These include SFRP family genes (78), LHX6...
(130), p16 (131), TCF21 (74), p53 (104), and members of the Fanconi anemia/BRCA1 pathway (105). Also, there has been evidence for a positive correlation between methylation of Tissue Inhibitor of Metalloproteinase 3 (TIMP3) and Death Associated Protein Kinase 1 (DAPK1) (76). Because methylation is an early event, methylation markers could provide great promise for early detection and treatment (132).

Our laboratory utilizes a technique called Restriction Landmark Genomic Scanning (RLGS) for genomewide promoter methylation analysis (109). Previous RLGS analysis on several HNSCC patient DNAs revealed more methylation in metastatic HNSCC samples compared to primary tumors, and the methylated loci in metastatic vs. primary tumors within the same patient were found to be dissimilar (102). Also, RLGS analysis has been utilized to identify amplification of potential oncogenes in HNSCC (25). In our study, we used these RLGS profiles to identify potential tumor suppressor genes that are frequently methylated in HNSCC. This analysis provided 5 genes (IRX1, FUSSEL18, EBF3, SLC5A8, and SEPT9) that were epigenetically silenced in more than 10% of the patient samples analyzed. Interestingly, all of these genes have been previously found to exhibit tumor suppressor abilities in several malignancies, including brain, breast, and gastric cancer (133-142).

2.2 Materials and Methods

Cell lines. The human HNSCC cell lines (SCC22B, SCC11B, SCC17AS, SCC25, and SCC8) used in the study were provided by Dr. Thomas Carey from the University of Michigan. These were maintained in DMEM with 10% FBS and 1% Streptomycin/Penicillin antibiotics.
**Combined Bisulfite Restriction Analysis.** Genomic DNA (1 μg) in a volume of 50 μl was denatured by NaOH (final concentration, 3M) for 30 min at 37 °C. The denatured DNA was then treated with 30 μl of 10 mM hydroquinone and 520 μl of 3M sodium bisulfite at 50 °C overnight. The PCR products were purified with a purification kit (Qiagen, CA) and incubated with methylation sensitive enzymes (i.e. BstUI, HPYCH4IV, or TaqI). Digested DNA was then size-fractionated via polyacrylamide gel electrophoresis to detect the methylation status.

**MassARRAY Methylation Analysis.** Quantitative DNA methylation analysis was performed by MassARRAY technique. Briefly, 1 μg of HNSCC patient or cell line DNA was bisulfite-treated, *in vitro* transcribed, cleaved by RNase A, and subjected to MALDI-TOF mass spectrometry analysis to determine methylation patterns, as previously described (97). In order to normalize for any bias, standards (0%, 5%, 10%, 20%, 40%, 60%, 80%, and 100% SssI methylated) were included in the assay to generate an “observed to expected” plot. The equation of the best fitting line was then used to normalize the values for the HNSCC samples. Subsequent to normalization, tumor samples with more methylation than adjacent normal samples were considered methylated. Standard curves are displayed below in Figure 7.

**RNA isolation and cDNA synthesis.** RNA was isolated according to the manufacturer’s protocol. DNase treatment was performed on 1-2 μg of RNA by adding 2U of DNaseI (Invitrogen, Carlsbad, CA), 1 μl DNase buffer, and 0.4 μl RNase Out (Invitrogen, Carlsbad, CA) for 15 min at room temperature.
Figure 7. Standard curves for MassARRAY normalization. The graphs show the plots for average % methylation of the standards (0%, 5%, 10%, 20%, 40%, 60%, 80%, and 100% methylated) for the 5 genes. The observed % methylation (y-axis) vs. the expected % methylation (x-axis) is plotted, then the best fitting line and its correlating equation is calculated and used for normalizing that gene’s MassARRAY data to account for any bias. The slope of the lines is also shown (i.e. the $R^2$ value).
One microliter ethylenediaminetetraacetic acid (EDTA) was then added to the mix for 10 min at 65°C, followed by an incubation on ice for 5 min. Complementary DNA synthesis was performed with 2 μl random hexamers and 1 μl deoxynucleotide triphosphates (dNTPs) (10 mmol/L) for 5 min at 65°C then 2 min at 4°C; 2 μl of 10X buffer, 4 μl MgCl₂, 2 μl dithiothreitol (DTT), and 1 μl RNase Out was added for 2 min at 25°C; 100 U of SuperScript II (Invitrogen) for 50 min at 42°C; 15 min at 70°C, then transferred to 4°C.

5 aza-2’-deoxycytidine treatment. HNSCC cell lines were incubated for 96 hrs with 3 μM 5-aza-2’-deoxycytidine (5-aza-dC) (Sigma) with media changed every day. Treated cells were harvested for analysis 1 to 4 days after the procedure. Cells were suspended in Trizol for RNA isolation.

Real-time PCR (RT-PCR). Quantitative mRNA expression was measured using SYBR Green I (BioRad, Hercules, CA) in an I-Cycler (BioRad). Expression of glycosylphosphatidylinositol (GPI) was used as the internal control gene. For the RT-PCRs on the HNSCC patient samples, each tumor was normalized to the expression of its adjacent normal, which was set to a value of one. For the 5-aza-dC treated HNSCC cell lines, each treated cell line was normalized to its untreated counterpart, which was set to a value of one.

Plasmids. SLC5A8-pBABE was provided by Dr. Joe Costello (UCSF) (133).

Transfections. Stable transfections performed as previously described (74). Briefly, 120 hrs post-infection, cells were collected, RNA isolated, and cDNA synthesized for RT-PCR confirmation of overexpression.

Growth Curve. Cells were plated in triplicates in a 6 well plate for a 5 day growth curve analysis. Trypsinized cells were suspended in 2 mls PBS, and 500 μl was counted.
Colony Formation Assay. One thousand cells were plated in triplicates in 60 cm plates. After 10 days, the media was removed, cells were washed with PBS, fixed with 2:1 methanol:acetic acid for 15 minutes, and stained with 0.1% crystal violet PBS for 30 minutes at RT. Cells were washed with water, and colonies were counted.

Statistical Analysis. The statistical significance was calculated by unpaired Student’s $t$ test, and $P<0.05$ was considered to be statistically significant.

2.3 Results

2.3.1 RLGS analysis in HNSCC patient samples. Global methylation analysis via RLGS has previously been performed on 42 HNSCC patient samples (25, 102, 109). However, these studies focused only on general changes in methylation between primary and metastatic tumors and copy number changes. Therefore, these profiles were reevaluated to identify the specific genes (potential tumor suppressors) that are frequently methylated in HNSCC. This analysis yielded 23 different loci among the profiles that had decreased RLGS fragment intensity in more than 10% of the profiles (Table 2 and Figure 8). Eight of these loci were associated with a CpG island and a known gene: ALX3, HMX2, SLC5A8, SEPT9, FUSSEL18, EBF3, IRX1, GBX2 (Table 2). BLAT analysis revealed that 7/8 of these genes had CpG islands upstream of their transcription start sites, and SEPT9 contained several within the transcribed region (Figure 9). The RLGS analysis revealed that 69% (29/42) of the HNSCC patients had at least one of these 8 genes methylated. 31% (9/29) shared methylation between 2 genes, and 14% (4/29) shared methylation between 4 genes (2/29) or 6 (2/29) genes (Table 2).
Figure 8. Decreased intensity fragments identified by RLGS analysis. Three loci are shown as examples and are indicated by their RLGS identifier (i.e. 3D41, 4D8, and 3D57) and associated gene. The profiles on the left are generated from adjacent normal tissue, whereas the profiles on the right come from HNSCC tumor tissue. The arrow indicates the fragment of interest. Decreased intensity is assessed by comparing with the profile of its adjacent normal.
| Fragment | Chrom | Gene | Freq | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 | 39 | 40 | 41 | 42 |
|----------|-------|------|------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| 4E08     | 1p13.3| ALX3 | 0   |   |   |   |   |   |   |   |   |   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 2E04     | 16p11.2| NA   | 9   |   |   |   |   |   |   |   |   |   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 3E08     | 17q25.3| SEPT9| 9   |   |   |   |   |   |   |   |   |   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 3D07     | 10q26.13 | HMX2 | 8   |   |   |   |   |   |   |   |   |   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 3D11     | 12q23.2 | SLC5A8 | 7   |   |   |   |   |   |   |   |   |   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 3E25     | 16q12.1 |     | 8   |   |   |   |   |   |   |   |   |   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 2E64     | 12q24.21|     | 6   |   |   |   |   |   |   |   |   |   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 3F01     | 5p13.33 | IRX1  | 5   |   |   |   |   |   |   |   |   |   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 3E04     | 5p13.33 |     | 5   |   |   |   |   |   |   |   |   |   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 3E19     | 5p13.33 |     | 6   |   |   |   |   |   |   |   |   |   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 3F25     | 5p13.33 |     | 5   |   |   |   |   |   |   |   |   |   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 4E03     | 5p13.33 |     | 5   |   |   |   |   |   |   |   |   |   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 5E01     | 5p13.33 |     | 5   |   |   |   |   |   |   |   |   |   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 2E00     | 18q21.1 | FUSSEL18 | 4   |   |   |   |   |   |   |   |   |   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 2E54     | 10q26.3 | EBF3  | 4   |   |   |   |   |   |   |   |   |   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 3C21     | 5p15.33 |     | 5   |   |   |   |   |   |   |   |   |   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 3C03     | 5p15.33 |     | 4   |   |   |   |   |   |   |   |   |   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 3E06     | 5p15.33 |     | 4   |   |   |   |   |   |   |   |   |   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 3E10     | 5p15.33 |     | 4   |   |   |   |   |   |   |   |   |   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |

**Table 2. Complete summary of RLGS analysis in HNSCC.**
The table shows the different RLGS fragments lost in at least 10% of the profiles. Chromosomal locations are provided for the cloned fragments, as well as any associated genes. “Freq” is the number of profiles in which that fragment displayed decreased intensity compared to normal. All patient profiles are listed by unique patient numbers, 1 through 42, in order to show shared methylation events within a given patient.
Figure 9. Depiction of BS primer and NotI/RV clone locations relative to the associated genes. The arrow depicts the transcription start site. The open, white box represents the COBRA product, the black box indicates the MassARRAY product, and the black line represents the NotI/RV clone, with the black triangle indicating the NotI site. The dashed line reveals the location of the associated CpG island. SEPT9 is the only gene not drawn to exact scale due to its large size.
2.3.2 Confirmation of DNA methylation by High-throughput MassARRAY DNA methylation analysis. The MassARRAY technique was employed in order to attain a broad and quantitative DNA methylation analysis of the gene promoters. Primers were designed near or upstream of the transcription start site and/or within the associated CpG island (Figure 9). The genes with more than 20% methylation incidence according to a preliminary COBRA analysis (Figure 10a) were selected for the MassARRAY (i.e. SEPT9, FUSSEL18, SLC5A8, EBF3, and IRX1). The MassARRAY analysis revealed 56% (20/36) of the tumor samples with SEPT9 methylation; 49% (17/35) with methylation for FUSSEL18; 32% (13/41) SLC5A8 methylation; 50% (28/56) EBF3 methylation in tumors; and 45% (21/47) showed IRX1 methylation (Figure 10b). The methylation results from COBRA and MassARRAY are compared in Figure 10c. Interestingly, 53% (21/40) of the methylated tumor samples that were shared between the genes revealed methylation in at least 2 of the genes tested (most commonly between IRX1 and EBF3). Also, 15% of the samples contained shared methylation between 3 of the candidate genes (most commonly between IRX1, SLC5A8, and EBF3) (Table 3). However, there did not appear to be any correlation with tumor location, age, or gender (Table 3).

