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Copy number gain and oncogenic activity of *YWHAZ*/14-3-3ζ in head and neck squamous cell carcinoma

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Abstract

Gene amplification, a common mechanism for oncogene activation in cancers, has been used in the discovery of novel oncogenes. Low level copy number gains are frequently observed in head and neck squamous cell carcinomas (HNSCCs) where numerous amplification events and potential oncogenes have already been reported. Recently, we applied restriction landmark genome scanning (RLGS) to study gene amplifications in HNSCC and located novel and uncharacterized regions in primary tumor samples. Gain on chromosome 8q22.3, the location of *YWHAZ* (14-3-3ζ), is found in 30-40% HNSCC cases. Data obtained from fluorescence *in situ* hybridization (FISH) and immunohistochemistry on HNSCC tissue microarrays confirmed frequent low-level *YWHAZ* copy number gain and protein overexpression. *YWHAZ* mRNA was frequently upregulated in patients' tumor tissues. Furthermore, *YWHAZ* RNAi significantly suppressed the growth rate of HNSCC cell lines, and overexpression of *YWHAZ* in HaCaT immortalized human skin keratinocytes promotes overgrowth, as well as morphological changes. Reduced *YWHAZ* levels increased the G1/G0-phase proportion, decreased the S-phase proportion and the rate of DNA synthesis. Based on this evidence, we suggest that *YWHAZ* is a candidate proto-oncogene and deserves further investigation into its role in HNSCC carcinogenesis.

Keywords

HNSCC; amplification; oncogene; *YWHAZ*/14-3-3ζ

Introduction

DNA amplification is a common genetic alteration found in many cancers 1. Subchromosomal DNA amplification results in extra copies of specific regions, potentially containing growth-promoting oncogenes. DNA amplification results in overexpression of the target genes located in the amplified regions, and thus confers a growth advantage 2. Overexpression of oncogenes results in neoplastic transformation and confers an advantage

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either by providing resistance to apoptotic stimuli 3 or by promoting accelerated cell growth 4. Amplification and overexpression of oncogenes is an early event in carcinogenesis 1 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 for *MYC* in oesophageal cancer 5 or *EGFR* in lung cancer 6.

In an earlier study we used restriction landmark genomic scanning (RLGS) to locate novel and known chromosomal regions displaying copy number gains 7. In these regions, we found oncogenes which are well-connected to HNSCC carcinogenesis, including the PI-3 kinase catalytic α subunit (*PIK3CA*) on chromosome 3q26.3, c-Myc (*MYC*) on 8q24.21 or cyclin D1 (*CCND1*) on 11q13.3. In addition, other novel and frequent gains on chromosome 3q29 and 8q22.3 were identified and confirmed. Each of these alterations was observed in approximately 30-40% HNSCC cases and considered a frequent alteration. Oncogenes in these regions have not yet been identified. Interestingly, RLGS fragment 2C13 on 8q22.3, identified as a frequently gained sequence is located in the 5' exon of tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta (*YWHAZ*), a 14-3-3 zeta isoform.

The 14-3-3 gene family was first discovered in 1967 during classification of cow brain proteins 8. Currently seven distinct isoforms (β , γ , ϵ , η , σ , τ , and ζ) encoded by seven distinct genes have been identified in mammalian systems 9. 14-3-3 family members are 28-33 kDa proteins which can homo- or hetero-dimerize 10. Dimerized 14-3-3 molecules bind to specific phosphor-serine/threonine (pS/-T)-containing motifs, two of which are RSXpSXP and RXXXpSXP 11, 12. Many molecules regulating cell signaling (*PKC*, *RAF1*, *CTTNB*) and apoptosis (*BAD*, *BAX*) are known that bind to 14-3-3 proteins 13-17, which make them master-regulators of these regulators. Evidence for involvement in cancer comes from studies on 14-3-3 σ (stratifin, *YWHAS* or *SFN*), which is frequently hypermethylated and down-regulated in various types of cancers 18-20 but overexpressed in others, possibly in response to cellular stress 21-23. Its cooperation with several regulators to block cell cycle progression makes 14-3-3 σ an ideal tumor-suppressor gene 24-26.

