IDENTIFICATION OF FREQUENT GAINS OF DNA COPY NUMBER AND CHARACTERIZATION OF POTENTIAL NOVEL ONCOGENES IN HEAD AND NECK SQUAMOUS CELL CARCINOMA

DISSERTATION

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ABSTRACT

Head and neck cancers account for 5% of cancer occurrences in the United States. Ninety-five percent of head and neck cancers are squamous cell carcinomas (HNSCC), which arise from the squamous cells of the upper aerodigestive tract. Largely, HNSCC has been attributed to carcinogen exposure from tobacco and alcohol consumption. The overall 5-year survival rate is over 50%. Treatment of HNSCC involves a combination of surgery and/or chemotherapy and radiation. Although HNSCC is not the most frequent human malignancy, nor the deadliest, patients suffer not only physical disfigurements from their surgical treatment, but often their speech, swallowing and breathing abilities are impeded.

Carcinogen exposure has been associated with changes in normal gene expression, contributing to the uncontrolled growth of cells and their transformation to cancer. One mechanism that results in this dysregulated
gene expression is DNA amplification, a common mechanism for oncogene activation in cancers and has been used in the discovery of novel oncogenes. DNA amplification is frequently observed in head and neck squamous cell carcinomas (HNSCCs) where numerous amplification events and potential oncogenes have already been reported. Identifying the oncogenes and mechanisms responsible for carcinogenesis can help us to better understand the underlying biological processes of the disease.

Chapter 1 describes the nature of head and neck cancer, and the significance of DNA amplification in cancer. Due to selective pressure, cancer cells selectively keep and multiply target oncogenes, in addition to other “bystander” genes that do not necessarily contribute to carcinogenesis. The possible mechanisms of DNA amplification will also be discussed.

In chapter 2, we applied restriction landmark genomic scanning (RLGS) to study gene amplification in HNSCC and to locate novel amplified and uncharacterized regions in primary tumor samples. Our analysis is highly consistent with published reports and generates new research interests.

Chapter 3 represents a follow-up study on one gene of interest, YWHAZ (14-3-3ζ, KCIP-1). We verify YWHAZ overrepresentation at the levels of DNA, mRNA, and protein. Furthermore, down- and up-regulation of YWHAZ
significantly alters cell growth. Based on this, we suggest that YWHAZ deserves further investigation into its role in HNSCC carcinogenesis.

Chapter 4 describes a search for oncogenes along the novel 3q29 amplicon. Transferrin receptor arises to be the first gene to look at because of its associations with many cancers. Our results suggest that the transferrin receptor may have some oncogenic activity. Chapter 5 summarizes our discoveries of chapters 2 to 4 and proposes work that needs to be done in the future. From the described significance of these results and others in the thesis where conclusions from other amplicons have not been made, it is my hope that additional insights will develop in future studies.
Dedicated to my family and all people who have supported me, and made me

a better person I can be by myself alone.
ACKNOWLEDGMENTS

At the age of 33, I guess this is it. This thesis marks the end of my student career, but just another beginning of endless learning opportunities from Mother Nature. We will never know enough.

As far as I can remember, I always wanted to be nothing other than a scientist. I regard scientists as smart people who can truly benefit mankind; by curing disease, landing on the moon, and changing what once was thought unchangeable. Nothing impresses me more than the joy of solving mysteries. I don’t know many people who are willing to make less money and work during the weekends. Maybe that’s why my mother sometimes teases and calls me a dreamer. Being a dreamer is difficult in this modern, money-oriented society, and that’s why I’d like to thank all the people who made my dream a bit more realistic.

First, I’d like to thank the people who help me to come here. My
experience at the National Taiwan University Hospital was invaluable. Dr. Chin-Tarng Lin and Dr. Tzu-Shou Jou showed me what it takes to be a scientist—hard work, a knowledgeable mind, and strong motivation. Thanks to Dr. David Bisaro, the Chair of the MCDB program, who gave me the chance to study at this prestigious university and program. He also gave me a warm hand when I was down and struggling here.

After a grueling start to my journey, I finally became a member of Dr. Christoph Plass’ lab. Now I believe it was a blissful opportunity in disguise. Christoph established a successful lab and recruited many great minds. I was taught the rigid and non-compromising logic in doing science, which I will carry on. Christoph expected high quality of research from us, and yet is very reasonable and caring for us. I am also happy that I didn’t have to sacrifice most of my family life and fun just to be a great scientist, because Christoph shows me it’s possible to have both. It makes me sad that research life after here will be harder because I don’t think I will work for a nicer boss again, unless I become one.

In this lab everyone works hard and helps each other. I also don’t think I can be in a warmer lab again. I’d like to especially thank Dr. Laura T. Smith, who took me under her wings when I rotated here four years ago. To all of my
colleagues: Kristi, Martin, Shih-shih, Aparna, Bjorn, Shu-huei, Yue-Zhong, Jie-Jun, Li, Chun-hui, Tada, Betsy, Katie, Matt, it’s my pleasure to work with you all.

My first paper would not be complete without the contributions from Ramaki and Dr. Raphael Wenger and Dr. Karl Kornacker. Dr. Carl Morrison and his great staff contributed to the crucial data in my YWHAZ story. I thank Dr. David Schuller and Dr. James Lang for providing research samples.

Finally, last but not least, I pay the ultimate gratitude to my family. My mother supports me all the way to do whatever I want to do. My brother and sister take good care of family responsibilities when I couldn’t in these years. For my own purpose, I missed the final farewells to my two grandparents, who brought me up. I have to be successful because I owe too much to them.

It has been six great years. I will stand up, proud from my accomplishments, from now on.
VITA

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CHAPTER 1

THE ROLE OF GENE AMPLIFICATIONS IN HEAD AND NECK SQUAMOUS CELL CARCINOMA (HNSCC)

1.1 Head and neck cancer

Cancer is the leading cause of death in United States and most modern countries. It is estimated that nearly 1.4 million Americans will be diagnosed of cancers in 2007\(^1\). Unlike other diseases caused by microbes, viruses or parasites, cancer is mainly a genetic disease, occurring by alterations on the DNA level. Besides sporadic cancers in which the initiating cancer cell acquires a genetic change, some cancers are heritable and characterized by a predisposing defect in a tumor suppressor gene\(^2\). Normal cells are under strict regulation to maintain the status quo between growth and death. Sometimes, however, cells bearing certain genetic defects may escape this balance. When compared to the original normal cells, cancer cells are generally immortal (evade programmed death), divide more rapidly, are resistant to most growth limitations and in more advanced stages may
develop metastatic ability (able to travel to new body sites and establish new colonies) \(^3\). Due to these characteristics, human cancers remain one of the top threats to human health.

Head and neck cancer refers to cancers arising from the head and neck regions, which include nasal cavity, lip, mouth, tongue, salivary glands, tonsil, pharynx and larynx (brain, esophageal or thyroid cancers are usually excluded). 95% of head and neck cancers are squamous cell carcinomas (HNSCCs), which arise from the mucosal surface (squamous cells) of the upper aerodigestive tract. Head and neck cancers account for 5% of cancer occurrences in United States \(^1\). It is estimated that 35,000 new cases will emerge and 7,500 will die of it in 2007 \(^1\). Men are more likely to suffer from head and neck cancers than women (gender ratio: of 2~2.5 to 1) \(^1\). Although the 5-year survival rate for HNSCC is nearly 60%, patients often suffer from the inconveniences and humiliation due to facial deformities after surgeries and treatments.

Chronic exposure to alcohol and tobacco are the most common risk factors of head and neck cancers. Individuals who smoke and drink heavily may increase their risk to 100 times that of non-smokers \(^4\). Inhalation of fine particles like asbestos, cement and wood dusts has been related to the
development of head and neck cancers. Infection of Epstein-Barr virus (EBV) and human papilloma virus (HPV) type 16 and 18 also increase the risk of nasopharyngeal and oral carcinomas, respectively. Hereditary factors could be playing roles since several genetic diseases, such as ataxia-telangiectasia (A-T), Li-Fraumeni Syndrome (LFS) and Bloom Syndrome (BS), are associated with higher risks of head and neck cancers. The responsible oncogenes for these diseases have been identified as ATM, TP53 and BLM respectively.

1.2 Gene amplification, oncogene and cancer

Despite a stable nature of and numerous repair mechanisms, the human genome is not a damage-proof material. Besides rare errors during DNA replication, it is constantly under the influence of environmental stress and countless sources of DNA damaging agents as common as sunlight, microwaves or household chemicals. Cells are equipped with some intrinsic protective and repairing mechanisms to prevent severe damage on pivotal genetic materials. For those cells whose damages are beyond repair, their apoptotic program could be activated and bad cells are eliminated. Despite of these fail-safe mechanisms, a few damaged cells still manage to escape,
survive and gain extra abilities. Ranging from the change of a single nucleotide to several megabases or even an entire chromosome arm, the unrepaired damages can lead to acquisition or loss of some genetic information (amplification or deletion), new gene products (fusion or truncation), or wrongful gene activation or silencing (epigenetics). Each cancer often bears its unique features of common DNA defects, which help scientists to identify and predict the tendency of cancers.

Gain of specific chromosomal regions is a very common event in all cancers. These non-random chromosomal gains are believed to represent oncogenes, which become activated and provide a selective growth advantage to the cell and in this way initiate or facilitate cell transformation (#REF). Oncogenes are vital to normal cell functions such as survival, growth and differentiation when their levels are in check. When amplified, the levels of their gene products may be also increased even with the regular transcription controls. Numerous oncogenes, such as N-MYC, ERBB2 and EGFR have been found and verified through the discoveries of their respective chromosomal amplifications 12-14.

Gene amplification exists in various forms. It can occur as extra whole chromosome copies (aneuploidy), as concentrated head-to-tail or inverted
tandem repeats within a small region, as copies randomly inserted into other chromosomes, or as tiny extrachromosomal elements (EEs) \(^{15}\). Multiple mechanisms contribute to gene amplification. Simple uneven sister chromatid segregation in mitosis is likely to generate aneuploidy in daughter cells. Other mechanisms involve more complexity in DNA damages, repair and recombination.

According to breakage-fusion-bridge (BFB) model proposed by McClintock \(^{16}\), the initiation event of tandem gene repeats is uncapping of DNA sequence by double-strand break or telomere erosion (breakage). After DNA replication, two uncapped sister chromatids are fused in their ends by DNA repair mechanism (fusion). In mitosis, the two end-fused chromatids are pulled toward two opposite centromeres (bridge). When the second breakage happens somewhere in the proximity of the bridge, one of the daughter cells will have two gene copies on one sister chromatid and the other has none. The process can be repeated and the gene copy number will increase if the carried gene confers growth advantage and favorable selection. The involvement of breakage in gene amplification is supported by the fact that some oncogenes are found near natural break-prone fragile sites. Many chromosomal and mitotic structures predicted by BFB models, including
inverted repeats \(^{17}\), fused sister chromatids and breakage of anaphase bridge \(^{18}\) are all observed \textit{in vivo}.

Extrachromosomal elements (EEs) refer to the circular chromosomal fragments which are too small in size to be considered a chromosome. Some extrachromosomal elements are random while others are non-random and highly regulated. One of them, double minutes (DMs), are closely related to cancers. In the form of double minutes, an oncogene can easily have dozens of copies. Double minutes carrying MDR2 or DHFR genes are often found in drug-resistant cancer cells \(^{19,20}\). The proven oncogene c-Myc amplification often exists in double minute form in cancers, and it has been shown to induce formation of more double minutes \(^{21}\). Thus, studying double minutes in cancers provide a convenient path to identify oncogenes.