2.3.3. mRNA expression of methylated genes is reduced in HNSCC tumor samples.

To assess whether methylation may provide decreased expression, RT-PCR analysis was performed on the 5 genes with the highest methylation incidence (see Figure 10). IRX1 showed decreased expression in 80% (12/15) of the tumors, SLC5A8 had 77% (10/13), EBF3 showed 46% (6/13), FUSSEL18 showed 44% (4/9) of tumors, and SEPT9 revealed decreased expression in 25% (3/12) of the tumor samples (* P<0.0430) (Figure 11).
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Table 3. Summary of tumor samples with methylation from MassARRAY analysis.
Figure 10. Methylation of candidate genes in HNSCC.

a. Gene methylation incidence in HNSCC tumors and cell lines by COBRA. The % of HNSCC samples showing methylation by COBRA analysis is summarized.

b. MassARRAY broad methylation analysis. Row = sample, column= CpG unit. The top section= normals, the middle section= tumors, and the bottom section= HNSCC cell lines. Increasing color intensity represents increasing methylation.

c. Comparison of methylation results using COBRA or MassARRAY. The graph reveals the percentages of methylation attained for the different genes tested using either COBRA or MassARRAY analysis.
Figure 11. Candidate mRNA expression in HNSCC tumors. The top 5 candidates from the methylation summary were subjected to quantitative RT-PCR analysis. Each bar represents the expression of HNSCC tumor or adjacent normal. The tumor expression is normalized to its matched adjacent normal, which is set as 1 (indicated by the dashed line). * P < 0.0430.
Figure 12. Candidate mRNA expression in HNSCC cell lines following demethylation. cDNA was synthesized from HNSCC cell lines with and without 1 μM 5-aza-dC for 96 hours. Quantitative RT-PCR was performed on the 4 candidates that showed more than 25% of the samples with decreased mRNA expression in HNSCC tumors compared to normals. Each bar represents the expression of the 5-aza-dC treated HNSCC cell lines normalized to the untreated HNSCC cell line, which is set as 1. * P < 0.050.
2.3.4 Demethylating treatment restores expression. Several HNSCC cell lines were treated with 5-aza-dC, and mRNA expression was compared between treated and untreated cell lines to analyze the capacity for demethylation to restore gene expression. RT-PCR was performed on the 4 genes that had originally shown decreased expression in more than 25% of the HNSCC tumor samples tested (Figure 11). This analysis revealed a significant restoration of mRNA expression in 4/5 cell lines for SLC5A8, 3/5 cell lines for FUSSEL18, and 2/5 cell lines for IRX1 and EBF3 (* P < 0.050) (Figure 12).

2.3.5 SLC5A8 tumor suppressor activity. The methylated genes identified in this study are possibly downregulated due to their tumor suppressor activity. In order to investigate this, one of the top methylated candidates (SLC5A8) was overexpressed in an HNSCC cell line. After validating overexpression (* P < .001) (Figure 13a), colony formation assays and growth curves were performed. The colony formation assay revealed a significant decrease in colonies in the presence of SLC5A8 overexpression (* P < 0.006) (Figure 13b). Also, growth curve analysis revealed a significant reduction in cell proliferation compared to the wild type SCC22B cells (* P < 0.005) (Figure 6c). We also found that increased SLC5A8 expression correlates with decreased SURVIVIN expression (an SLC5A8 target involved in apoptosis resistance (* P < 0.015) (Figure 13d and 14). Therefore, SLC5A8 overexpression appears to provide tumor suppressor activity in HNSCC.

2.4 Discussion

HNSCC is an aggressive cancer and exhibits poor survival outcome for patients in advanced stages (1). Early detection markers would provide great promise for timely diagnosis and treatment, but very few good markers are currently known (80).
Figure 13. Overexpressing SLC5A8 in HNSCC provides tumor suppressor activity.

a. cDNA was synthesized from HNSCC cell line SCC22B with and without SLC5A8-pBABE. Quantitative RT-PCR was performed to validate overexpression. * P < 0.001.

b. Colony formation assay was performed with SCC22B +/- SLC5A8 overexpression. 1,000 cells plated, grown 10 days, and colonies stained. * P < 0.006.

c. Growth curve analysis on wildtype and SLC5A8-overexpressing SCC22B cells. 5,000 cells were plated, and each day the plates were trypsinized and cells counted. * P < 0.005.

d. Quantitative RT-PCR was performed to check Survivin expression. * P < 0.015.
Deletions of 10q (which includes EBF3) and 14q have been shown to correlate with poor prognosis (134, 135), and increased expression in certain genes has been shown to indicate aggressiveness of disease (136-138). However, no methylation markers for HNSCC have been clinically developed (80). This study revealed a group of candidate tumor suppressor genes that are frequently methylated and downregulated in HNSCC, and demethylation was able to restore gene expression in HNSCC cell lines. SLC5A8 has previously been shown to act as a tumor suppressor in several cancer types (133, 139, 140), and overexpression of this candidate in an HNSCC cell line also revealed tumor suppressor activity. The other frequently methylated candidates elucidated in this study have also demonstrated tumor suppressor function in other malignancies, and some have previously been found to be methylated (141-143). However, fairly little is known about most of the candidate genes selected in this study, with the exception of SLC5A8.

FUSSEL18 has been characterized in recent years as a homolog of the Ski family that is predominantly expressed in the brain and is downregulated in brain cancer (144). It has also evidenced tumor suppressor activity in breast cancer (141). SEPT9 has been found to be methylated or deleted in ovarian cancer (142, 145), and a MLL-SEPT9 fusion protein has been discovered in MDS and AML (146, 147). It has also been detected as a candidate tumor suppressor in breast cancer (145). EBF3 is a gene involved in adipogenesis and neuronal maturation (148-151). It has been shown to be deleted or methylated in 73% of brain cancer, not to mention that it is silenced in brain, colorectal, liver, breast, and bone cancers (143). Also, EBF3 and SEPT9 are both involved in microtubule assembly (151, 152). IRX1 is a gene involved in digit, heart, and lung development (153-155). It is one of
3 genes recently found to reside within a minimally deleted region in gastric cancer (156) and is differentially expressed in seminomas (157). SLC5A8 is a sodium-coupled monocarboxylate transporter that is expressed in a variety of tissues (158, 159), and has been shown to be epigenetically silenced in several different types of cancer (133)(139)(160)(140). SLC5A8 may provide tumor suppressor activity through its role in cell apoptosis, because recently it has been found to be required for pyruvate to inhibit HDACs and induce apoptosis (160). Finally, EBF3 (143), SEPT9 (142), SLC5A8 (160), and IRX1 (161) have all been shown to be involved in apoptosis.

TGF-β signaling has been found to be disrupted in HNSCC progression (162-168); therefore, this pathway has been targeted for therapy (169-171). Aberrant TGF-B expression and defects in Smad2 and 4 result in loss of TGF-B growth inhibition (162, 163), TGF-B mutations correlate with metastatic HNSCC, and certain proteins in HNSCC allow the cancerous cells to overcome TGF-B inhibition and proceed through the cell cycle (163, 166, 172). Aberrant expression of hypoxia-induced proteins has also been shown to have relevance for HNSCC progression and prognosis prediction (173-179). Overexpression of Hypoxia Inducible Factor 1 alpha (HIF-1α) has been shown to associate with poor prognosis and treatment failure in HNSCC (180, 181). VEGF, an activator of HIF-1α, has also been found to be overexpressed in HNSCC (182).

There has been much evidence generated in support of synergistic cooperation between the hypoxia and TGF-B signaling pathways (183-185). Hypoxia can induce TGF-B (184), which in turn activates Smad proteins (184). Smads activate proteins responsible for blocking cell cycle progression (i.e. p16) (186-188).
Also, during tumor and inflammatory responses, increased TGF-B can induce Smad proteins to repress PHD2 (the protein which degrades HIF-1α), indirectly stabilizing HIF-1α (185). HIF-1α can then activate TGF-B and Survivin gene transcription by binding to their promoters, the latter of which results in apoptosis resistance (184, 189).

Interestingly, the candidates elucidated in our study are involved in these pathways (Figure 14). FUSSEL18 has been shown to bind Smad 2 and 3 and inhibit TGF-B signaling (144). Also, SEPT9 interacts with HIF-1α to prevent its ubiquitination and degradation, which can allow TGF-B feedback activation (184, 190). IRX-1 is a downstream target of Smad 2-mediated transcriptional activation (191), and SLC5A8 downregulates Survivin (HIF-1α’s target) to induce apoptosis (160) (which was also shown in our study). Although no evidence exists for involvement of EBF3 in TGF-B signaling, an EBF associated zinc-finger protein has been found to bind with Smad1 and Smad4 proteins (192-194). Therefore, epigenetic downregulation of these candidate genes may be critical in order to disrupt their roles in TGF-B signaling, allowing uncontrolled proliferation and apoptosis resistance for HNSCC progression.

The research discussed in this study may lend the opportunity for improved HNSCC screening and detection. However, more comprehensive studies with large patient sets should be performed before clinically implementing these markers. Methylation of the genes described in this study may occur at an early stage, providing great promise for earlier HNSCC detection.
Figure 14. Working model of candidate genes’ involvement in TGF-B signaling with relevance for HNSCC progression. The candidates are shown in their interactions with different components of the pathway. Hypoxia initiates TGF-B signaling, which activates Smad proteins that can be bound by FUSSEL18. Smad then inhibits cell cycle progression through p16 activation, activates IRX-1, or indirectly activates HIF-1α through PHD2 repression. HIF-1α, which can be bound by SEPT9, allows feedback activation of TGF-B or apoptosis resistance through activation of Survivin. However, SLC5A8 can repress Survivin to restore apoptosis.
Therefore, increased efforts should be taken to clinically implement saliva-based methylation screening assays, which would be non-invasive, inexpensive, and readily accessible (195). MS-PCR, has been shown to provide 82% sensitivity and 100% specificity for methylation screening purposes using a variety of substrates, including saliva (195-199). However, the MassARRAY analysis used in this study has promise for even greater sensitivity (i.e. detects $\geq 5\%$ methylation), and it provides the opportunity for quantitative methylation analysis of several CpGs in large sample sets (97). In conclusion, demethylating treatment has already proven successful in treating solid tumors (86, 200); therefore, the methylated candidates revealed in this study deserve further attention in order to discern their potential worth for HNSCC diagnosis and prognosis prediction.