In this study we test the hypothesis that *YWHAZ* may be the target gene in this chromosomal region conferring a growth advantage to cells carrying this gain. Besides general studies of the 14-3-3 gene family, the detailed knowledge about *YWHAZ* function is limited. Previous reports showed overexpression of *YWHAZ* in oral, stomach cancers and lung adenocarcinomas 27-31 with the latter tumor type also showing gene amplification. In this work we report the overall over-representation of *YWHAZ* at the DNA, mRNA and protein level in HNSCC patients. Here we show that it has the capability to control cell growth when being negatively or positively manipulated and also affects the cell cycle. How the activated gene could contribute to tumor progression is discussed.

Materials and methods

HNSCC patient samples

Primary HNSCC derived from the nasal cavity, oral cavity, larynx and pharynx were obtained from surgeries performed at The Ohio State University and delivered to the Cooperative Human Tissue Network and our lab. All normal specimens were harvested from morphologically normal appearing tissue located at least 3 cm distant from the tumor and were used as the normal control tissue for comparisons with the tumor. Quality control hematoxylin and eosin sections were performed for both normal and primary tumor and reviewed by a pathologist (CM). 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

genomic DNA preparation were described, and the patient IDs were given in our previous study 7.

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 the standard TRIzol protocol of RNA extraction (Invitrogen, Carlsbad, CA). Following DNase I (Invitrogen) treatment to remove genomic DNA, 1-2 μ g of total RNA was incubated with primers of 0.5 μ g oligo-deoxythymidine (dT) and 2 μ g 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 at -20°C.

YWHAZ and glycosylphosphatidylinositol (GPI, for internal control) cDNA levels were measured using SYBR Green I (Bio-Rad, Hercules, CA) in a Bio-Rad 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 (Δ Ct) from all normal tissues was defined as basal level (BL). Normalized *YWHAZ* cDNA levels in tumors were calculated using the formula $1.9^{-(\Delta C_{tTumor-BL})}$. (1.9 is the amplification fold per cycle determined by calibrating experiments). We defined the normal *YWHAZ* expression range as 0.5 to 2 folds of BL. Primer sequences are listed in Table 1.

Construction of HNSCC tissue microarray (TMA)

The tissue microarray was constituted of 33 samples from previously studied samples 7. 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.

Fluorescence 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) which was verified by PCR to contain the *YWHAZ* gene. FISH slides were prepared and scanned as previously described 32. A ratio of the total number of *YWHAZ* signals to the total number of CEP8 signals in at least 60 interphase nuclei with no 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 a *YWHAZ*:CEP8 ratio of ≥ 2 , were considered consistent with gains of the *YWHAZ* gene. Tumor cells displaying at least two centromeric chromosome 8 signals and an equal number of *YWHAZ* signals, with a *YWHAZ*:CEP8 ratio of less than 2, were considered consistent with no gain 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 xylene and graded ethanol solutions to water. All slides were quenched for 5 min in a 3% hydrogen peroxide solution in water to block for endogenous peroxidase. Antigen retrieval was performed by incubating slides in a solution

of citrate buffer in a vegetable steamer. Slides were then placed on a Dako Autostainer, immunostaining system, for immunohistochemistry. Primary rabbit polyclonal anti-*YWHAZ* antibody clone C-16 (Santa Cruz Biotechnology, Santa Cruz, CA) was diluted 1:1500 and incubated for 60 min. 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.

Cell lines and culture

Four HNSCC cell lines (UMSCC-8, -9, -11B and -22B) and one immortalized human skin keratinocyte (HaCaT) were maintained and grown in Dulbecco's Modified Eagle's Medium (DMEM) with 10% Fetal Bovine Serum (FBS) and 1% streptomycin/penicillin (S/P) antibiotics.

RNAi transfection

Scrambled negative control RNAi and *YWHAZ* RNAi previously published 31 were ordered through Qiagen (Valencia, CA). Lipofectamine 2000 transfection reagent (Invitrogen) was mixed with RNAi in a 1:1 ratio (v/v) at room temperature for 20 min. Cells were incubated in serum-free DMEM with 60nM negative control or *YWHAZ* RNAi for 6 hours, then the medium was replaced with regular 10% FBS DMEM. The RNAi sequence of choice did not affect the levels of the 14-3-3 β and τ isoforms which had been tested previously 31. RNAi sequences are listed in Table 1.