Several cytogenetic techniques with various perspectives and resolutions are utilized to identify abnormal amplification in cancers. Early stage of detections starts with classical G- or C-banding patterns and spectral karyotyping (SKY) to detect homogenous staining regions (HSR) and extrachromosomal elements by microscope. More advanced techniques such as comparative genomic hybridization (CGH) and more precise array-based CGH provide researchers more global and quantitative distributions of
amplification events. Fluorescent *in situ* hybridization (FISH) is often used when closing in on one or several specific genes.

### 1.3 Gene amplifications in HNSCC

Each cancer type possesses a unique pattern of gene amplifications. For example, amplification of the AML1 oncogene is often observed in acute myeloid leukemia but there is no evidence of amplification in HNSCC. The most frequent amplification events in HNSCC are on chromosome 3q26.3, 8q24 and 11q13.3, which are also shared in several other cancers. One or several genes on these regions are known to possess oncogenic activities, such as phosphatidylinositol 3-kinase catalytic alpha subunit (PIK3CA), c-myc (MYCC), and cyclin D1 (CCND1). Other reported frequent amplifications are listed in Table 1.1.

The elevated level of a single oncogene is insufficient to transform normal cells. The process of transformation involves too many cytological and genetic changes to be driven by a single oncogene. Multiple oncogene amplifications accompanied by tumor-suppressor gene loss must take place in cancer cells and they are in a bigger network of cell growth and apoptosis regulations. Figure 1.1 lays out the known components and interactions.
between them, many of which are proven oncogenes or tumor-suppressor genes. In the aspect of HNSCC, several genes in the same pathway are found co-amplified. Coupled with defects in other pathways controlling repair or growth inhibition, the transforming cells can increase their proliferative capacity and metastasize.

Understanding which oncogenes are responsible for HNSCC can help us to find therapeutic targets. Recently FDA-approved anti-tumor agent Erbitux (cetuximab) antagonizes overexpressed EGFR in HNSCC and other cancers. Another drug, Iressa (gefitinib), targets on mutated EGFR. Due to the constant evolution of cancer cells, finding new drugs is a never-ending quest. Thus, identifying new oncogenes is also a never-ending task. I hope that this research can benefit the treatment of HNSCC and other cancers.
Table 1.1 Meta-analysis of chromosomal gains in HNSCC.

Abbreviations: CC, classical cytogenetic analysis; CGH, comparative genomic hybridization; srg, small region of gain. The table is modified from the review by Dr. Gollin.²⁶
Figure 1.1 Schematic representation of receptor tyrosine kinase-mediated signaling.

Factors found to be amplified in HNSCCs are colored green and those found to be frequent lost or mutated are colored red. Black lines indicate direct activation (arrowheads) or inhibition (bars), blue lines indicate transcriptional activation and dotted lines indirect effects. The original figure \(^{22}\) is modified to reflect the real events of amplification and loss in HNSCC.
CHAPTER 2
IDENTIFICATION OF NOVEL AMPLICONS IN HEAD AND NECK SQUAMOUS CELL CARCINOMA

Published in Oncogene 2006 Mar 2;25(9):1424-33 by Lin M, Smith LT, Smiraglia DJ, Kazhiyur-Mannar R, Lang JC, Schuller DE, Kornacker K, Wenger R and Plass C

2.1 Introduction

Gain of DNA copy numbers, or DNA amplification, is a common genetic alteration found in most types of cancers \(^{27}\). Subchromosomal DNA amplification results in extra copies of specific regions, potentially containing oncogenes. DNA amplification often results in overexpression of the target gene located in the amplified region, and thus confers a growth advantage \(^{28}\). Overexpression of oncogenes results in neoplastic transformation and provides a growth advantage either by providing resistance to apoptotic stimuli \(^{29}\) or by promoting accelerated cell growth \(^{30}\). Amplification and
overexpression of oncogenes is an early event in carcinogenesis 31 and thus can be used as a diagnostic marker for early detection of tumor cells or to predict outcome of a clinical therapy. Furthermore, activated oncogenes provide potential targets for cancer therapies 32.

Head and neck cancer is a collective term for cancers located in nasal and oral cavities, pharynx, and larynx. About 95% of head and neck cancers are squamous cell carcinomas (HNSCC). In the United States, head and neck squamous cell carcinoma comprises about 5% of all malignancies and claims ~20,000 lives of Americans every year 33. Gene amplifications in HNSCC have been frequently observed using comparative genomic hybridization (CGH) 34,35, fluorescent in situ hybridization (FISH) 36,37 and recently array-based CGH 38,39. The most frequent amplifications in HNSCC include copy number gains of chromosomes 3q26 34,40,41, 8q24 34,41 and 11q13 35. Oncogenes in these regions include phosphatidylinositol 3-kinase (PI-3K) catalytic alpha subunit (PIK3CA), c-myc (MYCC), and cyclin D1 (CCND1), respectively 35,40,42. Many other less-frequent amplicons have been reported, but candidate oncogenes remain undiscovered.

In this study we utilized Restriction Landmark Genomic Scanning (RLGS) as a tool for a comprehensive identification of amplicons in HNSCC. RLGS
allows us to detect low-level amplification events and may help to pinpoint smaller amplicons that have been missed by other techniques. DNA is digested using restriction enzymes that leave sequence overhangs at one end of the DNA. These ends are then filled in with radioactive nucleotides. The intensities of RLGS fragments correlate directly with the copy number of a sequence, allowing the reproducible detection of a two-fold increase (duplication) \(^{43}\). Furthermore, the restriction landmark enzymes sites frequently reside within the CpG islands, which are GC-rich sequences found in the upstream regulating region of 50-60% genes \(^{44}\). This characteristic makes it possible to effectively pinpoint the genes within an amplicon. With these two advantages, RLGS has successfully identified amplicons including several oncogenes, e.g. CDK6 in glioblastomas \(^{45}\), MYCL in ovarian carcinomas \(^{46}\) and PIK3CA and IAP1/IAP2 in lung carcinomas \(^{47}\). This study is the first study to use RLGS for the identification of DNA amplifications in HNSCC and to compare these results with published reports utilizing other genome assays.
2.2 Materials and Methods

2.2.1 HNSCC cell lines and patient samples

Primary head and neck squamous cell carcinomas (HNSCC) derived from the nasal cavity (n=2), oral cavity (n=20), larynx (n=7), pharynx (n=7) and undocumented (n=5) were obtained from the Cooperative Human Tissue Network. Surgery was performed on all patients at The Ohio State University. All normal specimens were harvested from morphologically normal appearing tissue located at least 3 cm from the tumor margin and were used as the normal control tissue for comparisons with the tumor tissues. Histopathological characterization was performed for both normal and primary tumor. Samples were snap frozen in liquid nitrogen and stored at –80°C prior to DNA isolation. The study was performed under a protocol approved by the Institutional Review Board of The Ohio State University.

2.2.2 Genomic DNA preparation

The protocol for extraction of genomic DNA from normal adjacent and cancerous tissues has been described previously. In brief, frozen tissue samples were crushed and incubated in 15-20ml lysis buffer in the presence of 100µg/ml proteinase K at 55°C for 1 hour or until the buffer becomes
homogenous. Two phenol:chloroform:isoamyl alcohol extractions were performed using Phase Lock Gel tubes (Eppendorf). DNA was dialyzed overnight in 10mM Tris and RNase-treated for two hours at 37°C. Following ethanol precipitation DNA was stored in TE buffer.

2.2.3 Restriction Landmark Genomic Scanning (RLGS)

We followed the published protocol of 47. In brief, extracted genomic DNAs were incubated with DNA polymerase I (Boehringer Mannheim) for blocking the randomly sheared ends. DNA samples were digested with NotI (Promega) for two hours and restriction ends were labeled using $[\alpha^{32}P]dGTP$ and $[\alpha^{32}P]dCTP$ together with Sequenase (Amersham), followed by an EcoRV (Promega) digestion. The labeled NotI-EcoRV fragments were separated in 0.8% agarose gels. Separated DNA fragments were further digested with HinfI (New England Biolabs). Then restriction fragments were further separated in a second-dimension 5% polyacrylamide gel. The acrylamide gels were then transferred onto 43x35cm filter papers, dried, and exposed to the X-ray films (Kodak) for 5-10 days.
2.2.4 Analysis of RLGS gel pictures

All shown RLGS fragments are given ID numbers according to the “RLGS Master Profile” (http://pandora.med.ohio-state.edu/masterRLGS). We compared the intensities of RLGS fragments from tumor tissues to those from normal adjacent tissues from the same individuals. Analyses were done by CONIME, customized computer software developed by Dr. Wenger of Ohio State University (Wenger et al. manuscript in preparation). We used the software to mark any mismatches on intensity, including gain and loss, by automatic and manual picks. These mismatches were further examined by visual inspection of original gels.

2.2.5 RLGS spot cloning

RLGS fragments were cloned utilizing the NotI-EcoRV clone libraries\textsuperscript{45,47,48}. Alternatively known NotI-EcoRV fragments from these libraries were used in RLGS mixing gels to identify corresponding RLGS fragments.

2.2.6 Southern hybridization and quantification

For Southern hybridization, we selected seven pairs of patient samples to digest with NotI and EcoRV at 37°C for two hours. These digested DNA
samples were electrophoresized in 0.8% agarose with 40 Volts for 16 hours. Later DNA fragments were transferred into HyBond membrane and fixed. Our hybridization probes were designed to hybridize specific NotI-EcoRV fragments, identified in the RLGS analysis. We used PCR to generate these probes. Primer sets of probes for RLGS fragments are listed in Table 2.1. Either 10ng genomic DNA, 1µl for 5’ primer and 3’ primer each, 5µl 10X PCR buffer (Invitrogen), 1.5µl 25mM MgCl$_2$, 1µl 10mM dNTPs, 0.5µl Taq polymerase (Invitrogen), 39µl H$_2$O were used (one asterisk in Table 2.1) or, for GC-rich sequences, 10ng genomic DNA, 2µl for 5’ primer and 3’ primer each, 5µl 10X special buffer for GC-rich sequence $^{49}$, 1.25µl 25mM DMSO, 2.5µl 10mM dNTPs, 0.4µl Taq polymerase, 35.85µl H$_2$O (two asterisks in Table 2.1). PCR cycles: 95°C for 5 minutes; 95°C for 30 seconds, individual Tm for 30 seconds, 72°C for 30 seconds for 35 cycles; 72°C for 10 minutes; preserve at 4°C. Probes were end-labeled by [$\alpha$-$^{32}$P]dCTP with Prime-it II kit (Stratagene). Blots were pre-hybridized in pre-hybridization buffer (0.5M phosphate buffer / 7% SDS) at 65°C for 3 hours, co-incubated with salmon sperm DNA for blocking. Then the probes were added and incubated for 16 hours. Washes were low-stringency buffer (0.2M phosphate buffer / 0.1% SDS) for 30 minutes and following high-stringency buffer (0.02M phosphate
buffer / 0.1% SDS) for 15 minutes. Labeled blots were sealed and exposed on PhosphorImager screens for five days.