2.5 Acknowledgements

The authors thank Dr. Thomas Carey for the HNSCC cell lines (SCC22B, SCC25, SCC8, SCC11B, SCC17AS). Thank you to the members of the Plass lab for thoughtful discussions. This work was supported in part by a grant from NIDCR, DE13123 (CP) and an AGGRS scholarship (KB).
CHAPTER 3
C/EBPα EPIGENETIC DOWNREGULATION IN HNSCC

3.1 Background

Head and neck squamous cell carcinoma (HNSCC) is the 6th most common cancer in the world (1). It is primarily caused by exposure to alcohol and tobacco products, which influence major pathways of cell proliferation (127). Surgery is performed only in cases of locally advanced HNSCC, and following surgical treatment, many HNSCC patients are prone to relapse and ultimately systemic dissemination (47). Neck dissection followed by chemotherapy may allow substantial regional control, but very few patients achieve long-term remission (3). Therefore, early detection and better disease prediction via genetic and epigenetic biomarkers becomes crucial.

Epigenetic modifications, especially in the form of DNA methylation, have emerged as a relevant factor in the disease progression of HNSCC. Despite the fact that global DNA methylation screens demonstrated relatively lower levels of promoter methylation in HNSCC as compared to other malignancies (102), several epigenetically silenced cancer genes have been described in HNSCC. However, none of the epigenetically regulated genes have been identified as poor prognostic markers in HNSCC (108). In a recent study, microarray expression profiling was performed on 40 HNSCC
tumor samples to identify gene expression differences correlating with poor prognosis (117). C/EBPα was one of the genes analyzed, and the authors found 78% (31/40) of the HNSCC tumor samples had C/EBPα downregulation compared to the expression in matched normals (117). Furthermore, a significant correlation was found between C/EBPα downregulation and patients with extensive lymph node metastasis (117).

Decreased C/EBPα expression could contribute to the tumor cells’ characteristics of uncontrolled proliferation and loss of differentiation. This has already been demonstrated in acute myeloid leukemia (AML), where mutations in the coding region of C/EBPα have been found to relieve the protein of its ability to block cell cycle progression (201). Also, C/EBPα provides an antiproliferative role in lung cancer (113), and we have recently shown that its downregulation is conducted by epigenetic mechanisms (114). Therefore, we hypothesized that C/EBPα might play a role in tumor and metastatic suppression in HNSCC.

3.2 Material and methods

Patient samples and cell lines. Frozen tumor tissues and adjacent normal tissue from HNSCC patients were attained from The Ohio State University Medical Center via the Cooperative Human Tissue Network. Surgery was performed on all patients at The Ohio State University Medical Center. All sample collections were done according to the National Institutes of Health guidelines and under a protocol approved by The Ohio State University’s Institutional Review Board. Control samples were collected from morphologically normal tissue located at least 3 cm from the tumor margin. Histopathological evaluation was performed on all samples for verification.
The HNSCC cell lines SCC4, SCC5, SCC8, SCC11B, SCC17AS, SCC22B, and SCC25 were provided by Dr. Carey, University of Michigan. These were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) with 10% Fetal Bovine Serum (FBS) and 1% streptomycin/penicillin (S/P) antibiotics.

**Immunohistochemistry.** Immunohistochemical staining was performed as previously described (114), including 3 tissue microarrays containing HNSCC normal, tumor, or metastatic samples. Tissue microarrays contained triplicates of each primary tumor or metastatic tissue sample. The tumor array contained 48 patient samples, the metastatic tissue array contained 34 samples (shared in the tumor array), and the normal array contained 47 matching adjacent normal samples. Scoring was done using the following index: 0) no staining, 1) faint staining in < 10% cells, 2) light staining in 10-50% of cells or medium staining in <10% cells, 3) light staining in all cells or dark staining in 10% cells, 4) medium staining in 10-50% of cells, 6) medium staining in all cells or dark staining in 10-50% of cells, and 9) dark staining in all cells. HNSCC tumor or metastatic tumor samples had to contain an index of at least 2 scores below its paired normal to be considered downregulated. One tumor sample was without a matching normal for comparison, and 14 tumor/normal pairs lacked matching metastatic samples. The magnification used was 400X (40X objective x 10X eyepiece).

**Migration Assay.** 400 μl of 10%FBS DMEM was plated in a 24-well plate. Millicell Culture Plate Inserts (Millipore, Billerica, MA) were placed into each well, and 5,000 or 20,000 cells were suspended in 300 μl DMEM without FBS. As a control, chambers with no cells were prepared similarly. After 20 hrs, the media was aspirated and chambers
washed with Dulbecco’s Phosphate-Buffered Saline (PBS). 250 μl of 0.05% crystal violet 3.7% formaldehyde was placed in the upper and outer chambers. Cells were allowed to incubate for 30 min, then the crystal violet was removed, and the wells were washed with water. After allowing 15 min for drying, 80 μl of water was used to moisten a cotton tip applicator. The applicator was then used to rub the membrane underneath the chamber, broken close to the tip and placed in a reaction tube with 500 μl 100% methanol. Tubes were vortexed well and incubated for 30 min. 100 μl of the methanol was taken into a 96-well plate for OD reading at 570 nm. 250 μl of 80% methanol was placed inside the chamber, incubated on shaker for 30 min at room temperature, and 100 μl was again taken for OD at 570 nm. Percent migration was determined by the following calculation: 100 x [(OD migrated cells-OD background)/( OD migrated cells-OD background) + (OD nonmigrated cells-OD background)].

5-aza-2’-deoxycytidine treatment. HNSCC cell lines were incubated for 96 hrs with 3 μM 5-aza-2’-deoxycytidine (5-aza-dC) (Sigma), with medium and drug changed daily. Treated cells were harvested for analysis after the procedure. Cells were suspended in Trizol for RNA isolation or Laemmeli buffer (62.5mM TrisCl (pH 6.8), 2%SDS, 10% glycerol, 0.1% bromophenol blue, 300 mM 2-mercaptoethanol) for western analysis.

RNA isolation and cDNA synthesis. RNA was isolated according to the Qiagen protocol. DNase treatment was performed on 1-2 μg of RNA by adding 2U of DNaseI (Invitrogen, Carlsbad, CA), 1 μl DNase buffer, and 0.4 μl RNase Out (Invitrogen, Carlsbad, CA) for 15 min at room temperature. 1 μl ethylenediaminetetraacetic acid (EDTA) was then added to the mix for 10 min at 65°C, followed by an incubation on ice.
for 5 min. cDNA synthesis was performed by the following reaction: 2 μl random hexamers and 1 μl deoxynucleotide triphosphates (dNTPs) (10 mmol/L) for 5 min at 65°C then 2 min at 4°C; 2 μl of 10X buffer, 4 μl MgCl2, 2 μl dithiothreitol (DTT). Then, 1 μl RNase Out was added for 2 min at 25°C, 100 U of SuperScript II (Invitrogen) for 50 min at 42°C, 15 min at 70°C, and then transferred to 4°C.

**Real-time PCR (RT-PCR).** Quantitative C/EBPα mRNA expression was measured using SYBR Green I (BioRad, Hercules, CA) in an I-Cycler (BioRad). Expression of glycosylphosphatidylinositol (GPI) was used as the internal control gene. We additionally performed PCR on DNaseI treated, “-” RT samples in order to ensure that no DNA contamination was present in the RNA extract (given the fact that C/EBPα is an intronless gene). No amplification of PCR product was seen in these samples, indicating the absence of contaminating genomic DNA in the DNaseI treated RNA extracts.

**Sequenom Methylation Analysis.** Quantitative DNA methylation analysis was performed by Sequenom, Inc. (San Diego, CA). 1 μg of HNSCC patient or cell line DNA was bisulfite-treated, in vitro transcribed, cleaved by RNase A, and subjected to MALDI-TOF mass spectrometry analysis to determine methylation patterns as previously described (97).

**Chromatin Immunoprecipitation assay (ChIP).** ChIP was performed according to the Upstate Cell Signaling Solutions Protocol. Quantitative PCR for the ChIP assay was done with 3 μl of ChIP eluate and primers surrounding the promoter binding sites. Promoter enrichment was assessed by normalization of threshold crossing of the samples compared to the threshold crossing of the negative control (no antibody).
**Plasmid constructs and oligos.** The pBABE-CEBPα construct was generously provided by Dr. Gokhan Hotamisligil (Harvard University) and previously described (202). The pRS retroviral vector from Origene (Rockville, MD) was used for stable silencing experiments. The sequence targeted for stable silencing was borrowed from a previous publication (203).

**Transfections.** Stable transfections were performed as previously described (74). 120 hrs post-infection, the target cells were collected, counted, and suspended in Laemmeli buffer for western blot confirmation of silencing. For the transient silencing experiment, 1.6 x 10^5 C/EBPα-overexpressing SCC22B cells were plated per well in a 6-well plate. The transfections were done in duplicates. The negative control transfection mix contained 100 μl EC-R buffer (Qiagen), 14.4 μl RNAiFect (Qiagen), and DMEM + 10% FBS. The siRNA transfection mix contained 4.8 μl 20 μM siRNA stock, 95.2 μl EC-R buffer (Qiagen), 14.4 μl RNAiFect (Qiagen), and DMEM + 10% FBS for a final concentration of 30 nM. The transfection media was left on the cells for 72 hrs. Then the cells were trypsinized, counted, and suspended in Laemmeli buffer for western analysis.

**Colony formation assay.** This was performed as previously described (74).

**Growth Curve.** Cells were counted using a cell-coulter counter. 1.4 x 10^4 cells were plated in triplicates on 6-well culture plates. Cells were then permitted to grow three days before taking counts, after which cells were washed, trypsinized, and counted every 24 hrs for 4 days. For each count, 500 μl of trypsin was added to each well for 3 min, after which the cells were suspended in 2 mls of PBS, and 500 μl was counted using a Coulter counter.
**COBRA.** Genomic DNA (1μg) in a volume of 50 μl was denatured by NaOH (final concentration, 3M) for 30 min at 37 °C. The denatured DNA was then treated with 30 μl of 10 mM hydroquinone and 520 μl of 3M sodium bisulfite at 50 °C overnight. The PCR conditions were initiated with a denaturing step of 95 °C for 10 min followed by 36 cycles of 96 °C for 30 s, 59°C for 30 s, and 72 °C for 30 s and were concluded with 72 °C for 10 min. The PCR products were purified with a purification kit (Qiagen, CA) and incubated with BstUI at 60 °C for 4 h. Digested DNA was then size-fractionated via polyacrylamide gel electrophoresis to detect the methylation status.

**Bisulfite Sequencing.** Promoter region of bisulfite-treated HNSCC cell lines and patient samples were analyzed for methylation as previously described (114).

**Loss of heterozygousity (LOH) analysis.** 122 HNSCC patient adjacent normal, stroma, and epithelial tumor samples were subjected to LOH analysis using D19S433 and D19S245 markers. Sequencing of the PCR products showed whether the tumor sample had homozygosity, retention of heterozygosity, or loss of heterozygosity when compared to normal.

**Northern Blot analysis.** The northern blot analysis was performed as previously described (114), but instead with 8 μg of RNA. 6 pairs of HNSCC normal/tumor patient samples (also included in the MassARRAY methylation analysis) were loaded onto the blot in order of increasing methylation. Glyeraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control. Image Quant was used to determine C/EBPα expression in tumor compared to its matching normal (which was set as 1).
Western Blot analysis. The western blots were performed as previously described (114). After C/EBPα detection, the blot was stripped with BioRad stripping buffer in 37˚C for 5 min and reprobed with a 1:500 dilution of α-tubulin antibody (1:3,000 secondary mouse antibody). A 1:250 dilution was used for involucrin (Biomed, Foster City, CA), and a 1:3,000 secondary rabbit.