Western blotting

Cells from 100-mm dishes were trypsinized, harvested, and lysed in lysis buffer containing 0.1% NP-40 and phosphatase inhibitor cocktails. 10 μ g of cell lysates were electrophoresed in a 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. Following two additional washes signals were developed with Supersignal (Pierce Biotechnology, Rockford, IL) and detected on Blue Autoradiography Film (ISC BioExpress, Kaysville, UT). Antibody concentrations were as follows: 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; mouse anti-E-cadherin (Santa Cruz Biotechnology), 1:100; mouse anti-N-cadherin (Santa Cruz Biotechnology), 1:200; mouse anti-vimentin (Santa Cruz Biotechnology), 1:100; goat anti-rabbit & anti-mouse, HRP-conjugated (Bio-Rad), 1:5000.

Cell growth curve assay

Twenty thousand HNSCC or HaCaT cells were seeded into 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) daily for up to 5 days. Each set of experiments was performed in duplicate at least twice.

Establishment of *YWHAZ*-overexpressing HaCaT strains

The *YWHAZ*-HA fragment from plasmid pcDNA3-*YWHAZ*-HA 33 was excised by BamHI and EcoRI, and then cloned into pBABE retroviral vector. The 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.

Flow cytometry and BrdU-incorporation assay

Flow cytometry was used to determine DNA content of cells in exponential phase (propidium iodide, PI) and the rate of DNA synthesis by BrdU (5'-bromo-2'-deoxyuridine)-incorporation assay. Ethanol-fixed cells were treated with 200µg/ml RNase A at 37°C and stained with 20µg/ml PI. As for BrdU- incorporation assay, we followed the manufacturer's FITC-BrdU Cell Proliferation Kit manual (BD Bioscience Pharmingen, San Diego CA). Cells were incubated with 10µM BrdU for 30 min, then harvested, permeabilized and fixed by provided reagents. 300µg/ml DNase I was used to nick genomic DNA for recognition by mouse FITC-conjugated anti-BrdU antibody. PI- or FITC-stained cells were analyzed with Beckman-Coulter flow cytometer and ModFit LT software (Verity Software House, Topsham, ME).

Results

RLGS and FISH analysis indicates gains of YWHAZ copies

Chromosome 8q22.3 represents an independent gained region of in HNSCC, breast and prostate cancers and is located distantly from the 8q24.21 amplicon which includes the *MYC* oncogene 34-36. Our previous analysis identified RLGS fragment 2C13 on chromosome 8q22.3 as a gained sequence in 14 out of 41 (~35%) tumor tissues in comparison to their normal counterparts 7. RLGS fragment 3D05, the neighboring fragment to 2C13, displays a similar frequency of enhancement concordant with 2C13 enhancement in the majority of cases. Both fragments reside within the 5' region of *YWHAZ*, a tyrosine 3 / tryptophan 5-monoxygenase (Supplemental Figure S1). The proximity of this gene to our RLGS fragments and the general characteristics of 14-3-3 proteins make *YWHAZ* our top candidate proto-oncogene. Figure 1a lays out the location of *YWHAZ* gene and its neighboring genes. Here we used FISH analysis to examine and confirm the status of DNA amplification (Figure 1b, Table 2). Polysomy 8 was detected in 33 out of 48 patients. 13 had low-level (*YWHAZ* /CEP8 = 2~4) and 3 had intermediate-level of copy number gain (*YWHAZ* /CEP8 = 4~10). No case of high-level copy number gain (ratio >10) was found. For comparison, the *MYC* locus on 8q24.21 showed low-level copy number gain in 10 patients, intermediate-level level of copy number gain in 5 and extremely high-level level copy number gain (*MYC*/CEP8 > 20) in 1 case (data not shown). The copy number variation of *YWHAZ* is better described as gain (low-level) instead of amplification (high-level). Overall, 33% (16/48) of the tumor samples carried extra *YWHAZ* gene copies, which was consistent with the percentage determined by RLGS analysis. Results from both RLGS and FISH analyses indicated that low-level gain of the *YWHAZ* locus is observed in 30-40% HNSCC cases.