The screens were scanned and quantified to evaluate the increase fold. Our internal control is the intensity of RLGS fragment 2E12, which demonstrates no intensity changes in any of our samples. The definition for increase fold is \( \frac{\text{Count}_{\text{Spot}}/\text{Count}_{2E12}}{\text{Tumor}} / \left( \frac{\text{Count}_{\text{Spot}}/\text{Count}_{2E12}}{\text{NAT}} \right) \).

2.2.7 HNSCC mRNA expression profiles by microarray

mRNA expression profiles were computed from raw HG-U133A probe intensities using RMAExpress with quantile scaling and background correction. RMAExpress program used in analyzing microarray data was written by Dr. Benjamin M. Bolstad. The CEL files were provided by Patrick M. Gaffney and have been described. mRNA expression profiles of fourteen normal head and neck tissues and forty HNSCC patient samples were obtained (independent studies from our sample database). The median of the log₂ value of fourteen normal tissues’ mRNA expression levels was regarded as one-fold, so that anything greater than two-fold (log₂ ratio>1) would be considered up-regulation. 95% of mRNA expression levels in normal tissues are located within 2-fold (log₂ value=1) and 0.5-fold (log₂ value=-1).
<table>
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<th>Reverse primer</th>
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<th>Target (bp)</th>
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**Table 2.1 Primer pairs used for the amplification of Southern probes.**

Number of asterisks in Tm indicates individual PCR component sets which are listed in Materials and Methods.
2.3 Results

2.3.1 A genome-wide search for DNA amplification events in HNSCC

We used pairs of primary tumor and normal adjacent tissues from HNSCC patient samples for RLGS analysis to eliminate differences among individuals. 41 pairs of \textit{NotI-EcoRV-Hinfl} were generated for the study. RLGS gels were analyzed for enhanced fragments only. Two types of enhancement were observed. Type 1 enhancements represent those RLGS fragments, which are present in both normal and tumor profiles, however the tumor profile shows the stronger signal intensity. Type 2 enhancements represent those RLGS fragments, which exist exclusively in tumor tissues and thus are not found in the RLGS profiles of normal tissues (see Figure 2.1A and B for examples). Sequences for a total 715 \textit{NotI-EcoRV} RLGS fragments were available to us from prior work \cite{45,47,48} or isolated more recently (Plass et al., unpublished data). Table 2.2 lists 63 enhanced RLGS fragments that have been cloned from the \textit{NotI-EcoRV} profile. Enhancements were seen in chromosomal regions: 1p32.3, 1q41, 2q21.1, 3p21.31, 3q21.2-3, 3q23, 3q26.1-q27.1, 3q29, 4q35.1, 5q31.3, 6p25.2, 6q27, 7p22.2, 7p14.3, 7q31.1, 8p11.23, 8q21-q24.21, 9q32-q33.2, 10p12.1, 10q21.3, 10q24.32, 11q13.3-4, 12p12.1-p11.22, 12q13.13, 14q32.32, 16q24.1, 17q11.2, 17q25.1, 19q13.11-13, 20p11.23,
20q11.21-22, 20q12 and 20q13.33. A total 11 out of 63 identified sequences are located within chromosomal region 3q26.1-3q27.1, a region which represents the most frequently amplified region in HNSCC \(^{34,40}\). Six sequences are located on chromosome 3q29, which represents the second most frequent amplification event in our study. Four sequences are from chromosome 11q13.3 a known amplified region in HNSCC, which ranks third in frequency in our study.
Figure 2.1 Examples of RLGS fragment enhancements detected in head and neck squamous cell carcinoma.

A. Type 1 enhancement represents RLGS fragments that are present in the normal profile and demonstrate increased enhancement in the tumor. Arrowheads indicate RLGS fragment 3B35 (MYCC locus) in RLGS section from patient 25. Sections from normal adjacent tissue (25N) and tumor (25T) tissue are shown.

B. Type 2 enhancement represents RLGS fragments in the tumor profile with complete absence of an RLGS fragment in the normal tissue profile. Arrowheads indicate fragment 3FX gained in patient 126 tumor (126T) as compared to PT126 normal adjacent tissue (126N).

C. Southern hybridization for human tandem repetitive sequence L09552. The NotI-EcoRV fragment for 3FX is ~2.8Kb, which is only detected in PT126 tumor sample. Among three HSNCC lines tested, fragment 3FX is hypomethylated in SCC9 confirmed by RLGS and Southern hybridization.

Abbreviations: RV: EcoRV-digested; NRV, NotI-EcoRV-digested.
### Table 2.2 Summary of enhanced NotI-EcoRV RLGS fragments in HNSCC

A white square indicates no change in copy number, gray indicates an increase from 1.5- to 4- fold and black indicates an enhancement >4-fold. A question mark indicates that the particular RLGS fragment was not analyzable on the gel and a (R) indicates a reduction of fragment intensity.
2.3.2 Confirmation of DNA amplification by Southern blotting

To confirm enhancements detected by RLGS, we used Southern blotting of 17 selected RLGS sequences specific for several chromosomal regions (Figure 2.2 and 2.4B). Southern band intensities of the test fragments were compared to the intensity of a control fragment located on chromosome 21q22.3, to give the relative intensity of the test fragment. This control sequence is derived from a confirmed single copy RLGS fragment (2E12), located outside of amplicons identified in this sample set. Following hybridization, the relative test fragment intensities were compared between normal and tumor DNA. A 1.4 fold or greater increase in relative test fragment intensity in the tumor DNA was considered to represent DNA amplification.

We tested a total of 17 sequences in seven pairs of patient samples (n=6 for sporadic amplification events in Figure 2.2 and n=5 for 3q26.1-3q29 region in Figure 2.4B) and found concordance of RLGS data with Southern data in 67 out of total 77 events (87%). The 10 discrepancies were fragments either not scored by RLGS (n=8), or scored by RLGS but not confirmed in Southern (n=2). Furthermore, Southern blot results show that events over two-fold amplification are not common, with the exception of copy number gain of sequences from the 3q26.1-q29 region in PT91T. The cases of discordances
likely represent the fact that the amplifications are of low enough level that their detection by the two methods is at the threshold of experimental errors, as well as to several technical limitations including (1) polymorphisms in the \textit{Hinfl} restriction site, resulting in different migration patterns of an RLGS sequence, (2) overlapping RLGS fragments that may have caused misinterpretation, or (3) lower quality of RLGS section analyzed. Interestingly, RLGS fragment 2E52, which is reduced in the tumor sample of PT173, shows also reduction to 0.42 fold intensity as measured by Southern blot analysis. RLGS fragment 3F55 (residing along chromosome 8q13.1) shows amplification in two out of six tumor samples. Fragment 2C13 (8q22.3) is amplified in two cases whereas enhancement was not detected by RLGS in PT126. DNA amplification in RLGS fragment 2E02 (9q32) is verified in two cases. Three cases show DNA copy number increase in RLGS fragment 2E52 (14q32.32); although two of them were missed in the RLGS analysis. 3D46 (17q25.1) is indeed amplified in three HNSCC cases, and amplification of 2D38 (20q13.33) in two cases is confirmed. Only one case shows amplification on 2D68. The absence of 2D68 fragments in both normal and tumor samples of PT117 in Southern analysis matches the same absence of RLGS fragment 2D68 in their corresponding RLGS gels.
We noticed RLGS fragment 3FX that appears as an enhanced RLGS fragment in 71% (40 in 56) of all cancerous tissues but never in the RLGS profiles of its normal adjacent tissue (Figure 2.1B). The sequences of this type 2 enhancement had previously been reported in lung cancer and neuroblastoma and represents a human tandem repetitive element (accession number L09552) on 8q21.2.\textsuperscript{47,52} Our Southern data confirmed that fragment 3FX is the consequence of a normally-methylated L09552 becoming hypomethylated after transformation (Figure 2.1C).
Figure 2.2 Southern hybridization of seven RLGS fragments amplified in HNSCC patients.

Each RLGS fragment number is listed together with chromosomal location. Six patient sample pairs, 110N/T, 126N/T, 91N/T, 99N/T, 167N/T and 173N/T were used for the Southern analysis and digested with NotI and EcoRV. For RLGS fragment 2D68, sample pair 117N/T was used instead of 167N/T. The intensity in the tumor sample (T) was compared to the corresponding normal sample (N). Fragment 2E12 was used as control (Control) fragment. The numbers underneath the Southern bands indicate fold increases. Increases over 1.4 are considered amplification. Gray shading indicates that the corresponding RLGS fragment was scored as enhanced.
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2.3.3 Novel DNA amplification events detected by RLGS in HNSCC

Table 2.3 summarizes the DNA amplification data obtained by RLGS analysis and compares them with those described in the literature. The RLGS findings are highly consistent with previous reports on the most frequently amplified regions. Gains of 3q26-27, 7p, 8q24, 11q13, 12p, 17q, 18p, 20p, and 20q, which are documented in most references, are also detected by RLGS. In addition, the RLGS analysis identified several novel amplification events. They are RLGS fragment 3E13 on chromosome 2q21.1 found amplified in 4 cases, 2C62 (3p21.31) in 12 cases, 4B44 and 3B32 (3q21.3) in 5 and 7 cases, 5G03 (3q23) in 5 cases, 4B25-3F38 (3q29) in 10-16 cases, 3C19 (4q35.1) in 6 cases, 2D73 (5q31.3) in 4 cases, 4F46 (6q27) in 8 cases, 2C33 (7p22.2) in 9 cases, 2B56 (7q31.3) in 8 cases, 2E02 (9q32) in 4 cases, 3E70 (9q33.2) in 6 cases, 3C31 (10p12.1) in 4 cases, 4C06 (10q21.3) in 4 cases, 2D68 (10q24.32) in 3 cases, 2F71 (16q24.1) in 6 cases, and 3D46 (17q25.1) in 5 cases. For those reported but not further characterized regions, we identified RLGS fragments 3B09 (1p32.3) in 6 cases, 2D70 (1q41) in 13 cases, 5F33 (8p11.23) in 5 cases, 3F55 (8q13.1) in 4 cases, 2C13 and 3D05 (8q22.3) in 14 and 12 cases, 2D60-2C59 (19q13.1) in 4-7 cases and 2E21 (20p11.23) in 10 cases. Their exact locations are listed in Table 2.2.
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Table 2.3 Summary of amplification events found in head and neck squamous cell carcinomas either by RLGS or based on current literature.

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Table 2.3 (continued)

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* Maximum 3 references are listed.
2.3.4 Consecutive RLGS fragments can be used to determine amplicon structure

RLGS fragments co-localizing within a short chromosomal region and showing similar levels of enhancement are likely amplified together. Figure 2.3 represents RLGS fragments from chromosome 11q13.3, a well-documented amplicon in HNSCC. Four RLGS fragments, 2F51, 6FX, 2F63 and 6G27, are found within a ~755 Kbp region. RLGS fragments 2F51 and 6FX represent the promoter region of CCND1, and 6G27 represents the NotI site found in the promoter of EMS1. Both, CCND1 and EMS1, are oncogenes described in previous studies \(^{59,60}\). RLGS fragments 2F51 and 6FX are cleavage products from a single NotI-NotI restriction fragment. Among 41 patient samples, both 2F51 and 6FX are always co-amplified (found in 10 cases or 24%), while 2F63 amplification was observed in 9 cases (22%) and 6G27 amplification occurs in 11 cases (27%). Most importantly in the majority of samples all four RLGS fragments are enhanced to the same level (see Figure 3, PT29 for example). In PT40, 2F51/6FX and 6G27 are amplified, but not 2F63 (Figure 3) suggesting the presence of two potential core amplicons. Amplification of 6G27 but not 2F51/6FX and 2F63 is seen in PT16 (Figure 2.3). We do not have cases showing 2F51/6FX amplification alone. This data indicates that
CCND1 amplification is not necessarily associated with EMS1 amplification.