Mutation Analysis. The coding region of C/EBPα was amplified in 49 HNSCC tumor samples and 10 HNSCC cell lines using 4 sets of primers. Primer sequences and conditions were previously described (204). Mutation screening was done by comparing the resulting sequences with the known normal sequence on BLAT.

Animals and in vivo Assay. Three to four week old male nude mice [Taconic (NCRNU-M), Germantown, NY] were maintained in a pathogen-free environment. Injections were performed as previously described (74). Tumors were palpable after 17 and 26 days from the 8.5 and 3 million cell injections, respectively. Volumes were calculated by the following formula: V= (LxW^2)π/6.

Statistical Analysis. The statistical significance of the results was calculated by unpaired Student’s t test, and P<0.05 was considered to be statistically significant.

3.3 Results

3.3.1 C/EBPα downregulation in HNSCC

The previous publication by Roepman et al. demonstrated that C/EBPα is among the transcripts significantly downregulated in poor prognosis HNSCC patients (117). Therefore, we first wanted to validate decreased C/EBPα expression levels in our HNSCC patient samples via immunohistochemistry analysis. Strong C/EBPα expression was
detectable in epithelial cells in 39/47 (83%) of the normal tissue sections, but it is
downregulated in 33/48 (69%) of the tumor samples and 23/34 (68%) of the metastatic
tumor samples (Figure 15a and Table 4). 52.2% (12/23) of the paired metastatic and tumor
samples shared similar levels of downregulation, 43.5% (10/23) of the metastatic samples
showed even greater downregulation than the matched tumor samples, and 4.3% (1/23) of
the tumors showed greater downregulation than the matched metastatic samples (Table 4).

3.3.2 C/EBPα deletion in HNSCC

We next investigated possible mechanisms for C/EBPα downregulation, first evaluating
the genetic mechanisms. We assessed the incidence of deletions in this genomic region in
laser-captured-microdissected (LCM) HNSCC samples by utilizing markers D19S245
(200kb downstream of C/EBPα) and D19S433 (2 Mb upstream of C/EBPα). This analysis
revealed LOH in 30/49 (61%) of HNSCC patient tumor epithelium samples at D19S433,
41/60 (68%) LOH at D19S245, and 8/29 (28%) LOH at both markers. Interestingly, the
highest LOH incidence was attained from D19S245, which is the marker closest to the
C/EBPα locus (Table 5).

3.3.3 C/EBPα mutations in HNSCC

C/EBPα mutations have been associated with increased proliferation of myeloid
precursors in acute myeloid leukemia by creating a truncated isoform lacking the
transactivator domain (204). Therefore, we investigated the possibility of C/EBPα
mutations in HNSCC. Mutation screening was accomplished via sequencing the C/EBPα
coding region from 19 HNSCC tumor samples, 10 HNSCC cell lines, and 30 LCM
samples (which had also been included in the LOH analysis).
Figure 15. Immunohistochemistry and DNA methylation analysis in HNSCC.

A. Tissue microarrays contained tumor (“T”), adjacent normal (“N”), or metastatic tumor (“M”) tissues stained with C/EBPα antibody. Magnification is 400X.


C. Bisulfite sequencing analysis for HNSCC tumors and their matching normal tissue DNAs. Open circles= unmethylated, closed circles= methylated CpGs. Each row= individual clone. T= tumor; N= normal.
Table 4. Summary of IHC analysis, demographic, and methylation status of patient samples. L=larynx, T=tongue, P=pharynx, O=oral cavity, A=Adenoid.
Table 5. Summary of LOH analysis on HNSCC samples.

Black squares represent loss of heterozygosity; white squares represent retention of heterozygosity. Total sample numbers applicable to each incident vary due to the analyzable sample set (i.e. failure of PCR or homozygosity). D19S433 is 2MB upstream of the C/EBPα locus (38.5MB), and D19S245 is 200KB downstream of the C/EBPα locus.

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<td>LOH = 30/49 (61%)</td>
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<td>LOH = 41/60 (68%)</td>
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Samples included in the mutation analysis were generally those that had no promoter methylation and were from 5 different anatomical regions encompassed by HNSCC: pharynx, larynx, oral cavity, adenoids, and tongue. Mutation analysis on these samples revealed no mutations (Table 6).
3.3.4 C/EBPα upstream methylation

Recently, our laboratory has shown that C/EBPα is epigenetically regulated in lung cancer (114). Therefore, we investigated epigenetic regulation of C/EBPα a gene associated with a dense CpG island, in HNSCC. In order to first locate the region of methylation, we required a broad methylation analysis of the upstream and promoter-proximal regions of C/EBPα. Therefore, we performed quantitative DNA methylation analysis by using the MassARRAY technique that combines in vitro transcription, RNase A cleavage, and MALDI-TOF mass spectrometry on HNSCC DNAs (97). This strategy provided information on upstream region 1 (-1450 bp to -1120 bp), promoter-proximal region 2 (-580bp to -297bp), and promoter-associated region 3 (+164bp to +379bp). All three of these regions are encompassed within a CpG island (i.e. the sequence contains a GC content of ≥50% over a length greater than 200bp). The MassARRAY analysis of region 1 demonstrated an average of 25% methylation in the 28 HNSCC tumor samples, an average of 10% methylation in the 5 HNSCC adjacent normal samples, and an average of 87% methylation in the 6 HNSCC cell lines. Lower methylation seen in the HNSCC tumor samples compared to the HNSCC cell lines is most likely due to the potential for contaminating normal tissue in the tumor specimens. Virtually no methylation was seen in regions 2 and 3 (Figure 15b). Interestingly, the sequence of the methylated region 1 is extremely conserved between humans, mice, rats, dogs, and opossums according to UCSC Genome Browser on the March 2006 assembly. This sequence may provide promoter or enhancer activity, which is supported by previous promoter studies that have revealed increasing promoter activity by including more upstream sequence (114, 205).
Subsequent to the broad methylation analysis, we desired to further define where
DNA methylation is occurring in this region. Although MassARRAY allows a large-scale
methylation analysis, information on some CpG sites is lost due to the nature of the
technique (i.e. cleavage products of the same size are indistinguishable from one another).
In contrast, bisulfite sequencing provides methylation data on each CpG within the
sequenced region. Therefore, we performed bisulfite sequencing on 4 tumor samples (and
their adjacent normals) that had shown methylation in the MassARRAY analysis.
Bisulfite sequencing revealed consistent methylation in all 4 tumor samples in upstream
region 1 not seen in their corresponding normal tissues (or “germline DNA”) (Figure 15c).
These tumor samples showed decreased C/EBPα mRNA expression (compared to
adjacent normal tissue DNAs) by quantitative-RT PCR (data not shown). Furthermore,
two of the four samples had been included on the tissue microarray for the IHC analysis,
which revealed significant C/EBPα downregulation in these samples (Table 4). This
bisulfite sequencing data closely correlates with the broad methylation analysis (Figure
15b), and it reveals that methylation exists only in upstream region 1.

In order to correlate the incidence of LOH and methylation, 23 samples that
exhibited LOH at D19S245 (the marker closest to the C/EBPα locus) were also tested for
C/EBPα promoter methylation in the upstream region 1. COBRA analysis revealed
methylation in 65% of these samples (15/23) (data not shown). Bisulfite sequencing was
performed on 5 of these tumor samples which showed partial methylation ranging from
~15 to 50% (Figure 16).
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Table 6. Summary of mutation analysis, demographics, and LOH data.
3.3.5 C/EBPα reexpression with demethylation treatment

We next wanted to verify that C/EBPα promoter methylation is capable of decreasing C/EBPα expression. We performed northern analysis on 6 normal/tumor HNSCC patient pairs which had shown increasing methylation according to the MassARRAY analysis. Quantification of the northern analysis revealed a strong correlation between increasing promoter methylation and decreased mRNA expression (Figure 17a). Quantitative RT-PCR on 18 normal/tumor HNSCC patient pairs also revealed a correlation between increasing methylation (according to the MassARRAY) and decreased C/EBPα mRNA expression (Figure 18). Next, we used 3 HNSCC cell lines that all exhibited high amounts of methylation in the upstream C/EBPα sequence (SCC11B, SCC22B, and SCC25) (Figure 15b and 15c). We added 3 μM of the demethylating drug 5-aza-dC to the media of HNSCC cell lines for 96 hrs in order to see if demethylation allows C/EBPα reexpression. After treatment, cells were harvested and analyzed for changes in C/EBPα methylation and expression compared to the untreated cells. Bisulfite sequencing on SCC17AS cells before and after 5-aza-dC treatment revealed a decrease in upstream C/EBPα methylation (93 to 56%) (Figure 17b). Also, quantitative RT-PCR and western blot analysis revealed an increase in C/EBPα expression. SCC11B, SCC22B, and SCC25 all showed a significant increase in C/EBPα mRNA expression (p<0.018, p<0.008, and p<0.001, respectively), (Figure 17c), and western blot analysis on two HNSCC cell lines (SCC11B and SCC17AS) showed an increase in C/EBPα protein expression (Figure 17d), which suggests epigenetic control of the gene.
3.3.6 C/EBPα overexpression provides tumor suppressor activity

In order to assess C/EBPα’s potential for tumor suppressor activity in HNSCC, we overexpressed C/EBPα in a HNSCC cell line (SCC22B). After protein overexpression was confirmed via Western analysis (Figure 19a), growth curve analysis and colony formation assays were performed. In comparison to the pBABE-transfected cells and SCC22B untransfected cells, C/EBPα-transfectants exhibited a significant decrease in proliferation (21 hr vs. 50 hr average doubling time, respectively) and colony formation (Figure 19b and c). Next, we used a migration assay to investigate C/EBPα’s ability to inhibit metastasis. The migration assay showed a significant decrease in migration of C/EBPα-transfectants compared with the pBABE-transfectants (Figure 19d). To validate the in vitro tumor suppressor activity, we performed in vivo analysis using nude mice. We executed two separate in vivo experiments using 3 and 8.5 million cells for the injections. The experiment using 3 million cells yielded 6 out of 10 mice with tumors from WT cells, and 1 of these 6 also formed a tumor from the C/EBPα-overexpressing cells. We performed IHC on the tumors of the mouse that formed tumors from both cell types. As expected, this analysis revealed increased C/EBPα expression in the tumor from the C/EBPα–overexpressing SCC22B cells (Figure 20a). The experiment using 8.5 million cells yielded 10 out of 10 mice with tumors from the pBABE-transfected SCC22B cells. However, none of these mice formed tumors from the C/EBPα-pBABE-transfected SCC22B cells (Figure 20b and c).
Figure 16. Bisulfite sequencing analysis for LOH HNSCC tumors and normals. The arrow = transcription start site, diagram is drawn to scale, bisulfite-sequenced region is indicated on this diagram. Open = unmethylated; closed = methylated. Each row represents an individual clone. Overall percentage methylation is shown next to each tumor and normal.
3.3.7 C/EBPα reexpression allows differentiation in HNSCC

One of C/EBPα’s main functions is cellular differentiation. With differentiation inducing agents, C/EBPα expression has been shown to increase in keratinocytes (206). Keratinocytes account for ~90% of epidermal cells, which allows them to serve as the normal cell population control for HNSCC. Increased C/EBPα expression leads to elevated involucrin expression (a squamous cell differentiation marker in keratinocytes) (206). Therefore, to check whether demethylation and increased C/EBPα expression induces differentiation, we used an involucrin antibody which showed increased involucrin expression subsequent to demethylation treatment. This analysis suggests a correlation between increased C/EBPα expression and increased involucrin expression (Figure 21a). Furthermore, C/EBPα binding within the involucrin promoter was previously found to be required for its transcription (207), so we investigated whether increased involucrin expression would result from C/EBPα reexpression. To do this, we performed ChIP analysis on SCC22B and SCC17AS cells with and without 5-aza-dC treatment. Quantitative PCR was then done on the ChIP eluate, using primers surrounding the C/EBPα binding site in the involucrin promoter. This revealed a significantly greater amount of involucrin promoter pulldown following 5-aza-dC treatment (Figure 21b). This suggests that C/EBPα reexpression allows C/EBPα to bind to the involucrin promoter and activate its transcription. Although we did not exclude the possibility for methylation of involucrin, it is very unlikely that it is directly controlled by methylation [since it contains very few CGs (8 in 1,500bp upstream of the TSS) and lacks a CpG island].
Figure 17. *C/EBPα* expression analysis for methylated HNSCC patients and cell lines. 