YWHAZ mRNA and protein are up-regulated in HNSCC patient samples

To determine whether *YWHAZ* is a frequently-upregulated gene in HNSCC, we used a second independent panel of HNSCC tissue samples to verify elevated *YWHAZ* mRNA levels by semi-quantitative RT-PCR (Figure 1c). *YWHAZ* expression in 23 out of 44 tumor tissues was up-regulated (at least 2 fold over basal level) compared to only 3 out of 41 normal tissues (p-value = 0.0024). Sub-categorization by tumor locations showed that *YWHAZ* mRNA levels were more than 2-fold higher in 14 out of 17 tongue and 6 out of 8 oral cavity tumors, but in only 3 out of 10 pharyngeal and none of 9 laryngeal tumors. These data are consistent with previous reports on elevated *YWHAZ* mRNA levels in HNSCC 37.

To evaluate *YWHAZ* protein levels in normal and tumor tissues, the same TMA set used in the FISH analysis was stained with an anti-*YWHAZ* antibody (Figure 1d). *YWHAZ* protein staining in the cytoplasm was ubiquitous and more intense in tumors than in normal tissues. Light staining of *YWHAZ* in normal cells is seemingly within or around nuclei. Some hyperplastic and dysplastic cells in normal tissues were also positive for *YWHAZ*. Interestingly, in tumor sample PT91, whose *YWHAZ* copy number gain was confirmed by FISH, there was no difference in *YWHAZ* staining intensity between normal and tumor samples. A total of 37 out of 48 (77%) patients' tumor samples showed overexpression (Table 2). After further categorization, 8 cases had neither copy number gain nor overexpression, 3 had copy number gain but no detectable overexpression, 24 had overexpression but no copy number gain and 13 had both copy number gain and overexpression. These data suggest that cancer cells can overexpress *YWHAZ* protein frequently through mechanisms other than copy number gain.

Down- and up-regulation of *YWHAZ* greatly affects cell growth

We wonder what effects it will have if *YWHAZ* level is reduced in established cell lines. The effects by temporary knockdown of *YWHAZ* by RNAi were examined in three HNSCC (UMSCC-8, -11B and -22B) and HaCaT cell lines (Figure 2a). *YWHAZ* RNAi successfully reduced protein levels in all four tested cell lines to various degrees. Our RNAi sequence of choice reduced *YWHAZ* levels greatly in UMSCC-8 and UMSCC-22B, but mildly in UMSCC-11B and HaCaT, approximately to 0.5 fold compared to their original levels in those cell lines. In UMSCC-22B line, the *YWHAZ* RNAi was able to inhibit the protein level for at least 4 days, sufficient time for short-term experiments (Figure 2b). In a 5-day interval, *YWHAZ* RNAi also greatly suppressed the cell population in all three tested cell lines, even in UMSCC-11B and HaCaT cells, which still expressed *YWHAZ* at ~0.5 fold relative to original levels (Figure 2c). UMSCC-8 was not used here because the optimized condition for transfection for the other three cell lines did not work on UMSCC-8.

To evaluate the oncogenic potentials of *YWHAZ*, we also established the non-cancerous HaCaT cell line with ectopically-transfected *YWHAZ*. Previously, *HRAS*, *BCL2*, *MYC*, *TERT* and *CCND1* have been overexpressed ectopically in HaCaT, an immortalized skin keratinocyte line 38⁴⁰. It is a closer and easy-to-acquire model to squamous cells of head and neck than widely-used human or mouse fibroblast cell lines. Although HaCaT, along with all tested HNSCC cell lines, already had abundant endogenous *YWHAZ* protein, our *YWHAZ*-transfected HaCaT cells expressed roughly twice the amount of vector-transfected control (Figure 3a). HaCaT cells with nearly doubled *YWHAZ* level attained a population density nearly twice that of vector control after 5 days (Figure 3b). There was no immediate morphological difference between normal and *YWHAZ*-overexpressing HaCaT cells after 10 passages (p10) following the initial transfection (data not shown). However, 10 passages later (p20), we observed that all *YWHAZ*-overexpressing cells underwent morphological changes, from flatter original HaCaT cells to spindle-shaped smaller cells (Figure 3c). These cells were also highly clustered and piled, possibly due to rapid proliferation and lack of contact inhibition. They were more easily detached by trypsin during passages than original HaCaT cells, suggesting a weaker attachment toward the collagen-coated plates. Western blotting revealed decreased levels of E-cadherin and upregulation of N-cadherin and vimentin with sustaining overexpression of *YWHAZ*, supporting that these cells have undergone epithelial-mesenchymal transition (EMT), which is considered a step toward metastasis (Figure 3d). These experiments demonstrated that inhibition and overexpression of *YWHAZ* have immediate and profound impact on cell growth and morphology.