The presence of two separate amplicons in this chromosomal region with either CCND1 or EMS1 functioning as oncogenes has previously been investigated using differential PCR and it was shown that EMS1, but not cyclin D1 amplification correlates with poor prognosis.\textsuperscript{60}
**Figure 2.3 Subclassification of 11q13.3 amplification events.**

Left vertical line represents the relative location of RLGS fragments (2F51, 6FX, 2F63 and 6G27, highlighted) in the 11q13.3 region. Intensity of fragment 6FX in reference genomic DNA is so weak that not identified and given ID on the original master profile. Base-pair numbers are given according to UCSC Genome Bioinformatics website [http://genome.ucsc.edu](http://genome.ucsc.edu), May 2004 freeze. CEN, centromeric. TEL, telomeric. CCND1, FGF19/4/3 and EMS1 are known genes. Arrows in tumor samples (T) indicate the enhancement compared to the same fragment in normal samples (N). Panel A, B and C are sections of the actual RLGS gels obtained from patients 29, 40 and 16. Panel A, all four RLGS fragments are enhanced. Panel B, all but 2F63 are enhanced. Panel C, only 6G27 is enhanced.
2.3.5 Subclassification of amplicons on chromosome 3q26-q29

We have identified 19 RLGS markers spanning chromosomal region 3q26.1-q29 (eleven on 3q26.1-q27.1, which are 5E10, 6E06, 3D17, 3D21, 1G20, 5E06, 2F72, 3E58, 2E46, 3E46 and 3E24, two on 3q28, 5F39 and 5F07, and six in 3q29, which are 4E12, 4B25, 5D03, 2C38, 4F31, 3F38 (Figure 2.4A). The two most frequent amplification events were scored for RLGS fragments 3D17, (with 21 amplifications out of 41 samples tested) and in the neighboring fragment, 3D21, (17 out of 41 amplification events). Interestingly, this region includes PIK3CA, a known oncogene, confirming previously published data. Numerous patient samples demonstrate consecutive DNA amplification in several markers in this region suggesting that the whole 3q26-3q29 region is amplified as a single amplicon (e.g. PT12, PT24). However, in other patients (PT110, PT174 and PT175) we found only amplification in the 3q29 markers suggesting the presence of a smaller amplicon independently existing in this region. This assumption is further supported by the finding of two amplified regions in PT2, one on 3q26.32 (RLGS fragments 3D17 and 3D21) and the other one on 3q29 (5D03, 2C38 and 4F31) separated by sequences that are not amplified (5E06 to 4B25, 8 consecutive spots).
In addition, there is evidence that the amplicon on 3q26.32-q27.1 may be composed of two separate amplicons, as well. Several patients including PT2 and PT71 show amplification of 3q26.32 marker (3D17 and 3D21) without amplification of a further telomeric marker in the 3q27.1 region. However, PT16, PT45, PT50 and PT56 demonstrate only amplification of the 3q27.1 marker (PT16 also possesses an amplification on 3F38). In conclusion, it seems that the amplification data suggests the presence of three separate amplicons on chromosome 3q, region 1 on 3q26.32 encompasses the known oncogene PIK3CA, whereas for region 2 (3q27.1) and region 3 (3q29) no prevalent candidate oncogene has been reported in HNSCC (Figure 2.4A). The RLGS data was further validated by Southern blot analysis using RLGS sequences from different regions on patient samples and matched normal controls (Figure 2.4B). PT91 and PT114 show consecutive amplifications of regions 1, 2 and 3, whereas PT167 shows only amplification in region 3. PT99 showed amplification of markers in region 1, 2 and 3, separated by non-amplified fragments (1G20, 5E06, 5F39 and 4E12). PT126 does not show any amplification in the sequences tested by Southern analysis however RLGS indicated amplification in fragments 2E46 and 3E46.
Figure 2.4 Subclassification of 3q26.1-3q29 amplification events.

A. Amplified RLGS fragments on the 3q26.1-q29 amplicon in HNSCC patient. Left vertical line illustrates the relative location of RLGS fragments in the 3q26.1-q29 region. CEN, centromeric; TEL, telomeric. Base-pair numbers are given according to UCSC Genome Bioinformatics website http://genome.ucsc.edu, May 2004 freeze. Arrows and arrowheads are used to point out these spots-to-see. Three patient sample pairs are used to demonstrate segmental amplifications. In PT91T, all fragments are enhanced. In PT16T, only 3E24 (3q27.1) and 3F38 (3q29) are enhanced. In PT71T, only 3D17 and 3D21 (3q26.32) are enhanced.

B. Results of ten RLGS fragments on chromosome 3q26.1-q29 in five patient sample pairs. NotI/EcoRV-digested genomic DNAs of five patient pairs were used. RLGS fragment, 2E12, serves as the internal control. Relative increases are summarized in the right panel. Intensities over 1.4 are considered amplification. Dark gray indicates enhancement on actual RLGS gels, and light gray indicates that the RLGS fragments were not analyzable in the particular gels.
Figure 2.4 (continued)
2.3.6 DNA amplification events correlate with mRNA overexpression in 11q13.3 and 3q29 regions

In an attempt to correlate DNA amplification with gene overexpression, we evaluated mRNA levels of identified genes in two regions, the established amplicon on 11q13.3 and the newly identified amplicon on 3q29. Expression levels of all known genes in these two regions were reexamined using published microarray data. Figure 2.5 shows the frequency of increased expression relative to the chromosomal locations of the respective gene. CCND1 and EMS1 are upregulated in 37.5% cases. Gene PPF11A located about 43 Kbp centromeric to EMS1, is upregulated in 37.5-65% cases (the frequencies vary depending on hybridization probes). Two adjacent genes ORAOV2 and FADD, ~200 Kbp centromeric to EMS1, are both upregulated in 30% and 40% cases. Interestingly, the expression level of FGF4 and FGF3 are not elevated in the 40 tumors analyzed. Thus mRNA expression levels on 11q13.3 support the idea of a discontinuous amplicon as described by DNA amplification in PT40 and previously suggested by Rodrigo et al. Figure 2.5B represents gene upregulation in the novel 3q29 amplicon. Genes located at the postulated junction of this amplicon with the 3q28 region (gene FGF12 to HES1) show no or only low frequency of overexpression, whereas multiple
peaks of increased expression frequencies in 3q29 region are seen for genes (67.5% for CPN2, 47.5%-95% for AFURS1, 35-45% for MUC4, 80-85% for TFRC, 32.5-60% for KIAA0794, 17.5-80% for PAK2 and 72.5-85% for NCBP2). This supports our assumption that 3q29 represents a novel amplicon in HNSCC.
Figure 2.5 Analysis of microarray data on the 11q13.3 and 3q29 regions.

A. 11q13.3 region, covering 3.0 Mbps. B. 3q29 region, covering 6.5 Mbps. X-axis indicates the chromosomal locations, and Y-axis indicates the frequencies of upregulation (over two-fold increase to the median of normal level). Key genes mentioned in Results and Discussion are noted with arrows. The horizontal red line indicates 30%.
2.4 Discussion

In this study we used RLGS on 41 primary head and neck squamous cell carcinoma to detect DNA amplification. Due to the quantitative nature of the radioactive labeling of NotI restriction ends, this assay allows the detection of low-copy number amplification. We identified a total of 33 amplified regions, and confirmed ten of them by quantitative Southern blot analysis. Comparing the amplified regions with published amplification data demonstrated that the majority of amplicons detected by other techniques such as comparative genomic hybridization (CGH) are also identified by RLGS. However, 24 novel amplified sequences were detected that may have been missed in the past. Interestingly some of these novel amplicons (1q41, 7p22, 8p11.23, 8q13.1, 8q22.3, 10q21.3, 10q24.32, 19q13.1, 20p11.23) have been described in other human malignancies. For example, amplification of chromosome 1q41 was described in breast cancers.\(^1\) Copy number gain of 7p22 has been observed in colorectal carcinomas.\(^2\) DNA gain of 8q13 is reported in lung squamous cell carcinoma, breast cancers and esophageal carcinoma.\(^3\) Chromosome 8q22.3 amplification is also associated with transitional cell carcinoma of the bladder\(^4\) and 10q21 and 10q24 gains are detected in cervical carcinomas.\(^5\) Amplification of three consecutive RLGS fragments on
19q13.1 (2D60-2D65-2C59), is seen in 4-7 cases and amplification of this chromosomal segment has been reported in bladder, cervical carcinoma, ovarian and pancreatic tumor cell lines \(^{65-67}\). Finally, amplification of 20p11.23 has been demonstrated also in esophageal squamous cell carcinoma and breast cancers \(^{61,68}\). Altogether this data suggests that common, but yet unknown, oncogenes are activated by DNA amplification in HNSCC.

One of the interesting observations in this study was the identification and reclassification of amplicons. DNA amplification of the 11q13.3 region has been intensively studied in HNSCC and various other types of human malignancies. CCND1 is thought to be the target oncogene in this region, that when overexpressed confers a growth advantage to the tumor cell. Along with CCND1 are three fibroblast growth factor (FGF) family members (FGF-19/-3/-4) as well as EMS1, also called cortactin, an actin-associated scaffolding protein that regulates cell migration \(^{69}\). These five genes are frequently co-amplified in our data set, but there is one case of discontinuous amplicon (PT40) and one case with EMS1 amplification alone (PT16). We demonstrated by RLGS that amplification of CCND1 and EMS1 loci are not necessarily related, suggesting at least two separate amplicons in this short region. The data supports earlier work by Rodrigo’s work \(^{60}\) indicating that
EMS1 and CCND1 amplification could occur separately. Interestingly, the authors found that not CCND1 amplification but EMS1 predicts early recurrence and reduced survival in squamous cell carcinoma in HNSCC and thus the prognostic significance previously attributed to CCND1 amplification may be attributable to its colocalization with EMS1 on the same amplicon. The phenomenon of independent amplification on 11q13.3 has also been observed in the studies in primary breast cancers. Studies in bladder cancers by Bringuier et al. discovered that expression of both CCND1 and EMS1 is highly elevated with amplifications in tumors, but FGF3/4 are not expressed. Bringuier's report is consistent with our microarray data in HNSCC. In conclusion both CCND1 and EMS1 may provide different functions in HNSCC carcinogenesis.

DNA amplification of 3q26-q27 is the most frequently found genetic alteration in HNSCC. This was confirmed by the RLGS and Southern data provided here. However, more interestingly, our data support the hypothesis that 3q29 commonly co-amplified with the 3q26-q27 region can be a separate amplicon. Furthermore, 3q26-q27 was thought to represent a single amplicon. This idea is supported by 17 cases of co-amplification of consecutive RLGS fragments in this region. However, there are four cases in which 3q26.32 is
amplified alone and six cases of 3q27.1 amplification (without 3q26 co-amplification). We suggest that these two regions cannot be regarded as a single amplification event in HNSCC. In summary, there are at least three independent amplicons on 3q26-qter: 3q26.32, 3q27.1 and 3q29. These three amplicons should be studied separately.