**a.** Northern on 6 HNSCC patient pairs with increasing methylation. Quantification is shown beneath each lane (normalized with GAPDH and normal, which is set as 1). The MassARRAY methylation according to is listed below the quantification.

**b.** Bisulfite sequencing SCC17AS cells. Open circles = unmethylated CpGs; closed circles = methylated CpGs. Each row represents an individual clone.

**c.** RT-PCR analysis on treated or untreated HNSCC cells. Each *C/EBPα* expression level was normalized to its internal control (* p-value <0.0177; ** <0.0083; *** <0.0010).

**d.** Western analysis on SCC11B or SCC17AS cells + and - treatment. Blot was probed with *C/EBPα* (sc-61) and α-tubulin (control for loading) antibodies.
Figure 18. Quantitative RT-PCR analysis on HNSCC patients. The RNA was subjected to DNase treatment and cDNA synthesis. Each \( C/EBP\alpha \) expression level was normalized to its internal control (GPI expression). Each tumor \( C/EBP\alpha \) expression level was normalized to its adjacent normal (which was set as 1). The samples are listed in order of increasing amounts of methylation (according to MassARRAY).
Figure 19. Analysis of C/EBPα tumor suppressor activity in a HNSCC cell line.

A. The western blot contained lysates from untransfected SCC22B cells and C/EBPα-transfected SCC22B cells. 50,000, 150,000, and 300,000 cells of each lysate were loaded.

B. $1 \times 10^4$ SCC22B untransfected, pBABE-transfected, or C/EBPα-transfected cells were plated in triplicates and then counted daily. (** = p-value < $9.39 \times 10^{-6}$.)

C. Colony formation was compared between C/EBPα-“+”, pBABE-transfected, and untransfected SCC22B cells. (p-value <0.002738.)

D. Cell migration was compared between C/EBPα-overexpressing SCC22B cells and pBABE-transfected SCC22B cells. (* = p-value <0.018.)
Figure 20. *C/EBPα* overexpression *in vivo*.

A. The tumors analyzed were from the mouse that developed tumors from both cell types. Tumor tissues were stained with *C/EBPα* antibody (sc-61). Magnification is 400X. “W” represents WT SCC22B, and “C” represents SCC22B cells overexpressing *C/EBPα*.

B. The 10 mice injected with pBABE-transfected (left flank) and *C/EBPα*-overexpressing SCC22B cells (right flank) are shown.

C. The average tumor volume of the SCC22B cells vs. the average tumor volume from the SCC22B cells overexpressing *C/EBPα* is plotted. Volumes are plotted from 18 days through 38 days post-injection. Volumes were calculated by the following formula: \( V = \frac{(L \times W^2) \pi}{6} \) (208). (* = p-value < 0.009385.)
Figure 21. Analysis of C/EBPα increasing involucrin expression in HNSCC.

A. Involucrin expression compared between HNSCC cell lines with and without 5-aza-2'-deoxycytidine using western analysis with an antibody against involucrin. B. ChIP was performed by using C/EBPα antibody on HNSCC cells before and after demethylating treatment. Quantitative PCR was done by using involucrin promoter primers on the ChIP eluate, normalized to the negative control. (* p-value <0.0126; ** <0.00381.)
C. Microscope images [overexpressing (+); WT (–)]. Quantitation of % differentiated in 10 frames. Magnification= 100X. Spindle formation= differentiated (* p< 0.00035.). Furthermore, differences in cell morphology were seen through microscopic evaluation in C/EBPα-overexpressing cell lines. The C/EBPα-overexpressing SCC22B are much larger in size compared with the untransfected SCC22B cells (possibly due to accumulation of granules and/or vacuoles, which are often increased in differentiated cells) (113). Using previously accepted criteria for differentiation determination (i.e. spindle formation) (113), C/EBPα-overexpressing cells evidence a higher frequency of differentiated cells than the untransfected SCC22B cells (16% vs. 5%, respectively)(Figure 21c).

3.4 Discussion

C/EBPα, a gene involved in cellular differentiation and cell cycle control (110), has been found to exhibit tumor suppressor activity in non-small cell lung cancer (113) and AML (201). More recently, a publication revealed C/EBPα as one of 102 metastasis predictive genes in HNSCC which exhibit distinct expression profiles (from their non-metastatic counterpart) at the primary tumor stage (209). This is likely due to the requirement for C/EBPα’s activation of differentiation genes (i.e. involucrin) in keratinocytes (207).

In our study, we have shown for the first time not only that C/EBPα overexpression provides tumor suppressor activity (and metastasis inhibition) in vitro and in vivo in HNSCC, but also it is epigenetically downregulated by upstream methylation. It seems surprising that we found no methylation around the transcription start site and core promoter, but this supports previous findings of C/EBPα epigenetic regulation in lung
cancer (114). Therefore, it may be assumed that upstream methylation is capable of decreasing expression, but the core promoter remains unmethylated due to a requirement of basal expression for tumor cell viability (114). Deletions within chromosome 19 have not been previously discovered in HNSCC (210), and very few cases (i.e. RASSF1A and TCF21) have been documented thus far (37, 74) as having frequent co-occurrence of both DNA methylation and LOH in HNSCC, as we witnessed with C/EBPα. Although, there are several examples in other cancer types (211-215).

The findings of this study are of critical importance, because HNSCC often has a fatal clinical course with very little chance of recovery following operation (3). Currently, there are relatively few good markers for poor prognosis HNSCC detection. Loss of BRCA2 correlates with a significant decrease in survival time, and certain chromosomal deletions (i.e. 10q and 14q) were found to be associated with poor prognosis (134, 135, 216). Increased gene expression (i.e. NBS1, FGFR, Ki-67, HER2, MMP2 and MMP9, and cyclin D1) has been shown to indicate the aggressiveness of the disease (136-138, 217-219), and elevated alpha B-crystallin expression has been found to be a more sensitive marker for HNSCC recurrence than the combination of SPARC, PAI-1, and uPA (220). However, there is still not a DNA methylation marker for poor prognosis (108). DNA methylation occurs early in carcinogenesis, making it a good early indicator (221). Consequently, the discovery of C/EBPα downregulation in poor prognosis patients (117) combined with our findings of epigenetic regulation of the gene has great importance for detection of HNSCC. This suggests there may be great promise for using DNA methylation as a biomarker in clinical screening, which has already been proposed in other cancer types (196, 208, 220, 221). This could ultimately lead to better detection (either by
IHC or by quantitative DNA methylation analysis) and treatment of the disease, increasing the rate of survival (196).

However, \( C/EBP\alpha \) methylation in HNSCC should be validated in a large sample set before pursuing its diagnostic potential (220). Furthermore, \( C/EBP\alpha \) methylation in other tumor types (besides lung cancer and HNSCC) is worthy of investigation and could yield increased prognostic value and therapeutic intervention for other patients with this epigenetic alteration.

3.5 Acknowledgments

We would like to thank Dr. Gokhan Hotamisligil for the \( C/EBP\alpha \) overexpression plasmid. The HNSCC cell lines were kindly provided by Dr. Thomas Carey, University of Michigan. We thank members of the Plass lab for their thoughtful discussions. This work was supported in part by an AGGRS scholarship (KB).
CHAPTER 4
AP2 ALPHA SUPPRESSES FULL LENGTH C/EBP ALPHA IN HNSCC

4.1 Background

C/EBPα is a transcription factor involved in cell cycle regulation and cellular differentiation in hepatocytes and adipocytes (110). It promotes anti-proliferation through inhibition of CDK2, 4, and 6 and repression of S-phase gene transcription (222). C/EBPα is alternatively translated via leaky translation, creating p42C/EBPα and p30C/EBPα proteins (111). Although both isoforms are able to bind to C/CAAT elements in target promoters, only the larger isoform contains the anti-mitotic activity (222). In this way, the truncated isoform behaves as a dominant negative isoform.

Recent studies suggest putative tumor suppressor function of C/EBPα not only in leukemia (201) but also in solid tumors, such as lung cancer (113). C/EBPα was found to be downregulated in 78% (31/40) of HNSCC samples in a microarray profiling study (117). Furthermore, there was a significant correlation between C/EBPα downregulation and poor prognosis patients with extensive lymph node metastasis (117). Subsequently, tumor suppressor activity in HNSCC was demonstrated, and epigenetic alterations were shown to play a major role in altering C/EBPα expression in tumor samples (223).
AP2α has been previously shown to act as a transcriptional suppressor for C/EBPα promoter activity in adipocytes, hepatocytes, and keratinocytes by binding to the core promoter (119, 123, 124). An inverse correlation between AP2α and C/EBPα expression is required for adipocyte differentiation: AP2α expression decreases and C/EBPα expression increases during differentiation (119). Decreased C/EBPα expression has been observed in HNSCC, correlating with decreased cellular differentiation (223). Therefore, AP2α may also provide transcriptional suppression of C/EBPα in HNSCC. In this study, we demonstrate with C/EBPα promoter assays and ChIP analysis that upstream AP2α binding inhibits SP1 binding and suppresses C/EBPα transcription in HNSCC. Also, AP2α silencing using stable shRNA reveals restored promoter activity and increased p42 C/EBPα protein expression.

4.2 Materials and Methods

Cell lines. The human HNSCC cell lines used in the study (SCC11B, 17AS, 22B, and 25) were maintained in DMEM with 10% FBS and 1% Streptomycin/Penicillin antibiotics. The Hacat immortalized keratinocytes were maintained in keratinocyte growth medium with 10% FBS and 1% Streptomycin/Penicillin antibiotics.