YWHAZ inhibition affects cell cycle and DNA synthesis

Based on results from the inhibition and overexpression experiments, we wanted to determine whether *YWHAZ* regulates cell populations by accelerating cell division or blocking apoptosis through flow cytometry experiments. First, we observed the effects of *YWHAZ* RNAi on DNA content index, an indicator of cell cycle progression and apoptosis. Cells were treated with 60 nM scrambled or *YWHAZ* RNAi for 3 days before being fixed and stained by PI. The H1299 lung adenocarcinoma cell line was used as a positive control, whose growth was also inhibited by the same *YWHAZ* RNAi oligomer in previous study 31. Figure 4a showed that the proportion of S phase was reduced and that of G1/G0 phase was elevated in both *YWHAZ* RNAi-transfected HaCaT and H1299 cells in contrast to the negative controls. No differences were observed in the percentages of either sub-G1 (apoptotic body) or G2/M proportion (metaphase) between scrambled and *YWHAZ* RNAi-transfected cells. This result suggests that reduced *YWHAZ* level results in more cells staying in diploid state and fewer in S phase. Second, we performed the alternative BrdU-incorporation assay to determine the proportion of cells entering S phase. Both HaCaT and H1299 cells transfected with *YWHAZ* RNAi incorporated less BrdU than those with the negative controls (Figure 4b), supporting that a smaller proportion of cells underwent *de novo* DNA synthesis. However, both DNA content index and BrdU-incorporation assay were unable to detect any differences between original and *YWHAZ*-overexpressing HaCaT cells (data not shown). These findings supported that *YWHAZ* regulates cell population by accelerating cell cycle progression instead of blocking apoptosis.

Discussion

In this study we showed that gain on chromosome 8q22.3 is frequently observed in 30-40% of HNSCC patients. This frequency is similar to that observed for three other major amplicons (3q26.3, 8q24.21 and 11q13.3) harboring among other potential candidate oncogenes, three major proven oncogenes (*PIK3CA*, *MYC* and *CCND1*), respectively 7. Frequent copy number gain of a specific gene may imply its importance in carcinogenesis, since the cells are inclined to preserve 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 multiplied and preserved in such a high rate. This idea of *YWHAZ* being an initiator is supported by the observations in early oral pre-malignant lesions 37. We have seen the oncogenic potentials of *PIK3CA*, *MYC* and *CCND1* in cancers including HNSCC, and our study suggests that *YWHAZ* should be an additional major gene to study in HNSCC carcinogenesis.

In addition to *YWHAZ*, other genes are also gained and overexpressed in the proximity of 8q22.3 in HNSCC. There is a possibility that *YWHAZ* is not the sole proto-oncogene in this chromosomal region as described also for other amplification events 41. Gene *EDD* (*UBR5*), the human orthologue of the hyperplastic discs tumor suppressor gene, is gained and overexpressed in multiple cancers 42. *EDD* is only ~1.3 Mbp away from *YWHAZ*. Another gained and overexpressed gene *LRP12* is ~3.5 Mb away 43. *BAALC* (brain and acute leukemia, cytoplasmic, ~2.2 Mb away) is found to be associated with acute myeloid leukemia (AML) 44. Eight consecutive genes starting from *AZIN1* to *RIMS2* (Figure 1a) are upregulated in HNSCC based on the re-analysis of existing cDNA microarray data (data not shown), though we do not have the information about their status of copy number variation, as well as the characterization of their oncogenic potentials. On the other hand, the 14-3-3 family is known to be involved in signal transduction, which is one of the major reasons we studied it first, along with its extreme proximity to our RLGS fragments. Whether other genes are merely co-amplified with *YWHAZ* or they also possess oncogenic potential is open for debate, but hereby we have clearly demonstrated the oncogenic activity of *YWHAZ*.