There are many implications of the presence of oncogenes on these three proposed amplicons. PI3KCA, the PI-3 kinase catalytic alpha subunit, is adjacent to fragment 3D21 of 3q26.32. Amplification and overexpression of PIK3CA is found not only in HNSCC, but also in cervical, ovarian, breast, and lung squamous cell carcinomas. Our data further suggests that another potentially independent amplicon exists on 3q27.1, marked by RLGS fragments 2F72 to 3E24, the 345 Kbp region including at least sixteen genes are located. Two genes in this region, Disheveled 3 (DVL3) and EIF4G1, are amplified in hypopharyngeal cancers. Many genes ranging from PSARL to POLR2H in 3q27.1 also show frequent co-upregulation of mRNA levels (unpublished data). For the novel 3q29 amplification event, a gene named mucin 4 (MUC4) was first found to be associated with pancreatic cancers. MUC4 is upregulated in 35-45% HNSCC cases in our microarray data. In addition, a cluster of genes including KIAA0794, PAK2, CENP5 and NCBP2
are frequently co-upregulated (also in Figure 2.5B). Future studies will show if MUC4 or other candidate oncogenes play indeed roles in HNSCC carcinogenesis.
CHAPTER 3

YWHAZ/14-3-3ζ IS AMPLIFIED, OVEREXPRESSED AND CAPABLE OF REGULATING CELL GROWTH IN HEAD AND NECK SQUAMOUS CELL CARCINOMA

In preparation, authored by Lin M, Morrison C, Jones S, Mohamed N, Basher J and Plass C

3.1 Introduction

Gain of DNA copy numbers, or DNA amplification, is a common genetic alteration found in many cancers. Subchromosomal DNA amplification results in extra copies of specific regions, potentially containing oncogenes. DNA amplification results in overexpression of the target genes located in the amplified regions, and thus confers a growth advantage. Overexpression of oncogenes results in neoplastic transformation and provides a growth advantage either by providing resistance to apoptotic stimuli or by promoting accelerated cell growth. Amplification and overexpression of
oncogenes is an early event in carcinogenesis and thus can be used as a diagnostic marker for early detection of tumor cells or to predict outcome of a clinical therapy. Furthermore, activated oncogenes provide potential targets for cancer therapies as shown in oesophageal cancer.

In an earlier study we used restriction landmark genomic scanning (RLGS) to locate novel and known amplified chromosomal regions. In these amplified regions, we found oncogenes which are well-connected to HNSCC carcinogenesis, such as PI-3 kinase catalytic α subunit (PIK3CA) on chromosome 3q26.3, c-Myc (MYCC) on 8q24.21 and cyclin D1 (CCND1) on 11q13.3. In addition, other novel and frequent amplicons, such as on chromosome 3q29 and 8q22.3 were identified and confirmed. Each of those alterations were observed in approximately 30-40% HNSCC cases and are thus considered a frequent alteration. The oncogenes in these regions have not been identified so far. Interestingly, RLGS fragment 2C13 on 8q22.3, identified as frequently amplified sequence is located in the 5' exon of gene YWHAZ, a 14-3-3 zeta isoform (a.k.a KCIP-1) which thus became a candidate oncogene.

The 14-3-3 gene family was first discovered in 1967 during classification of cow brain proteins. Currently seven distinct isoforms (β, γ, ε, η, σ, τ and ζ)
encoded by seven distinct genes have been identified in mammalian systems. 14-3-3 family members are 28-33 kDa proteins which can homo- or heterodimerize. Dimerized 14-3-3 molecules bind to specific phosphor-serine/threonine (pS/T)-containing motifs, two of which are RSXpSXP and RXXXpSXP. Many molecules regulating cell signaling (PKC, Raf-1, β-catenin) and apoptosis (Bad, Bax) are known that bind to 14-3-3 proteins, which make them master-regulators of these regulators. The majority of studies of 14-3-3 genes focused on embryonic development, neural functions and brain diseases, such as Alzheimer’s and Creutzfeldt-Jakob diseases (CJD). Evidence for its involvements with cancers comes from studies on 14-3-3σ, which is frequently hypermethylated and down-regulated in various types of cancers although overexpressed in some others, possibly in response to cellular stress. Its cooperation with several regulators to block cell cycle progression makes 14-3-3σ an ideal tumor-suppressor gene.

Previous reports show overexpression of YWHAZ in oral, stomach cancers and lung adenocarcinomas, the latter also shows gene amplification in lung tumor tissues. In this work we demonstrate the overall overrepresentations of YWHAZ in DNA, mRNA and protein levels in HNSCC.
patients, followed by its capabilities to control cell growth when being negatively or positively manipulated. Furthermore we examine its effects on cell cycle and discuss how the activated gene could contribute to tumor progression.

3.2 Materials and Methods

3.2.1 HNSCC patient samples, restriction landmark genomic scanning (RLGS) and analysis

Primary HNSCC derived from the nasal cavity, oral cavity, larynx and pharynx were obtained from the Cooperative Human Tissue Network. Surgery was performed on all patients at The Ohio State University. All normal specimens were harvested from morphologically normal appearing tissue located at least 3 cm from the tumor margin and were used as the normal control tissue for comparisons with the tumor tissues. Histopathological characterization was performed for both normal and primary tumor. Samples were snap frozen in liquid nitrogen and stored at −80°C prior to DNA isolation. The study was performed under a protocol approved by the Institutional Review Board of The Ohio State University. The detailed protocols for following genomic DNA preparation for RLGS are described in our previous
study 76.

3.2.2 RNA extraction, cDNA synthesis and semi-quantitative real-time RT-PCR

Total RNAs of an independent panel from additional 44 pairs of patients’ normal and tumor tissues were extracted according to standard TRIzol protocol of RNA extraction (Invitrogen, Carlsbad, CA). 1-2µg of total RNA after DNase I treatment (Invitrogen) was incubated with primers of 0.5µg oligo-deoxynucleotides (dT) and 2µg random hexamers, and deoxynucleotide triphosphates (dNTPs) (10mM) for 5 min at 65°C in supplied RT-PCR buffer (Invitrogen). 50U of SuperScript II (Invitrogen) was added for 50 min at 42°C, followed by heat inactivation at 70°C for 15 min. Remaining RNA templates were removed by 37°C, 20-min RNase H incubation (Invitrogen). cDNAs are stored in -20°C.

YWHAZ and glycosylphosphatidylinositol (GPI, for internal control) cDNA levels were measured using SYBR Green I (BioRad, Hercules, CA) in a BioRad I-Cycler. Data were acquired in the format of cycle number crossing the software-generated threshold (Ct). The average of differences in Ct between YWHAZ and GPI (ΔCt) from all normal tissues is defined as basal
level (BL). Normalized YWHAZ cDNA levels in tumors were calculated using the formula $1.9^{\Delta Ct_{\text{Tumor}-BL}}$. (1.9 is the amplification fold per cycle determined by calibrating experiments). We defined the normal YWHAZ level as 0.5 to 2 folds of BL, where 34 out of 44 normal tissues fall in. 7 are below and 3 are above the normal range.

### 3.2.3 Construction of HNSCC tissue microarray (TMA)

The tissue microarray is constituted of 33 samples from previously studied samples. Triplicate 1-mm tissue cores from each of 48 formalin-fixed paraffin embedded donor blocks of HNSCC tumors and their normal counterparts were precisely arrayed into a new recipient paraffin block. Specimens for controls consisted of multiple cores of normal tissue from 10 different organs including heart, colon, kidney, adrenal, ovary, myometrium, brain, thyroid, lung, and prostate.

### 3.2.4 Fluorescent *in situ* hybridization (FISH) and immunohistochemical staining

FISH for YWHAZ was done using the BAC clone RP11-302J23 (chr8: 102,004,801-102,195,725, UCSC Genome Bioinformatics, Mar 2006 release)
from centromere to telomere. FISH slides were prepared and scanned as previously described. A ratio of the total number of YWHAZ signals to the total number of CEP8 signals in at least 60 interphase nuclei with not overlapping nuclei in the tumor cells was determined. Cells with no signals or with signals of only one color were disregarded. Tumor cells displaying at least two centromeric chromosome 8 signals and multiple YWHAZ signals, with an YWHAZ:CEP8 ratio of ≥2, were considered consistent with amplification of the YWHAZ gene. Tumor cells displaying at least two centromeric chromosome 8 signals and an equal number of YWHAZ signals, with an YWHAZ:CEP8 ratio of less than 2, were considered consistent with no amplification of the YWHAZ gene. Tumor cells displaying multiple CEP8 signals and an approximately equal number of YWHAZ signals, with a somewhat random distribution of both, were considered polysomic chromosome 8.

Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded TMA sections. Paraffin embedded tissue was cut at 4 microns and placed on positively charged slides. Slides with specimens were then placed in a 60°C oven for 1 hour, cooled, and deparaffinized and rehydrated through xylenes and graded ethanol solutions to water. All slides were quenched for 5 minutes in a 3% hydrogen peroxide solution in water to block for endogenous
peroxidase. Slides were antigen retrieved in a solution of citrate buffer in a vegetable steamer. Slides were then placed on a Dako Autostainer, immunostaining system, for use with immunohistochemistry. Primary rabbit polyclonal anti-YWHAZ antibody clone C-16 (Santa Cruz Biotechnology, Santa Cruz, CA) was diluted 1:1500 and incubated for 60 minutes. Detection system used was a labeled polymer system, Envision Plus Dual Link (Dako code number K4061). Staining was visualized with DAB chromogen (Dako code number K3468). Slides were then counterstained in Richard Allen hematoxylin, dehydrated through graded ethanol solutions and coverslipped.

3.2.5 Cell lines and culture

Four HNSCC cell lines (UMSCC-8, -9, -11B and -22B, gifts from Dr. Thomas Carey, University of Michigan) and one immortalized human skin keratinocyte (HaCaT, a gift from Dr. Andrzej Slominski, University of Tennessee Health Science Center) are maintained and grown in Dulbecco’s Modified Eagle’s Medium (DMEM) with 10% Fetal Bovine Serum (FBS) and 1% streptomycin/penicillin (S/P) antibiotics.
3.2.6 RNAi transfection

We acquired the YWHAZ RNAi sequence information from the dissertation of Dr. Christoph Neal. Allstar negative control (GGG-UAU-CGA-CGA-UUA-CA-AUU-dTdT) and YWHAZ (AGU-UCU-UGA-UCC-CCA-AUG-C-dTdT) RNAi were ordered through Qiagen (Valencia, CA). Lipofectamine 2000 transfection reagent (Invitrogen) was mixed with RNAi in the 1:1 ratio (v/v) at room temperature for 20 minutes. Cells were incubated with serum-free DMEM of 60nM negative control or YWHAZ RNAi for 6 hours, then replaced with regular 10% FBS DMEM.