Plasmids and oligonucleotides. The promoter constructs used in the luciferase assay were cloned into the multiple cloning site of pGL3 basic. The promoter sequences spanned from +4 bp (relative to the C/EBPα transcription start site) to -889 bp, -1013 bp, -1256 bp, and -1423 bp. The suppressor constructs contained -1423 bp to -1357 bp (“Sup 1”), -1357 bp to -1258 bp (“Sup 2”), and -1402 bp to -1329 bp (“No Sup”). The E2F3a promoter construct contained 2kb upstream E2F3a promoter sequence removed from
pGL2 basic (224) and cloned into the HindIII site within the MCS of pGL3 basic. The full suppressor construct contained -1423 bp to -1258 bp of C/EBPα sequence adjacent to the E2F3a promoter in pGL3. The USF and SP1 mutant promoter constructs were made by site mutagenesis as previously described (114). The control, NFκB responsive promoter, contains 3 NFκB binding sites in pGL3 basic (225). The dominant negative NFκB, pcDNA IκBα, contains cDNA coding for IκB that is mutant at the phosphorylation sites necessary for its degradation (i.e. Ser 32 and Ser 36 changed to Ala) (225). The C/EBPα overexpression vector was provided by Dr. Gokhan Hotamisligil (The Harvard School of Public Health). The 30kDa C/EBPα overexpression vector was generated by removing 316 bp of the 5’ cDNA sequence upstream of the 30 kDa translation start site using endogenous SacII restriction sites. The pRS retroviral vector from Origene was used for stable silencing experiments. The AP2α shRNA construct contained an oligo sequence cloned into the BamHI/HindIII sites. The hairpin sequence targeted against C/EBPα (227 bp past the 42kDa translation start site) was cloned into the BamHI and HindIII sites of the plasmid. The C/EBPα double-stranded RNA oligos (targeted 1,466 bp beyond the 42kDa translation start site) were purchased from Ambion for the transient silencing experiments.

**Transfections.** Stable transfections were performed as previously described (74). 120 hrs post-infection, the target cells were collected, counted, and suspended in Laemmeli buffer for western blot confirmation of silencing. For the stable silencing, 30 μg of pRS-C/EBPα along with 60 μl of superfect and 210 μl of DMEM without FBS were incubated together for 10 minutes. This was then suspended in 8 ml of DMEM + 10% FBS and added to a
p150 cm plate of Phoenix cells at 50% confluency. After 4 hrs, the media was replaced with 16 ml of DMEM + 10% FBS. After 48 hrs, 8 ml Phoenix media was centrifuged with 8 μl of polybrene and added to 1.5 million target cells on a p150 cm plate. 120 hrs post-infection, the target cells were collected, counted and suspended in laemelli buffer for western. For the transient silencing experiment, 160,000 C/EBPα-overexpressing SCC22B cells were plated per well in a 6-well plate. The transfections were done in duplicates. The negative control transfection mix contained 100 μl EC-R buffer (Qiagen), 14.4 μl RNAifect (Qiagen), and 2,885.6 μl DMEM + 10% FBS. The 30 nM siRNA transfection mix contained 4.8 μl 20 μM siRNA stock, 95.2 μl EC-R buffer (Qiagen), 14.4 μl RNAifect (Qiagen), and 2,885.6 μl DMEM + 10% FBS. The transfection media was left on the cells for 72 hrs. Then the cells were trypsinized, counted, and suspended in laemelli buffer for western analysis.

**Bisulfite Sequencing.** -1423bp to -1121 bp upstream of C/EBPα’s transcription start site of bisulfite-treated HNSCC cell line SCC22B (“+” and “–” AP2α shRNA) was analyzed for methylation as previously described (114).

**Growth Curve.** 10,000 cells (SCC22B; SCC22B C/EBPα; and SCC22B C/EBPα C/EBPα shRNA) were plated in triplicates in a 6 well plate for a 4 day growth curve analysis. For each count, 500 μl of trypsin was added to each well for 3 minutes, after which the cells were suspended in 2 mls of PBS, and 500 μl was counted using a Coulter counter.

**Western Blot analysis.** The westerns were performed as previously described (114).
**5-aza-2’-deoxycytidine treatment.** HNSCC cell lines were incubated for 96 hrs with 3 μM 5-aza-2’-deoxycytidine (5-aza-dC) (Sigma) with medium changed every day. Treated cells were harvested for analysis 1~4 days after the procedure. Cells were trypsinized and suspended in 10 ml DMEM and 270 μl 37% formaldehyde for ChIP analysis.

**Chromatin Immunoprecipitation assay (ChIP).** ChIP was performed according to the Upstate Protocol. Cells were grown on p150 mm plates, and then the media was removed and replaced with 10 ml DMEM and 270 μl 37% formaldehyde. Plates were shaken at room temperature for 10 minutes then 1 ml of 1.25 M glycine was added for 5 minutes. Plates were then washed 3 times with cold PBS with 2 μl PIC/ml, centrifuging at 2500 rpm for 5 minutes at 4°C and resuspending the pellet after each wash. PBS was removed and cells were suspended in 7 ml of cell lysis buffer (85 mM KCl, 5 mM PIPES, 2 μl PIC/ml) and dounced on ice. Cells were then centrifuged again and suspended in 500 μl SDS lysis buffer (Upstate) with 1 μl PIC. The cells were then sonicated at setting 7 (Misonix) for 15 pulses of 20 seconds each. Debris was pelleted by brief centrifugation at 4°C, and the supernatant was placed in a new centrifuge tube. 60 μl of agarose beads were added, and the tubes were rotated for 1hr at 4°C. Tubes were then centrifuged, and the supernatant was placed in a clean microcentrifuge tube with 1800 μl ChIP dilution buffer. At this point, 20 μl from each tube was removed as input. Then 5 μg of antibody was added to the tubes and allowed to rotate at 4°C overnight. 60 μl agarose beads were added to the tubes and allowed to rotate 1 hr at 4°C. Tubes were then centrifuged, supernatant removed, and beads were washed with low salt, high salt, LiCl, and TE (2X) rotating for 4
minutes each. DNA was eluted from the beads with 500 μl eluate, and the crosslinks were reversed for 4 hrs at 65°C. The eluate was then treated with RNase, 0.5 M EDTA, 1 M Tris, and PK for 1 hr at 45°C. Samples were then PCR purified with QIAquick PCR purification kit. Quantitative PCR for the ChIP assay was done with 3 μl of ChIP eluate and primers surrounding the promoter binding sites. Promoter enrichment was assessed by normalization of threshold crossing of the samples compared to the threshold crossing of the negative control (no antibody).

**RNA isolation and cDNA synthesis.** RNA was isolated according to the Qiagen protocol. DNase treatment was performed on 1-2 μg of RNA by adding 2U of DNaseI (Invitrogen, Carlsbad, CA), 1 μl DNase buffer, and 0.4 μl RNase Out (Invitrogen, Carlsbad, CA) for 15 min at room temperature. 1 μl ethylenediaminetetraacetic acid (EDTA) was then added to the mix for 10 min at 65°C, followed by an incubation on ice for 5 min. cDNA synthesis was performed by the following reaction: 2 μl random hexamers and 1 μl deoxynucleotide triphosphates (dNTPs) (10 mmol/L) for 5 min at 65°C then 2 min at 4°C; 2 μl of 10X buffer, 4 μl MgCl₂, 2 μl dithiothreitol (DTT), and 1 μl RNase Out was added for 2 min at 25°C; 100 U of SuperScript II (Invitrogen) for 50 min at 42°C; 15 min at 70°C, then transferred to 4°C.

**Real-time PCR (RT-PCR).** Quantitative mRNA expression was measured using SYBR Green I (BioRad, Hercules, CA) in an I-Cycler (BioRad). Expression of glycosylphosphatidylinositol (GPI) was used as the internal control gene.
RT-PCRs on the HNSCC patient samples: each tumor was normalized to the expression of its adjacent normal, which was set as one. For the 5-aza-dC treated HNSCC cell lines, each treated cell line was normalized to its untreated counterpart, which was set as one.

**Luciferase Assay.** Keratinocytes or SCC22B cells (20,000) were plated in each well of a 24-well plate. 24 hrs later, the transfection was performed using the Promega manufacturer’s protocol. Briefly, 1 μg of the promoter-pGL3 plasmid DNA was combined with 4 μl superfect, 60 ng renilla TK plasmid, and 60 μl DMEM (without FBS). After complexing for 10 minutes, 200 μl 10% FBS DMEM was added. The transfection media was plated in triplicates, and 48 hrs later the media was removed, cells were lysed in 100 μl of passive lysis buffer for 40 minutes, and 20 μl of lysate was plated in the 96-well opaque plate and analyzed in the luminometer. The resulting luciferase/renilla ratios were normalized to pGL3 basic to obtain the fold increase. For the dominant negative NFKB experiment, the same protocol as mentioned above was used, except the transfection mixture contained also 1 μg of the pcDNA/IkBα.

**Statistical Analysis.** The statistical significance of the results was calculated by unpaired Student’s *t* test, and *P*<0.05 was considered to be statistically significant.

### 4.3 Results

#### 4.3.1 Greatest C/EBPα promoter activity -1256 bp upstream of the transcriptional start site.** Previous work demonstrated aberrant DNA methylation in the upstream regulatory region (-1399 to -1146 bp) of C/EBPα in HNSCC (223). To check whether the upstream methylation in HNSCC coincides with gene regulatory regions, we performed luciferase promoter assays using Hacat (normal keratinocytes) and SCC22B cell lines.
These assays revealed that the sequence from either -1013 to +4 bp (Hacat) or from -1256 to +4 bp (SCC22B) had the strongest promoter activity. Consistent with previous reports in lung cancer cells (114), -1423 to -1256 bp appears to contain a suppressor sequence (Figure 22a). The sequence between -1253 bp and -1146 bp in SCC22B was found to exhibit both high promoter activity and methylation in HNSCC.

Previous studies in lung cancer cell lines indicated SP1 and USF1 binding in this region and activation of C/EBPα (114, 118). We used ChIP analysis to investigate whether or not these proteins play a role in HNSCC cells. There was a significant increase in USF1 and USF2 bound DNA from the upstream C/EBPα sequence compared to the no-antibody control (P < 0.002) (Figure 23a), indicating that SP1 and USF1 sites are required for C/EBPα activation.