One of the important characteristics for *YWHAZ* is the ability to promote cell overgrowth when overexpressed as demonstrated in this study. However, overexpression of several proven oncogenes more often leads cells to senescence rather than overgrowth. Hyperactive mutant *RAS* (RasV12) and *MYC*-induced senescence in human or mouse fibroblasts is well documented and a powerful tool to study other oncogenes 45-47. Mounting evidence shows that whenever an oncogene is overrepresented, tumor suppressors such as *TP53* and *RB* may be activated and block further cell cycle progression, which is regarded as the intrinsic defense mechanism 48. Cells remain arrested until a second defect such as *TP53* mutation overcomes these defenses, then transform 49. Overexpressing *YWHAZ* is capable of transforming immortalized mouse NIH3T3 fibroblasts when co-transfected with RasV12 31. It appears that HaCaT cells possess some genomic alterations that allow *YWHAZ* to promote overgrowth.

The growth-promoting activity of *YWHAZ* has been demonstrated *in vitro*, but we did not address its potential effects on apoptosis. The idea of *YWHAZ* being anti-apoptotic is implicated in several reports. *YWHAZ* binds to pro-apoptotic *BAD* of the *BCL2* family member 16. High-level *YWHAZ* is known to protect cells from insults such as anti-tumor agents, anoikis and radiation 50-52. Based on our results from flow cytometry experiments, however, cells with reduced *YWHAZ* level seem simply less proliferative, not apoptotic since the sub-G1 proportion, the fraction of apoptotic bodies, was negligible in those cells. Lower *YWHAZ* level seems to increase cells' sensitivity to anti-tumor agents and insults, but functional apoptotic machinery may not be required for *YWHAZ* to regulate cell growth. This hypothesis can be supported or rejected by suppressing the *YWHAZ* level in HaCaT cells with impaired apoptotic machinery. If the hypothesis holds true, we still should be able to observe the suppression of cell growth even in these cells.

The oncogenic activity of *YWHAZ* may be attributed to its binding partners. *YWHAZ* binds to several general signaling molecules as do other 14-3-3 isoforms. *YWHAZ* can homo-dimerize or hetero-dimerize with the other five isoforms. The 14-3-3 σ isoform is an exception as it only homo-dimerizes with itself. It is a proven tumor suppressor, however, replacement of five key amino acids on 14-3-3 σ with the corresponding ones from *YWHAZ* allowed the mutated 14-3-3 σ to hetero-dimerize in a manner similar to *YWHAZ*, and also abrogated the capability to suppress HEK293 cell growth 53. Thus, there is possible that these two isoforms compete for the same targets, which include signaling and pro-apoptotic molecules such as *PKC*, *RAF1*, *CTTNB*, *BAD*, *CDC25B/C*, *AKT*, *GSK3B* (GSK-3 β) and *EGFR*, all of which are proto-oncogene candidates 13-17, 52, 54-56. Phosphor-serine 10 on the histone H3 peptide has also been shown to bind *YWHAZ*, probably affecting the acetylation of nearby lysines 9 and 14, which may lead to chromatin remodeling and gene activation 57. *ADAM22*, which is a number of transmembrane proteins that contain disintegrin-like and metalloproteinase-like domains, could be a gene of interest when *YWHAZ*-induced EMT mentioned earlier is taken into account 58. Due to the numerous binding partners of *YWHAZ*, there could be multiple mechanism(s) contributing to *YWHAZ*'s oncogenic activity and each will require further investigation in order to understand the oncogenic function of *YWHAZ*.