3.2.7 Western blotting

Cells from 100-mm dishes were trypsinized, harvested, and lysed in lysis buffer containing 0.1% NP-40 and phosphatase inhibitor cocktails. 10µg of cell lysates were electrophoresed in 12% polyacrylamide gel, then transferred onto Amersham Hybond ECL nitrocellulose membrane (GE Healthcare Bio-Sciences, Piscataway, NJ). For standard Western protocol, the membrane was blocked with 3% non-fat milk at room temperature for 2 hours. After one wash with TBS wash buffer (0.1% Tween-20), adequate primary antibodies were applied and incubated for 1 hour, following by two 15-min washes.
Peroxidase-conjugated secondary antibodies were incubated with the membrane for 1 hour. Another two washes later we used Supersignal (Pierce Biotechnology, Rockford, IL) to detect signals developed on Blue Autoradiography Film (ISC BioExpress, Kaysville, UT). Antibody concentrations: rabbit anti-YWHAZ (Santa Cruz Biotechnology), 1:1000; mouse anti-α-tubulin (Calbiochem, San Diego, CA), 1:1000; mouse anti-HA tag clone 6E2 (Cell Signaling Technology, Danvers, MA), 1:1000; goat anti-rabbit & anti-mouse, HRP-conjugated (Biorad), 1:5000.

3.2.8 Cell growth curve assay

Twenty thousand HNSCC or HaCaT cells were seeded onto 6-well plates or 60-mm dishes depending on the proliferating potential of individual cell lines. After treatment, cells were trypsinized and counted by cell counter (Beckman Coulter, Fullerton, CA) up to 5 days. Each experiment was done in duplicate set and at least twice.

3.2.9 Establishment of YWHAZ-overexpressing HaCaT strains

Plasmid pCDNA3-YWHAZ-HA is a kind gift from Dr. Joan E.B. Fox, Case Western Reserve University (originally designed by Dr. Charles Adams,
University of Pennsylvania). YWHAZ-HA fragment was excised by BamHI and EcoRI, and then cloned into pBABE retroviral vector (Dr. Gokhan Hotamisligil, Harvard University). pBABE-YWHAZ-HA plasmid was transfected into 40-50% confluent Phoenix packaging cells with Superfect transfection reagent (Qiagen) for 4 hours, then replaced with 10% FBS DMEM. After 48-hour incubation, the supernatant was collected and placed on 40-50% confluent HaCaT cells in the presence of 8µg/ml polybrene for 48 hours. Transfected cells were selected with 2µg/ml puromycin.

3.2.10 Flow cytometry and BrdU incorporation

Flow cytometry is used to determine DNA content of cells in exponential phase (propidium iodine, PI) and the rate of DNA synthesis by BrdU (5’-bromo-2’-deoxyuridine) incorporation assay. Ethanol-fixed cells are treated with 200µg/ml RNase A at 37°C and stained with 20µg/ml PI. As for BrdU incorporation assay, we follow the manufacturer’s FITC-BrdU Cell Proliferation Kit manual (BD Bioscience Pharmingen, San Diego CA). Cells are incubated with 10µM BrdU for 30 minutes, harvested and fixed. 30µg/ml DNase I is used to digest genomic DNA for mouse FITC-conjugated BrdU antibody. PI- or FITC- stained cells are analyzed with Beckman-Coulter flow cytometer and
3.3 Results

3.3.1 RLGS and FISH analysis indicates gains on YWHAZ copies

Our previous analysis identified that RLGS fragment 2C13 is enhanced in 14 out of 41 (~35%) tumor tissues compared to their normal counterparts. Examples of the enhanced RLGS fragments is given in Figure 3.1. A more detailed analysis on chromosome 8q can be found in our earlier work. RLGS fragment 3D05, the neighboring fragment to 2C13, also displays a similar frequency of enhancement and is often co-amplified with 2C13. Both fragments represent the 5’ proximity of gene YWHAZ, a tyrosine 3 / tryptophan 5-monoxygenase. Southern blotting confirmed low-level amplification of 2C13. Here, we used FISH analysis to examine the status of amplification in histopathological level (Table 3). Out of 48 patients, 33 have polysomy 8. 13 have low-level (YWHAZ/CEP8 = 2~4) and 3 have intermediate-levels of amplification (YWHAZ/CEP8 = 4~10). High-level amplification (ratio >10), as for examples for the BRCA1 amplicon seen in some breast cancer patients, was not found. For comparison, the C-MYC locus on 8q24.21 shows low-level amplification in 10 patients, intermediate-
level in 5 and extremely high-level (MYCC/CEP8 > 20) in only 1 (data not shown). Overall, 33% (16/48) of the tumor samples bear extra YWHAZ gene copies, very comparable to the percentage determined by RLGS analysis. Four amplification events were detected together by RLGS and FISH. Combination of RLGS and FISH analyses compensate limitations by each assay and demonstrate that amplification of YWHAZ locus accounts for 30-40% HNSCC cases.
Figure 3.1 YWHAZ gene copy gain in HNSCC.

RLGS analysis demonstrates 2C13 enhancement in four HNSCC tumor samples. Arrows indicate fragment 2C13.
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Table 3.1 Summary of FISH analysis for YWHAZ gene copy gain in HNSCC
Polysomy 8 is highlighted in yellow color in Polysomy 8 column. Plus and minus signs in RLGS 2C13 column refer to whether 2C13 is enhanced in tumor samples. NA, not analyzable. Yellow color in YWHAZ/CEP column as low level of amplification and orange as intermediate level.
3.3.2 YWHAZ mRNA and protein are up-regulated in HNSCC patient samples

We used a second independent panel of HNSCC tissue samples to verify elevated YWHAZ mRNA levels by semiquantitive RT-PCR (Figure 3.2). YWHAZ cDNAs in 23 out of 44 tumor tissues are up-regulated (at least 2 folds over basal level) comparing to only 3 out of 41 normal tissues (p-value = 0.0024). Further categorization by tumor locations shows that YWHAZ mRNA levels are more than 2-fold higher in 14 out of 17 tongue, 6 out of 8 oral cavity tumors, but only 3 out of 10 pharyngeal and none out of 9 laryngeal tumors. To evaluate YWHAZ protein levels, the same TMA set used in FISH analysis is stained with anti-YWHAZ antibody (Figure 3.2B). YWHAZ protein staining is ubiquitous and stronger in tumors than in normal tissues. Some hyperplastic cells in normal tissues are also positive for YWHAZ. Interestingly, in tumor sample PT91, whose YWHAZ amplification was confirmed by RLGS, there is no difference in YWHAZ staining between normal and tumor samples. We suspect that the YWHAZ gene in PT91 tumor might be mutated on the epitope for our antibody not to recognize.
Figure 3.2 YWHAZ mRNA and protein overexpression in HNSCC (continued).

A. mRNA levels in HNSCC patients’ normal and tumor samples. Red line indicates two folds and green line indicates 0.5 fold. YWHAZ mRNA levels of 34 out of 41 normal samples and 16 out of 44 tumors fall between 0.5 to 2 folds.

B. Immunohistochemistry of YWHAZ protein in four pairs of normal and tumor samples. YWHAZ protein is stained brown. Magnification is 100X.
Figure 3.2 (continued)

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3.3.3 Down- and up-regulation of YWHAZ greatly affect cell growth

The effects by temporary knockdown of YWHAZ by RNAi are examined in three HNSCC (SCC-8, -11B and -22B) and HaCaT cell lines (Figure 3.3A). YWHAZ RNAi successfully reduces protein expression in all four tested cell lines in various degrees. Our RNAi sequence of choice reduced YWHAZ levels greatly in SCC-8 and SCC-22B, but mildly on SCC-11B and HaCaT, approximately to 0.5 fold compared to their original levels in those cell lines. In SCC-22B line, the YWHAZ RNAi is able to inhibit protein level for at least 4 days, more than the time needed for short-term experiments (Figure 3.3B). In 5-day interval, YWHAZ RNAi greatly suppresses cell population in all three tested cell lines, even in SCC-11B and HaCaT which still have 0.5 fold of original YWHAZ levels (Figure 3.3C).

In the study by Neal et al.\textsuperscript{103}, YWHAZ protein has been overexpressed in MCF-7 breast cancer line, and there was no effect on cell or tumor progression \textit{in vitro} or \textit{in vivo} experiments. We suspect that this non-effectiveness might be partially explained by the malignant nature of cancer cells and decided to use an immortalized and non-cancerous keratinocyte line HaCaT instead of our HNSCC lines. Although HaCaT, along with all tested HNSCC cell lines, already have abundant endogenous YWHAZ protein, our
YWHAZ-transfected HaCaT cells expressed twice the amount of vector-transfected control (Figure 3.4A). HaCaT cells with nearly doubled YWHAZ level attain a population density nearly twice that of vector control after 5 days (Figure 3.4B). There was no morphological difference between normal and YWHAZ-reduced HaCaT. However, after approximately 10 passages following the initial experiment, we noticed that some YWHAZ-overexpressing cells undergo morphological changes, from flatter normal HaCaT morphology to spindel-shaped cells (Figure 3.4C, bottom row). These cells are also highly clustered, possibly due to rapid proliferation. They are more easily trypsinized during passages than normal HaCaT cells. These results demonstrate that inhibition and overexpression of YWHAZ have immediate and profound impacts on cell growth. It is still unclear whether YWHAZ can induce morphological changes at this point and what this change means.
Figure 3.3 YWHAZ RNAi inhibits cell population.

A. Western blotting of YWHAZ protein after 3-day treatment of scrambled (-) or YWHAZ (+) RNAi (both 60nM). α-tubulin serves as the internal control. YWHAZ RNAi reduces target protein levels in all four cell lines.

B. Time course of the inhibitory effect of YWHAZ RNAi in SCC-22B cell line. YWHAZ RNAi suppresses protein levels for at least four days.

C. Growth curve of three cell lines under YWHAZ RNAi. YWHAZ RNAi suppresses cell growth in all cell lines (starting 20K cells, 5-days interval).
Figure 3.3 (continued)
Figure 3.3 (continued)

**SCC22B growth**

![Graph showing cell number (10^5) over days for SCC22B growth with different conditions: No treatment, Scrambled RNAi, YWHAZ RNAi.]

**HaCaT growth**

![Graph showing cell number (10^5) over days for HaCaT growth with different conditions: No treatment, Scrambled RNAi, YWHAZ RNAi.]

Figure 3.4 YWHAZ overexpression in human keratinocyte line causes cell overgrowth and morphological changes.

A. Western blotting confirms HA-tagged YWHAZ ectopic overexpression in stable-transfected HaCaT cell line in spite of a high endogenous YWHAZ level.

B. YWHAZ-HA-transfected HaCaT line shows more rapid growth than negative vector control line in regular 10% FBS DMEM.

C. HaCaT cell growth and morphology. Top row, left to right: HaCaT cells with vector only (passage 10), with YWHAZ-HA (passage 10), and with YWHAZ RNAi. Bottom row, left to right: HaCaT cells with vector only (passage 20) and with YWHAZ (passage 20).
Figure 3.4 (continued)
Figure 3.4 (continued)
3.3.4 YWHAZ inhibition affects cell cycle and DNA synthesis

Based on results from the inhibition and overexpression experiments, we want to determine whether YWHAZ regulates cell population by accelerating cell cycle progression or blocking apoptosis. Flow cytometry is used to address this question. For DNA content index, cells were treated with 60 nM negative control RNAi or YWHAZ RNAi for 3 days before being fixed and stained by PI. H1299 lung adenocarcinoma cell line is used as a comparison as used in the study by Neal et al. Figure 3.5A shows that YWHAZ RNAi reduces the proportion of S phase and increases that of G1/G0 phase in both HaCaT and H1299 cell lines. H1299 shows more obvious changes than HaCaT. Besides, no change in sub-G1 (apoptotic body) or G2/M proportion is detected after YWHAZ RNAi treatment. We also used BrdU incorporation assay to measure the proportion of cells in S-phase for four cell lines (Figure 3.5B). Among four tested cell lines, YWHAZ RNAi strongly reduces the proportion of S-phase cells in SCC-11B and H1299 cells, and mildly in SCC-22B and HaCaT cells. These findings support that YWHAZ regulates cell population by accelerating cell cycle progression instead of blocking apoptosis.
Figure 3.5 YWHAZ RNAi affects cell cycle and DNA synthesis.