The importance of USF and SP1 binding sites was further validated by promoter analysis with C/EBPα promoter constructs containing USF and SP1 binding site mutations. The promoter analysis revealed that mutating the downstream USF1 binding site (-894 bp) did not decrease promoter activity (Figure 23b), but mutating the 2 upstream USF1 binding sites (-1201 and -1001 bp) significantly decreased C/EBPα promoter activity (P = 0.004 and 0.010, respectively) compared to the control -1256 bp construct (Figure 23b). It is important to note that the most upstream USF binding site (-1201 bp) is within the methylated region in this HNSCC cell line. The construct that contained mutations in the SP1 binding sites also revealed a significant reduction in promoter activity (P = 0.003) (Figure 23b). Therefore, it appears that upstream USF1 and SP1 binding is required for enhanced promoter activity.
Figure 22. C/EBPα methylation and promoter analyses in cell lines.

a. **C/EBPα promoter assay in Hacat and SCC22B.** Promoter activity (x-axis) is fold increase in the luciferase/renilla ratio in respect to the pGL3 vector only.  

b. **Quantitative ChIP PCR on HNSCC cells to detect pulldown +/- 5-aza-dC treatment.** The dashed line= the region analyzed for promoter pulldown. Promoter pulldown enrichment (SCC22B cells +/- 5-aza-2’-deoxycytidine treatment) by MBD2/3, dimethyl H3K9, or acetyl H3K9 antibodies was normalized to the negative control. * P = 0.0003; ** 0.0002; *** 3 x 10⁻⁶.  

c. **Suppressor promoter assay in SCC22B.** Promoter activity (x-axis)= fold increase in luciferase/renilla ratio in respect to the “-“ control. The suppressor construct = E2F3a/promoter construct.
Figure 23. USF1 and SP1 binding and transcriptional activation in SCC22B cell line.

a. C/EBPα promoter pulldown enrichment (in SCC22B cells) by USF1 and USF2 antibodies was normalized to the negative (no antibody) control. * P = 0.0020; ** P = 4 x 10^{-5}. b. Diagram of the control and mutated USF1 and SP1 promoter constructs. The top construct represents the location of the SP1 and USF1 binding sites in the sequence. The “X”’s in the mutant constructs indicate which site(s) was mutated. All of these promoter sequences were cloned into the pGL3 basic promoter construct. Promoter analysis of the indicated promoter constructs in SCC22B cell line. The fold increase in luciferase activity is normalized to the empty pGL3 basic plasmid. * P = 0.0100, ** P = 0.0040, *** P = 0.0030.
Figure 24. C/EBPα promoter activity with dominant negative NFκB present.

a. ChIP PCR in cells +/- 5-aza-2’-deoxycytidine. C/EBPα promoter pulldown enrichment by NFκB and cRel antibodies was normalized to the negative (no antibody) control. b. The C/EBPα promoter sequence where NFκB and cRel potential binding sites are located. The black lollipops illustrate the methylation in this region in HNSCC.

c. C/EBPα promoter assay in Hacat and SCC22B cell lines. Constructs have parts of the upstream C/EBPα sequence in pGL3 basic. Promoter activity (plotted on the y-axis) is measured as the fold increase in the luciferase/renilla ratio in respect to the negative control (pGL3 vector only). Each construct was tested with and without the dominant negative NFκB plasmid (“DN”). “NRP” (NFκB responsive promoter) is the positive control that the dominant negative NFκB is effective. * p= 0.051; ** p= 0.010.
Computational analysis also predicted NFκB and cRel binding sites in this region, and ChIP analysis revealed they both bind to their predicted binding sites within the upstream C/EBPα sequence (Figure 24a). Therefore, we next tested whether cRel and NFκB could affect C/EBPα transcription. We analyzed changes in C/EBPα promoter activity in the HNSCC cell line SCC22B and normal keratinocytes when introducing a construct that overexpresses a dominant negative form against cRel and NFκB (225). An increase in promoter activity would suggest suppressor activity of cRel and NFκB (binding sites shown in Figure 24b). This experiment showed that there was a significant increase in promoter activity in normal keratinocytes with the -1423 and -1618 promoter constructs (p= 0.051 and 0.010, respectively), but no increase in activity in the SCC22B cell line (Figure 24c). Therefore, NFκB and cRel do not appear to affect C/EBPα promoter activity in HNSCC.

4.3.2 MBD2/3 binding and histone H3Lys9 methylation are relieved by upstream demethylation of C/EBPα, replaced by histone H3 Lys9 acetylation.

Methylation is capable of recruiting repressive complexes that can inhibit subsequent binding and activation by transcription factors (226, 227). Therefore, it was investigated whether the upstream methylation seen in HNSCC patient samples and HNSCC cell lines may recruit proteins to C/EBPα’s upstream sequence. Bisulfite sequencing analysis revealed that SCC22B, a HNSCC cell line, is methylated between -1423 bp and -1146 bp (data not shown). The presence of methyl binding domain proteins (MBDs) and dimethyl H3K9 was investigated, because MBDs can be recruited to methylated CpG dinucleotides (226), and the dimethyl H3K9 histone tail modification can
coincide with DNA methylation and silenced transcription (228). HNSCC cells were treated with 5-aza-2’-deoxycytidine to investigate whether demethylation was able to relieve MBD binding, decrease histone H3K9 methylation, and increase histone H3K9 acetylation (which correlates with active transcription and the lack of methylation) (228). ChIP analysis was performed using MBD2/3, dimethyl H3K9, and acetyl H3K9 antibodies on SCC22B cells with and without 5-aza-2’deoxycytidine. Quantitative PCR revealed HNSCC cells treated with 5-aza-2’-deoxycytidine showed 5-fold decrease in MBD binding, a 2-fold decrease in dimethyl H3K9, and a >4-fold increase in acetyl H3K9 pulldown of the C/EBPα promoter compared to the untreated SCC22B cells (P = 0.0002, 3 x 10^-6, and 0.0003 respectively) (Figure 22b). This emphasizes the importance of DNA methylation in preparing the region for silencing by recruiting MBDs and allowing changes in histone modifications (i.e. replacement of acetyl H3K9 with dimethyl H3K9).

4.3.3 AP2α binds in the upstream promoter and suppresses C/EBPα promoter activity. In order to investigate whether the sequence between -1256 bp and -1423 bp contains true suppressor effects, the 167 bp fragment was cloned in front of a strong promoter, E2F3a, and subjected to a luciferase promoter assay in an HNSCC cell line, SCC22B. This analysis revealed that the “suppressor” fragment decreased E2F3a promoter activity by approximately 50% (Figure 22c).

The suppressor sequence was dissected in order to further define the region providing transcriptional suppression. Two suppressor constructs were created: “Sup1” contained the first portion (-1423bp to -1357bp), and “Sup2” contained the latter portion of the suppressor sequence (-1357bp to -1258bp)(Figure 25a). Promoter analysis of these 2 sequences revealed suppression was maintained with either truncated portion of the
suppressor sequence (Figure 25a). The “suppressor sequence” was analyzed for any similarities between the former and the latter halves. This revealed that both “Sup1” and “Sup2” contained an AP2α binding site (Figure 25a). Therefore, an additional construct was formed that contained a large portion of the suppressor sequence but lacked the AP2α sites (“No Sup”). Promoter analysis revealed that the “No Sup” was relieved of its ability to suppress E2F3a promoter activity (Figure 25a).

AP2α has previously been shown to suppress \( C/EBP\alpha \) promoter activity in adipocytes by binding close to the transcription start site (-311 bp) (118) and blocking SP1 binding. To investigate whether AP2α transcriptional suppression in HNSCC is by inhibiting adjacent SP1 binding to the upstream \( C/EBP\alpha \) sequence, AP2α was downregulated in a HNSCC cell line SCC22B (Figure 25b). ChIP analysis was performed using AP2α and SP1 antibodies in these SCC22B cells with and without AP2α shRNA. Quantitative PCR using primers surrounding the AP2α binding sites (-1423 to -1243 bp) revealed a significant ~8 fold increase in SP1 promoter pulldown after AP2α silencing (P = 0.0451) (Figure 25c). Furthermore, AP2α downregulation resulted in an increase in \( C/EBP\alpha \) mRNA and p42\(^{C/EBP\alpha}\) protein expression as determined by RT-PCR and western blot analysis, respectively (Figure 25b). Also, the promoter activity of the -1423 bp \( C/EBP\alpha \) promoter construct (which contains the 2 upstream AP2α sites) was significantly increased upon AP2α downregulation (Figure 25d). These data further validate that AP2α acts as a suppressor for \( C/EBP\alpha \) by inhibiting SP1 binding. Interestingly, AP2α silencing not only increased \( C/EBP\alpha \) expression, but also it changed the 42kDa:30kDa \( C/EBP\alpha \) isoform ratio (Figure 25b).
Figure 25. AP2α suppresses C/EBPα promoter activity. 

a. C/EBPα suppressor parts affect E2F3a promoter activity. When normalized to E2F3a, “No Sup” gave significantly more activity. * P = 0.0283. 

b. AP2α stable silencing and C/EBPα upregulation. The western and RT-PCR for SCC22B +/- AP2α shRNA. P = 0.0201 and 0.0121, respectively. 

c. SP1 binding inhibited by AP2α binding. RT-PCR on C/EBPα promoter with AP2α and SP1 antibodies in SCC22B +/- AP2α. * P = 0.0451. 

d. C/EBPα +/- AP2α silencing. The constructs contained -1423 bp of upstream C/EBPα sequence. Luciferase normalized to the negative control; promoter activity was significantly increased in the cell lysates with decreased AP2α expression. * P = 0.0475.
AP2α binding to the promoters of retinoic-acid responsive genes has previously been shown to initiate transcriptional silencing and recruit HDACs (126). Therefore, AP2α binding to the upstream C/EBPα sequence may initiate its transcriptional silencing and recruit DNA methyltransferase activity. In this manner, eliminating AP2α binding and C/EBPα downregulation via stable shRNA silencing may relieve upstream C/EBPα methylation. Bisulfite sequencing analysis was performed on SCC22B with and without AP2α downregulation via shRNA. This revealed a substantial decrease in methylation upon AP2α downregulation (Figure 26), which correlates with increased C/EBPα protein expression (Figure 25b). This suggests that AP2α binding to the upstream C/EBPα sequence may precede and facilitate methylation.

Because AP2α seems to be involved in methylation and suppression of C/EBPα (which is commonly downregulated by methylation in HNSCC), AP2α overexpression in HNSCC was investigated. Quantitative RT-PCR on HNSCC patient tumor and adjacent normal samples revealed AP2α mRNA overexpression in ~50% of the samples (Figure 27a). Furthermore, western blot analysis of several HNSCC cell lines revealed an inverse correlation between endogenous AP2α expression and full-length C/EBPα expression. Expression of the anti-mitotic p42kDaC/EBPα isoform was only evident in the absence of AP2α expression. In contrast, abundant expression of the dominant negative (p30kDaC/EBPα) correlated with the presence of AP2α expression (Figure 27b).
Figure 26. AP2α downregulation relieves C/EBPα upstream methylation.

Diagram depicts C/EBPα and its upstream sequence. CpG sites’ methylation status is shown by open (unmethylated) and closed (methylated) circles. The dashed line represents the region analyzed by bisulfite sequencing (-1423bp to -1121bp). Bisulfite sequencing analysis was performed on SCC22B cells before and after AP2α stable shRNA silencing. The region tested for methylation via bisulfite sequencing spans from -1423 bp to -1121 bp. Bisulfite sequencing was performed by comparing the sequences with 100% and 0% methylated DNA sequence (i.e. CG or TG at CpG sites, respectively). Open circles represent unmethylated CpGs, and closed circles represent methylated CpGs. Each row represents an individual clone. Percentage methylation is shown for the two cell types.
Figure 27. AP2α expression in HNSCC patient tumor samples and cell lines.

a. Quantitative RT-PCR on 13 HNSCC patient tumor and adjacent normal tissues was performed. The graph reveals the fold increase in AP2α expression in tumors normalized to their adjacent normal tissue.

b. Western blot analysis of C/EBPα and AP2α expression in HNSCC cell lines. Lysates of 5 HNSCC cell lines (8, 11B, 17AS, 22B, 25) were loaded on the blot and probed for AP2α and C/EBPα expression. α-tubulin is shown as the internal control.