Besides *YWHAZ* and 14-3-3 σ (stratifylin, *YWHAS* or *SFN*), two other 14-3-3 isoforms may be implicated in tumorigenesis. 14-3-3 τ (θ)/*YWHAB* on 2p25.1 was also frequently found gained in urothelial carcinoma as was *YWHAZ* 59. 14-3-3 β /*YWHAB* on 20q13.12 and *YWHAZ* are closer in the phylogenetic tree than any other isoforms (Supplemental Figure S2). Studies regarding tumorigenic functions of *YWHAB* also led to conclusions similar to our study 60. Considering our current knowledge about them, we speculate that *YWHAZ*, *YWHAB* and *YWHAB* may belong to the "pro-oncogenic" branch of the 14-3-3 family in contrast to the "anti-oncogenic" 14-3-3 σ . Their functions may be partly redundant, that is,

those tumor tissues which are negative for *YWHAZ* amplification or overexpression may be positive for *YWHAQ* or *YWHAB* amplification or overexpression. It will be very fruitful to conduct systematic studies regarding the roles of these three genes in tumorigenesis of multiple cancers.

At last, Dr. Tzivion's group observed that transgenic mice carrying extra *YWHAZ* gene copies developed various types of tumors by the age of 7-15 months in about 15% of the animals [61]. Furthermore, the level of *YWHAZ* protein in these transgenic mice was only 30% greater than that in normal ones. This observation corroborates with our observations: (1) *YWHAZ* copy number gain was only 2-4 fold in HNSCC patients; (2) knocking down *YWHAZ* production by merely 50% in HaCaT was sufficient to suppress cell growth; (3) doubling *YWHAZ* level was able to promote HaCaT overgrowth and in long-term culture, induced morphological changes and EMT. While high-copy numbers of proto-oncogenes such as *MYC* and *EGFR* are often seen in cancers, low-level copy number gain or overexpression may be sufficient for *YWHAZ* to facilitate cell transformation. From these observations, we believe that further research is warranted into the role of *YWHAZ* in tumor progression as well as its possible utility as a therapeutic target.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1.

YWHAZ copy number gain, overexpression of mRNA and protein in HNSCC.

(a) Schematic outline of identified genes on chromosome 8q22.2-8q22.3 region (100,000,001-106,000,000 bp) modified from the UCSC bioinformatic website. Filled-boxes represent exons. Arrows represent the direction of transcription. *YWHAZ* is highlighted by the red box.

(b) Low-level copy number gain of *YWHAZ* determined by FISH. The red signals (arrowhead) are BAC clone RP11-302J23 to detect *YWHAZ* copies. The green signals (arrow) are CEP8 probe to detect centromeres of chromosome 8 as the internal control.

(c) mRNA levels in HNSCC patients' normal and tumor samples. Red line indicates two fold 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 fold.

(d) Immunohistochemical staining of *YWHAZ* protein in three pairs of normal and tumor samples. *YWHAZ* protein is stained brown. Magnification is 100X and 400X (patient 32 only). PT numbers represent the patient IDs as shown in Table 2.

FIGURE 2.

Transient *YWHAZ* RNAi transfection inhibits cell proliferation.

(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 UM-SCC-22B cell line. *YWHAZ* RNAi suppresses protein levels for at least four days.

(c) Growth curve of UM-SCC11B, UM-SCC22B and HaCaT cell lines with *YWHAZ* RNAi. Symbols: no treatment, transfection reagent only (diamond); scrambled RNAi plus transfection reagent (square); *YWHAZ* RNAi plus transfection reagent (triangle). *YWHAZ* RNAi suppresses cell growth in all three cell lines (starting 20,000 cells, 5-days interval).

FIGURE 3.

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

(a) Western blotting confirms HA-tagged *YWHAZ* ectopic overexpression in stably-transfected HaCaT cell line in spite of a high endogenous *YWHAZ* level.

(b) Growth curve of stable pBABE vector- and *YWHAZ*-HA-transfected HaCaT cells.

Symbols: pBABE vector (diamond); *YWHAZ*-HA transfected (square). *YWHAZ*-transfected HaCaT cells show more rapid growth than vector control cells in regular 10% FBS DMEM.

(c) HaCaT cell growth and morphology. Top row, left to right: HaCaT cells with vector only (passage 20) and with *YWHAZ* (passage 20). 100X magnification. Bottom row, 400X magnification.

(d) Western blotting of EMT markers (E-cadherin, N-cadherin, vimentin) in vector control and *YWHAZ*-overexpressing HaCaT cells. Full length of E-cadherin is 120KDa. 80KDa fragment is the proteolytic product.