A. DNA content in H1299 and HaCaT cells treated with negative control RNAi or YWHAZ RNAi. The left peak represents the G0/G1 phase and the right peak represents the G2/M phase. S phase is between these two peaks. Percentage of each phase is calculated by ModFit LT software.

B. BrdU incorporation assay in SCC-11B, SCC-22B, H1299 and HaCaT cells. The x-axis is the intensity of BrdU signal and the y-axis is the number of cells. Red line indicates treatment by negative scrambled RNAi; green line indicates treatment by YWHAZ RNAi. The number represents the percentage of the BrdU-positive (S-phase) cells in each population.
3.4 Discussion

In this study we showed that chromosome 8q22.3 gain is seen in 30-40% of HNSCC patients. 30-40% is a significant number in HNSCC, for that 3 other major amplicons (3q26.3, 8q24.21 and 11q13.3) all have similar incidences of amplification with three major proven oncogenes (PIK3CA, MYCC and CCND1) located in them, respectively. It is logical to assume that frequent amplification of a specific gene suggests its importance in carcinogenesis since the cells are inclined to keep it during natural cycles of proliferation and death. It also implies that YWHAZ might be an initiator in the early stage of carcinogenesis instead of merely a downstream effector, which is unlikely to be selectively amplified and kept in such a high rate. We have seen the oncogenic potentials of PIK3CA, MYCC and CCND1 in cancers including HNSCC, and our studies suggest that YWHAZ should be an additional major gene to study in HNSCC carcinogenesis.

One of the important characteristics for YWHAZ is the ability to promote cell growth when overexpressed. However, overexpression of some proven oncogenes more likely leads cells to senescence rather than outgrowth. Hyperactive Ras form (RasV12) and c-Myc-induced senescence in human or mouse fibroblasts is well documented and a powerful tool to study other
Mounting evidence shows that whenever an oncogene is overrepresented, many cell cycle inhibitors will be activated and block further cell cycle progression, which is regarded as the intrinsic defense mechanism. Cells remain arrested until a second defect such as p53 mutation switches off these defenses, then transform. Overexpressing YWHAZ is capable of transforming immortalized mouse fibroblasts NIH3T3 when co-transfected with RasV12. It is probable that HaCaT may have provided needed components to allow YWHAZ to promote growth.

Despite our evidence, we are not sure if YWHAZ is sufficient to transform normal head and neck squamous cells by itself alone. The major concern comes from its high-level expression in all cell lines we used, including non-cancerous HaCaT cells. Since all cell lines are immortal, YWHAZ may have something to do with the crucial immortalization step in carcinogenesis. Also, because original HaCaT line with abundant YWHAZ is non-cancerous, there is a possibility that our YWHAZ-transfected HaCaT strain may not form tumors in xenografted mice either. Still, we think it is worth studying in near future. Another approach will be the introduction of YWHAZ into oncogene-induced senescent cells. Further investigation will be more complete with this system.
Other than YWHAZ, there are other genes which are amplified and overexpressed on the proximity of 8q22.3 in HNSCC. In HNSCC, gene EDD, the human orthologue of the hyperplastic discs tumor suppressor gene, is amplified and overexpressed in multiple cancers \(^{58}\). EDD is only \(~1.3\) Mb away from YWHAZ location. Another amplified and overexpressed gene LRP12 is \(~3.5\) Mb away \(^{39}\). BAALC (brain and acute leukemia, cytoplasmic, \(~2.2\) Mb away) is found to be associated with acute myeloid leukemia (AML). One or some of them may be oncogenes and co-amplified with YWHAZ.

There are many cases supporting that multiple oncogenes are found in a concentrated region, such as 11q13.3 mentioned earlier. Probably co-amplifications of a group of oncogenes nearby have synergistic effects on carcinogenesis.

The growth-promoting activity of YWHAZ has been briefly demonstrated, but one question about its functions, which is left unaddressed in this study, is its effects on apoptosis. Although it has not been performed yet, we are tempted to believe that YWHAZ is anti-apoptotic. High-level of YWHAZ is known to protect cells from insults such as radiation and anti-tumor agents \(^{110,111}\). YWHAZ binds to Bad and Bax, two of the pro-apoptotic BCL2 family members. Based on our growth experiments, we speculate that cells with
reduced YWHAZ level proliferate less not due to inducing apoptosis because the sub-G1 proportion is neglectable. We can not exclude the possibility that apoptosis is somehow involved in other cancer cell lines, but for YWHAZ to regulate cell growth, functional apoptotic machinery may not be required.

Since YWHAZ is a 14-3-3 protein which binds to many phosphorylated targets, it is crucial to look at what targets it binds for unlocking the mechanisms of promoting growth. 14-3-3σ, its sibling, is known to inhibit cell cycle. It is possible that 14-3-3ζ and σ heterodimerize and modulate each other’s oncogenic and anti-tumor abilities. Other than 14-3-3 members, YWHAZ can bind to key components on PI-3K, MAPK and Wnt pathways. Physical contact does not necessary lead to activation of a pathway, so we need to clarify which pathway YWHAZ really exerts on to control cell growth. Our initial plan is to mutate known binding sites to disrupt binding with specific molecules. By eliminating non-factors, the real factors may be revealed.

The last thought comes from an unpublished study mentioned in a review. The group produced transgenic mice with extra YWHAZ gene copies, and observed that ~15% of the mice developed various types of tumors by the age of 7-15 months. More interestingly, the level of expression of YWHAZ in transgenic mice is only 30% greater than that in normal ones. This
observation corroborates very well with our observations: (1) YWHAZ copy number gain is only 2-4 fold in HNSCC patients; (2) knocking out merely a half of YWHAZ production in HaCaT is sufficient to suppress its growth; (3) merely doubling YWHAZ level can promote HaCaT growth and in a long term, induce mysterious morphological changes. In conclusions, we are confident to say that YWHAZ has the potential to be one of the next major oncogenes worthy of further investigations.
CHAPTER 4

SEARCH FOR ONCOGENE CANDIDATES ON THE NOVEL 3q29 AMPLICON IN HEAD AND NECK SQUAMOUS CELL CARCINOMA

4.1 Introduction

Cancer is a disease of genetics. Cancer cell transformation arises from random and accumulating changes in the genome, which can be an alteration to the sequence (mutation), gain or loss of DNA copy number/information (amplification or deletion), or on several modified nucleotides and/or histones (epigenetics). Oncologists repeatedly observe specific alterations in specific types of cancers, implying that these changes may contribute to the initiation of transformation of a particular tumor type. Categorization and cataloguing of the information has helped in the past in the discoveries of oncogenes and tumor-suppressor genes.

Chromosome 3 is one of the most altered chromosomal regions affected in the carcinogenesis of head and neck squamous cell carcinoma (HNSCC). General descriptions are loss of chromosome 3p and gain of 3q. Proposed
tumor suppressors and oncogenes are dispersed throughout the entire chromosome 3 and include FHIT (3p14.2) and RASSF1A (3p21.31), both tumor suppressor genes, are frequently lost or epigenetically inactivated in HNSCC \(^{113-115}\). Confirmed oncogenes include PIK3CA (3q26.32) and p63 (3q28), both of which are frequently amplified and overexpressed oncogenes \(^{116,117}\).

Due to the fact that chromosome 3 bears 22 out of our 63 RLGS fragments in this region \(^{76}\), we were able to get better resolution on the pattern of DNA amplification. We also demonstrate the existence of multiple amplicons from 3q26 to 3qter. Among these amplicons, 3q29 is the least-studied region and no unanimous oncogene candidate has been proposed. We decide to further investigate the possibility of novel oncogenes within 3q29.

4.2 Methods and Materials

4.2.1 RNA extraction, cDNA synthesis and semi-quantitative real-time RT-PCR

Please refer to chapter 3.2.2.
4.2.2 Construction of HNSCC tissue microarray (TMA)

Please refer to chapter 3.2.3.

4.2.3 Fluorescent *in situ* hybridization (FISH) and immunohistochemical staining

FISH for transferrin receptor (TfR) was done using the BAC clone RP11-480A16 (chr3: 197,126,186-197,313,823, UCSC Genome Bioinformatics, Mar 2006 draft). BAC clone RP11-125E8 (chr 3: 185,459,118-185,625,821) was used for probing the EIF4G1 gene on the 3q27.1 amplicon for comparison. For immunohistochemistry, we use a 1:3000 diluted primary mouse monoclonal anti-TfR antibody clone H68.4 (Zymed, San Francisco, CA). For additional methodological details, please refer to chapter 3.2.4.

4.2.4 Cell lines and culture

Please refer to chapter 3.2.5

4.2.5 RNAi transfection

We acquired the TfR RNAi sequence information from Chen et al. 118. Allstar negative control (GGG-UAU-CGA-CGA-UAU-CAA-AUU-dTdT) and TfR
(GGU-UGC-AAA-UGC-UGA-AAG-C-dTdT) RNAi were ordered through Qiagen (Valencia, CA). For the details of transfection, please refer to chapter 3.2.6.

4.2.6 Western blotting

1:1000 diluted mouse anti-TfR antibody (Cell Signaling Technology) is used to detect TfR. 1:1000 diluted mouse anti-α-tubulin antibody (Calbiochem, San Diego, CA) is used as internal control. For rest of details, please refer to chapter 3.2.7.

4.2.7 Cell growth curve assay

Please refer to chapter 3.2.8.

4.3 Results

4.3.1 Screening for novel candidates oncogenes on chromosome 3q29 through mRNA expression profile

Approximately 30-40% HNSCC tumors bear enhancement on RLGS fragments 2C38 and 3F38 on chromosome 3q29 (Table 4). The closest known genes to 2C38 and 3F38 are centaurin beta 2 (CENTB2) and melanoma-
associated antigen p97 (MFI2). We further examined which genes on 3q29 are up-regulated along with their gene amplifications. Based on Dr. Kornacker’s analysis of mRNA expression profiles (see chapter 2.3.6 and Figure 2.5B), we prioritized six frequently-overexpressed genes out of 29 known candidate genes. These genes included CPN2, ATP13A3, MUC4, TFRC, PAK2 and NCBP2, listed in their order from centromere to telomere respectively (highlighted rows in Table 4.1). Interestingly, CENTB2 and MFI2 are not among them despite of their proximity to our two amplified RLGS fragments, demonstrating that gene amplification does not necessarily result in more mRNA transcription. Semi-quantitative RT-PCR was performed to verify overexpression of these six genes (Figure 4.1). So far we positively confirmed overexpression of 3 genes, including ATP13A3 (p-value=0.0147), TFRC (p-value=0.0003) and PAK2 (p-value=0.0039).