4.3.4 p42C/EBPα silencing in HNSCC restores high proliferative growth rate of the cells. C/EBPα mRNA is translated into two alternative isoforms, p42C/EBPα and p30C/EBPα. This is due to alternative translation initiation at the third in-frame AUG via leaky ribosomal scanning (111). This creates a smaller 30kDa protein that lacks part of the transactivation domain. Therefore, p30C/EBPα has been characterized as a transcriptional
activator that lacks antimitotic activity (229). However, the full length 42kDa C/EBPα protein has been shown to exhibit anti-proliferative activity in different cell types such as hepatocytes and adipocytes (230, 231). Therefore, it is not surprising that most of the HNSCC cells (with the exception of SCC8) do not normally express the anti-mitotic 42 kDa isoform (Figure 25b). However, it was interesting that removing the AP2α suppressor in these cells caused an abundant increase in the proportion of the anti-proliferative 42kDa isoform (Figure 25b).

Previously, C/EBPα overexpression in a HNSCC cell line revealed a significant decrease in cell proliferation (223). The growth rate of these overexpressing cells in the presence or absence of stable C/EBPα downregulation was compared to find whether stable silencing would be capable of restoring the normal proliferation rate of the SCC22B cells. Using a retroviral vector containing a hairpin sequence targeting C/EBPα, p42C/EBPα was downregulated by ~75% (according to semi-quantitation by Image Quant) in C/EBPα–overexpressing SCC22B cells (Figure 28a). Changes in proliferation were analyzed by growth curve analysis, which revealed that silencing the overexpressed p42C/EBPα restored the proliferation rate back to the normal SCC22B growth rate (Figure 28b).

**4.3.5 Silencing p30C/EBPα in HNSCC decreases the proliferation rate of the cells.**

Next, transient silencing was performed using a double-stranded RNA oligo. In contrast to the stable silencing experiment, now only the p30C/EBPα was transiently silenced (Figure 28c). When the cells were counted 72 hrs post-transfection, the cells that contained the 30 nM siRNA had a 40% decrease in cell growth compared to the mock-transfected negative control (Figure 28d).
Figure 28. C/EBPα silencing and p30 C/EBPα-overexpression in SCC22B cells.

a. Confirmation of C/EBPα silencing. Western showing C/EBPα-overexpressing SCC22B cells before and after stable C/EBPα silencing. The blot was first probed with C/EBPα followed by α-tubulin. Semi-quantitation was performed using Image Quant computer software, which provided that 75% downregulation of the 42 kDa isoform was attained.

b. Growth curve analysis of C/EBPα-overexpressing SCC22B cells after stable C/EBPα silencing. SCC22B; SCC22B C/EBPα-overexpressing cells; and SCC22B C/EBPα-overexpressing + C/EBPα shRNA were plated in triplicates in a 6 well plate. Growth was assessed by counting the cells every 24 hrs for 4 days. ** P = 0.0002.

c. Confirmation of 30kDa C/EBPα silencing. Western showing C/EBPα-overexpressing SCC22B cells with and without 30 nM siRNA.

d. Cell count after transient 30kDa C/EBPα silencing. Cells were collected and counted 72 hrs after transfection using a Coulter counter. ** p < .0007.

e. Confirmation of p30C/EBPα overexpression in SCC22B cells. Western blot showing SCC22B cells with and without p30C/EBPα. 150,000 cells were loaded in each lane. The blot was first probed with C/EBPα followed by α-tubulin to confirm equal loading.

f. Growth curve analysis of p30C/EBPα overexpressing SCC22B cells. 5,000 cells (SCC22B untransfected and SCC22B + p30C/EBPα) were plated in triplicates in a 6 well plate. Growth was assessed by counting the cells every 24 hrs for 4 days. For each count, 500 μl of trypsin was added to each well for 3 min, after which the cells were suspended in 2 mls of PBS, and 500 μl was counted using a Coulter counter. * p-value < 0.0007.
Figure 28. C/EBPα silencing and p30 C/EBPα-overexpression in SCC22B cells.

Silencing p30C/EBPα apparently relieves the binding competition with p42C/EBPα for C/EBPα binding sites, increasing the ability of C/EBPα to provide differentiation and decreased cell proliferation (110). These data suggest that AP2α may be required to foster the tumorigenic potential in HNSCC by specifically targeting the anti-mitotic isoform.
4.3.6 p30C/EBPα overexpression causes increased cell proliferation in HNSCC.

Previously, overexpressed p42C/EBPα in SCC22B revealed decreased cell proliferation and increased differentiation (223). In order to further validate the specificity of p42C/EBPα’s antiproliferative effect, p30C/EBPα was stably overexpressed in SCC22B (Figure 28e). Interestingly, not only did p30C/EBPα overexpression fail to provide the tumor suppressive qualities of p42C/EBPα overexpression, but also it actually provided ~2.5-fold increase in cell proliferation (P = 0.0007)(Figure 28f).

4.4 Discussion

C/EBPα is a transcription factor involved in cellular differentiation and cell cycle control, making it a good tumor suppressor candidate (110). In fact, it has already been shown that C/EBPα has tumor suppressor ability in lung cancer and AML (113, 201). Recently, it was shown for the first time that C/EBPα also exhibits tumor suppressor activity in HNSCC. Furthermore, it is epigenetically downregulated by upstream methylation (223). This study suggests that AP2α interferes with SP1 binding, resulting in decreased C/EBPα transcription, and ultimately upstream methylation (Figure 29). The consequent DNA methylation recruits MBD proteins and allows repressive histone modifications. In this way, AP2α initiates C/EBPα downregulation, which is then followed by stable silencing via DNA methylation and chromatin compaction. Furthermore, AP2α silencing effect appears to be specific for the anti-proliferative full length C/EBPα isoform. This may be due to alternate promoter usage for alternative transcription which has yet to be defined.
Figure 29. Working model depicting AP2α initiation of C/EBPα silencing in HNSCC.

I. SP1 and USF are able to bind to the unmethylated upstream binding sites and provide transcriptional activation for C/EBPα.

II. AP2α interferes with SP1 binding, decreasing transcriptional activation of C/EBPα.

III. Decreased transcriptional activation leads to upstream DNA methylation, inhibition of USF binding, and stable silencing.
Overexpression of p30\(^{\text{C/EBP}\alpha}\) and p42\(^{\text{C/EBP}\alpha}\) in adipocytes has been shown to provide very different proliferation outcomes (232). Furthermore, mutations in AML have been shown to cause upregulation of p30\(^{\text{C/EBP}\alpha}\) and revealed its ability to function as a dominant negative (229). Both isoforms can bind to C/CAAT binding sites within promoter sequences, but only p42\(^{\text{C/EBP}\alpha}\) is capable of providing differentiation and decreased cell proliferation (232). In this manner, p30\(^{\text{C/EBP}\alpha}\) opposes the p42\(^{\text{C/EBP}\alpha}\), because it competes for binding to targets and lacks the ability to function (229). Therefore, it is not surprising that p42\(^{\text{C/EBP}\alpha}\) and p30\(^{\text{C/EBP}\alpha}\) also revealed different proliferative properties in HNSCC, similar to adipocytes (232).

\(\text{C/EBP}\alpha\) is involved in a variety of physiological processes (i.e. cell cycle control, differentiation, metabolism, and inflammation) (110). Alternative translation of \(\text{C/EBP}\alpha\) mRNA in order to create different isoforms with different domains would accommodate the genetic diversity required for such a multi-functional protein (233). It has been found that the alternative isoforms of both \(\text{C/EBP}\alpha\) and \(\text{C/EBP}\beta\) fluctuate during aging and LPS response (111). For \(\text{C/EBP}\alpha\), the 42 kDa isoform decreases over time, whereas the amount of 30 kDa isoform remains constant (111). Interestingly, the isoform ratios during LPS response in a young liver mimic the patterns seen in result to aging (111). Also, previous experiments revealed that p42\(^{\text{C/EBP}\alpha}\) levels in aged livers were further diminished upon inflammatory insult, suggesting that both age and LPS response can somehow force the scanning ribosome to bypass the first translation start site (111).
p30\textsuperscript{C/EBP\alpha} has been found to be upregulated and act as a dominant negative in AML, relieving the block on cell cycle progression (229). \textit{p42\textsuperscript{C/EBP\alpha}} has been found to provide tumor suppressor qualities in several cancer types including lung cancer, AML, and more recently HNSCC (114, 234). It is reasonable to propose that carcinogenesis, like aging and inflammation, may also augment the normal \textit{C/EBP\alpha} isoform ratios and increase translation from the downstream start site. On the other hand, preliminary evidence exists to support that \textit{C/EBP\alpha} could be alternatively transcribed in HNSCC, such that different isoforms would be under the control of distinct promoters. In this context of alternate promoter usage, AP2\alpha binding and suppression could specifically affect transcription of the 42 kDa isoform. Therefore, further investigation is necessary to decipher how AP2\alpha specifically suppresses the 42 kDa isoform of \textit{C/EBP\alpha}. Regardless of the mechanism, the resulting increased p30\textsuperscript{C/EBP\alpha} and decreased p42\textsuperscript{C/EBP\alpha} levels provide for increased cell proliferation, which ultimately propagate tumorigenesis. In the case of HNSCC, the suppressor AP2\alpha appears to be involved in this change in \textit{C/EBP\alpha} isoform ratios.

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CHAPTER 5
FUTURE DIRECTIONS

HNSCC is a very aggressive cancer, and patients undergoing surgical procedures and chemotherapy still have poor chances of long-term remission (3). Despite efforts for advancing HNSCC treatment, there remains to be substantial improvement in diagnosis and prevention. Therefore, early detection and better disease prediction via genetic and epigenetic biomarkers becomes crucial. Epigenetic modifications, especially in the form of DNA methylation, have emerged as a relevant factor in the disease progression of HNSCC. Because DNA methylation is an early event, identifying methylated genes in HNSCC could expedite diagnosis of the disease by methylation screening methods.

The studies described in the preceeding chapters reveal the potential use of a methylated gene panel as prognosis HNSCC indicators. In some cases (i.e. $C/EBP\alpha$), downregulation of the gene has already been correlated with poor prognosis patients (i.e extensive lymph node metastasis) (117). Downregulation of the 5 frequently methylated genes discussed in Chapter 2 may play a part in disruption of TGF-B signaling in HNSCC progression, and functional studies of these genes and their role in HNSCC should be pursued to address this possibility. Further methylation screening in large sample sets for these markers would be necessary before proceeding with diagnostic implementation.
Identification of AP2α as an additional component of C/EBPα’s transcriptional suppression speaks to the complexity of the progression and pathogenesis of the disease. In this context, C/EBPα methylation appears to be propagated by AP2α transcriptional suppression. Therefore, it seems crucial not only to identify genes affected in different cancers, but also to investigate their transcriptional regulation. In this manner, targeting the upstream effectors may prove more advantageous in terms of treatment perspectives. In terms of AP2α regulation of C/EBPα in HNSCC, the mechanism for the change in 42/30 kDa C/EBPα isoforms remains to be determined. It seems feasible to propose the possibility of alternative promoter usage. In this way, the 42 kDa transcript would be driven by an upstream promoter containing the upstream AP2α binding sites, and the 30 kDa isoform would utilize the proximal promoter for transcriptional activation. This concept is only practical if the C/EBPα isoforms arise through alternative transcription, not alternative translation as is currently accepted. Therefore, the future direction of this work should focus on the possibility for different promoter usage and alternative transcription start sites for C/EBPα in HNSCC.


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