FIGURE 4.

The effects of *YWHAZ* RNAi on cell cycle and DNA synthesis.

(a) DNA content index in H1299 and HaCaT cells treated with scrambled 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 H1299 and HaCaT cells treated with scrambled RNAi or *YWHAZ* RNAi. The number represents the percentage of the BrdU-positive (S-phase) cells in each population. Each set is done in duplicate.

TABLE 1

Sequences of RT-PCR primers and RNAi

		Forward sequence	Reverse sequence
RT-PCR	YWHAZ	5'-ATG TAC TTG GAA AAA GGC CG-3'	5'-CCC TGC TCT TGA GGA GCT TA-3'
	GPI	5'-GAC CCC CAG TTC CAG AAG CTG-3'	5'-GCA TCA CGT CCT CCG TCA CC-3'
RNAi	Scrambled	5'-GGG UAU CGA CGA UUA CAA AUU dTdT-3'	5'-AAU UUG UAA UCG UCG AUA CCC dTdT-3'
	YWHAZ	5'-AGU UCU UGA UCC CCA AUG C dTdT-3'	5'-GCA UUG GGG AUC AAG AAC U dTdT-3'

TABLE 2

Summary of FISH analysis and immunohistochemistry for *YWHAZ* gene copy and protein level in HNSCC. Polysomy 8 is highlighted in yellow color in Polysomy 8 column. Yellow color in *YWHAZ*/CEP column as low level of amplification and orange as intermediate level. Symbols in IHC expression column: +++, high level; ++, intermediate level; +, low level; -, no staining.

Patient ID	Polysomy 8	RP11-302J23 (<i>YWHAZ</i>)/CEP8	<i>YWHAZ</i> amplification status	Expression level
4	yes	3.56	Low level	+++
7	no	<1.8	No amplification or deletion	++
8	yes	<1.8	No amplification or deletion	+++
11	yes	1.95	Indeterminate	+
12	yes	<1.8	No amplification or deletion	+++
13	yes	<1.8	No amplification or deletion	+++
14	yes	<1.8	No amplification or deletion	++
15	no	<1.8	No amplification or deletion	-
22	yes	<1.8	No amplification or deletion	++
23	no	<1.8	No amplification or deletion	-
24	no	2.35	Low level	++
25	no	5.94	Intermediate level	+++
27	yes	<1.8	No amplification or deletion	++
28	no	<1.8	No amplification or deletion	++
29	yes	2.41	Low level	+++
32	yes	<1.8	No amplification or deletion	+++
39	no	<1.8	No amplification or deletion	+
40	yes	<1.8	No amplification or deletion	+++
46	no	3.1	Low level	-
47	yes	2.41	Low level	++
49	yes	<1.8	No amplification or deletion	+++
50	yes	2.89	Low level	+++
54	yes	5.87	Intermediate level	+
55	yes	4.59	Intermediate level	+++
56	yes	3.84	Low level	++
71	no	<1.8	No amplification or deletion	+++
82	yes	2.89	Low level	+
91	yes	<1.8	No amplification or deletion	-
99	yes	<1.8	No amplification or deletion	-
106	yes	<1.8	No amplification or deletion	++
110	yes	<1.8	No amplification or deletion	-
114	yes	<1.8	No amplification or deletion	+++
115	no	<1.8	No amplification or deletion	+++
118	yes	3.05	Low level	++
126	yes	<1.8	No amplification or deletion	++
129	no	<1.8	No amplification or deletion	-

Patient ID	Polysomy 8	RP11-302J23 (YWHAZ)/CEP8	YWHAZ amplification status	Expression level
130	yes	2.25	Low level	+
132	no	<1.8	No amplification or deletion	+++
145	no	<1.8	No amplification or deletion	-
158	yes	2.56	Low level	-
159	yes	<1.8	No amplification or deletion	+
167	yes	<1.8	No amplification or deletion	+++
173	no	2.04	Indeterminate	-
180	yes	2.06	Indeterminate	+
192	yes	<1.8	No amplification or deletion	++
<193	no	3.22	Low level	-
201	yes	2.15	Indeterminate	+
220	yes	3.41	Low level	+++