ATP13A3 (or AFURS1), is a transmembrane cation ATPase type 13A3. It is found upregulated in senescent human parenchymal kidney cells and to our knowledge has no reported association with any cancer types. PAK2, a p21-activated kinase, functions as a downstream target of TGF-β pathway. PAK2, a p21-activated kinase, functions as a downstream target of TGF-β pathway. PAK1 on 11q14.1, a family member of PAK2, is closely associated with breast and hepatocellular carcinoma. The potential role of PAK2 in
carcinogenesis is still unclear. On the other hand, transferrin receptor (TfR, also CD71) is overexpressed in HNSCC and many types of cancers \textsuperscript{124,125}, and its role in cancers remains largely unknown. For subsequent studies, we decide to focus on the transferrin receptor gene.
Table 4.1 Summary of identified genes in 3q29

Genes which are overexpressed in more than 30% HNSCC patients are highlighted.

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Figure 4.1 mRNA levels of PAK2, ATP13A3 and TfR in HNSCC patients’ normal and tumor samples.

mRNA levels in HNSCC patients’ normal and tumor samples. Red line indicates two-fold and green line indicates 0.5-fold differences.
4.3.2 TfR copy number gain and elevated protein levels in HNSCC patients

Similar to what has been done in YWHAZ study (chapter 3), we also performed FISH to detect TfR gene copy numbers with the positive EIF4G1 amplicon on 3q27.1 as a comparison (Table 4.2). EIF4G1 copy number gains were detected by FISH in nearly 50% of the HNSCC samples and largely corroborate with RLGS data of fragment 3E46. TfR copy number gains, however, were only found in 7 out of 49 (14%) tumor samples despite of 30-40% on two RLGS fragments. Our RLGS analysis does not correlate well with the FISH analysis. The discrepancy may be due to either (1) the fact that the TfR copy number gain is underestimated due to technical limitation of FISH, or (2) TfR copy number gain is indeed not as prevalent as we proposed from RLGS analysis, or (3) the circumstance that the copy number is indeed increased however the NotI restriction site is methylated and thus the increase is not detected by RLGS. Unlike the YWHAZ gene, which has RLGS fragment 2C13 inside it, fragment 2C38 is ~0.6Mb upstream of TfR gene and 3F38 is ~0.9Mb downstream of it. Thus, RLGS results are indirect markers compared with direct FISH marker on amplification status of TfR gene.

TfR overexpression is very prevalent in HNSCC tumor samples to normal
samples (Figure 4.2). Only 3 out of 48 tested tumor samples failed to be stained with anti-TfR antibody. Not limited to plasma membrane, the TfR staining is detectable all over the cell structure, demonstrating the dysregulated overexpression.
Table 4.2 Summary of FISH analysis for TfR gene copy gain in HNSCC

Polysomy 3 is highlighted in yellow color in Polysomy 3 column. Plus and minus signs in RLGS columns refer to whether the fragment is enhanced in tumor samples. NA, unanalyzable. Yellow color in TfR/CEP3 column as low level of amplification and orange as intermediate level.
Figure 4.2 TfR overexpression in HNSCC samples.

Immunohistochemistry of TfR protein in four pairs of normal and tumor samples. TfR protein is stained brown. Magnification is 400X.
4.3.3 Reduction of transferin receptor protein mildly inhibits cell growth

The effects by temporary knockdown of TfR by RNAi are examined in 3 HNSCC (SCC-8, -11B and -22B) and HaCaT lines (Figure 4.3A). TfR RNAi successfully reduces protein expression in all four tested lines. TfR levels in all cell lines are greatly reduced by our sequence of choice. In SCC-22B line, the TfR RNAi is able to inhibit protein levels for at least 4 days, more than the time needed for short-term experiments (Figure 4.3B). In 5-day interval, TfR RNAi indeed suppresses cell growth in all 3 tested cell lines, but only mildly compared with the results in YWHAZ studies (Figure 3.3C). We also attempt to establish a TfR-overexpressing HaCaT cell line similar to the experiments done in the YWHAZ study, but unable to achieve TfR overexpression so far (data not shown).
Figure 4.3 TfR down-regulation cause mild growth inhibition.

A. Western blotting of TfR protein after 3-day treatment of scrambled (-) or TfR (+) RNAi (both 60nM). α-tubulin serves as the internal control. TfR RNAi reduces target protein levels in all four cell lines.

B. Time course of the inhibitory effect of TfR RNAi in SCC-22B cell line. TfR RNAi suppresses protein levels for at least four days.

C. Growth curve of three cell lines under TfR RNAi. TfR RNAi mildly suppresses cell growth in all cell lines (starting 20K cells, 5-days interval).
Figure 4.3 (continued)
Figure 4.3 (continued)

SCC22B growth

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<td>TIR RNAi</td>
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HaCaT growth

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<td>Scrambled RNAi</td>
<td>2</td>
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<tr>
<td>TIR RNAi</td>
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</table>
4.4 Discussion

In this study, we investigated possible oncogene(s) in the chromosome 3q29 I amplicon. Three genes, ATP13A3, PAK2 and TfR were prioritized. Because of a better knowledge about TfR functions as provided in the literature, this gene was chosen for further studies. Copy number gain of TfR is detected in some cases by FISH, although not as frequent as we expected from the RLGS results. Despite of TfR prevalent overexpression in HNSCC tumors, suppression by TfR RNAi only mildly inhibits cell growth. Thus, we remain conservative about the TfR oncogenic abilities in HNSCC carcinogenesis and its potential as a target for cancer therapy.

The applications of transferrin receptor in cancer diagnosis and treatment has been intensively studied. Transferrin receptor has been used as a prognosis marker in many cancers \(^\text{125-127}\). Growth of several tumor cell lines can be inhibited by adding anti-transferrin receptor antibody \(^\text{128-130}\). Introduction of human TfR gene into a rat mammary adenocarcina line can increase its aggressiveness \(^\text{131}\). It also has been used as an entry for several anti-tumor agents \(^\text{132-134}\). To add originality in the following study, we decide to explore the possible mechanism for TfR to induce carcinogenesis, if there is any.
Transferrin receptor is the surface receptor for transferrin, which binds to iron ions (ferrin) in blood. Iron ions along with transferrin are engulfed, processed and stored for future usage in the cytoplasm. Iron is an important prosthetic group for many biomolecules including heme group in hemoglobin. Deficiency of body iron is known to cause anemia, and overload of iron is highly involved with the initiation of colorectal and liver cancers. Patients with hereditary hemochromatosis (HH), a genetic iron-overload disorder, are at a higher risk of developing liver cancers and other malignancies.

Three hypotheses have been proposed to explain how iron overload may be associated with multiple cancers. The first hypothesis suggests that iron is a nutrition source, which is required by ribonucleotide reductase (RNR), converting NDPs (except for TDP) into deoxy-NDPs for DNA synthesis. Without iron cells can not proceed from G1 to S phase. The second hypothesis, the most popular among three, suggests that excess irons are metabolized into reactive oxidative species (ROS) including superoxide ($O_2^-$) and hydroxyl radical ($OH^-$), damage DNA, proteins and cellular organelles, and increase the risk of DNA double-strand break and mutation. The last one proposes that excess iron may activate transcription of some oncogenes. These hypotheses seem logically plausible, but there is no report proving
overexpression of transferrin receptor can result in iron overload inside cells.

Besides the transferrin receptor, ATP13A3 and PAK2 overexpression are also confirmed in this study. These genes are described in chapter 4.3.1. CPN2, MUC4 and NCBP2 have not been looked at yet due to limitation of time and resources. Knowledge about CPN2 and NCBP2 is very limited, and their primary physical functions (Table 4.1) do not seem related to carcinogenesis. MUC4, a heavily-glycosylated transmembrane protein, is a prognosis marker in pancreatic and lung cancers\textsuperscript{139,140}. It is a ligand for Erbb2 protein, another proven oncogene\textsuperscript{141,142}. Inhibition of MUC4 can impede pancreatic tumor cell growth and metastasis\textsuperscript{143,144}. However, it is down-regulated in prostate cancer\textsuperscript{145}. Because of the conflicting reports possibly due to cell types, we can not assume the role of MUC4 in HNSCC before more studies are conducted.
CHAPTER 5

CONCLUSIONS

5.1 Significance of study

In this study, we have accomplished the identification of several novel amplicons in head and neck squamous cell carcinoma by RLGS. Two amplicons on 8q22.3 and 3q29 top our list because of their prevalent amplification in HNSCC. Follow-up studies demonstrated their gene amplification and overexpression on the mRNA and protein levels in HNSCC patients. Their potential as oncogenes were demonstrated in HNSCC lines. The results show that YWHAZ, on 8q22.3, has great potential as an oncogene, while more work needs to be done regarding 3q29 region.

By definition, oncogenes are those that can initiate or facilitate the formation of tumors. The search for oncogene candidates can be through identifying gene amplification, mRNA and protein overexpression. We believe that our approach of locating genomic amplification is a step closer to identifying oncogenes, in contrast to the approach of utilizing data available
through mRNA or protein expression profiles generated from microarrays. This is in part because thousands of genes are activated or elevated in expression after cell transformation occurs, but most of them do not possess oncogenic activities. It must be beneficial for cells to retain and pass on a specific chromosomal region. Those genes, which do not contribute to cell survival or growth, are unlikely to be kept and thrive for generations. Thus, our approach leaves us a smaller pool of genes to focus our study on, but we recognize that there are other mechanisms that result in the same increased oncogene expression.

We also take higher standards to verify oncogenes in an *in vitro* system. Theoretically, decreasing the level of oncogenes is expected to inhibit growth or survival, and it is a common procedure for verifying oncogenes. However, decreasing the level of a vital, but non-oncogenic gene, can have the same effect of inhibiting growth or survival. Therefore, we decided to overexpress YWHAZ as a must-have experiment. We believe that a complete demonstration of oncogenes or tumor-suppressor genes requires both knock-out and knock-in approaches.
5.2 Future directions

The mechanism of YWHAZ oncogenic activities are still unclear. Although YWHAZ binds to key molecules in several pathways regulating growth and apoptosis, we do not know if without these pathways the oncogenic activities may be neutralized or not. A mutation analysis of YWHAZ may help to clarify the essential parts in its oncogenic potential. On the other hand, study about the regulation of YWHAZ is nearly none, if there is any. For a new oncogene with great abilities, the need for more studies is imminent.

So far, we are unable to provide a definite answer whether the transferrin receptor is an oncogene, unlike the YWHAZ study. Since the transferrin receptor manages endocytosis of iron, we suspect its potential oncogenic activities may be through managing iron metabolism. Numerous reports pointed out the associations between iron and cancer. Hereditary hemochromatosis (HH), a liver cancer-prone disorder, is caused by the mutations on gene HFE, which induces excess iron in body organs 146. Studies have shown that heavy ions including iron can cause genomic instability and transformation of human epithelial cells 147,148 and iron chelators can inhibit DNA double-strand breaks 149. Thus, the theme of iron overload causing cancers is not too far of a stretch for the transferrin receptor. One of
the missing links is that there is yet no evidence that overexpression of the transferrin receptor can increase cellular iron levels. Proving or disproving this link should be the next topic to do.

One of the legacies of this research is that we leave many other frequent amplicons unstudied. Gains of 1q41, 3p21.31, 3q21.3, 7p22.3 and 20p11.23 are detected in 20-30% HNSCC patients by our analysis. These regions were previously uncharacterized in oncology. This provides other researchers targets to look at. We expect new oncogenes sprouting out of these amplicons in the not-so-far future.
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