Title

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Journal

Molecular Cancer Research, 12(4)

ISSN

1541-7786

Authors

Li, Hua
Wawrose, John S
Gooding, William E
et al.

Publication Date

2014-04-01

DOI

10.1158/1541-7786.mcr-13-0396

Peer reviewed

Published in final edited form as:
Mol Cancer Res. 2014 April ; 12(4): 571–582. doi:10.1158/1541-7786.MCR-13-0396.

Genomic analysis of head and neck squamous cell carcinoma cell lines and human tumors: a rational approach to preclinical model selection

Hua Li[1], **John S. Wawrose**[1], **William E. Gooding**[2], **Levi A. Garraway**[3,4,5], **Vivian Wai Yan Lui**[1], **Noah D. Peyser**[1], and **Jennifer R. Grandis**[1,6,*]

[1]Department of Otolaryngology, University of Pittsburgh School of Medicine and University of Pittsburgh, Cancer Institute, Pittsburgh, Pennsylvania.

[2]Department of Biostatistics, University of Pittsburgh School of Medicine and University of Pittsburgh, Cancer Institute, Pittsburgh, Pennsylvania.

[3]The Broad Institute of Harvard and MIT, Cambridge, Massachusetts 02142, USA.

[4]Department of Medical Oncology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts 02115, USA.

[5]Center for Cancer Genome Discovery, Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts 02115, USA

[6]Department of Pharmacology & Chemical Biology, University of Pittsburgh School of Medicine and University of Pittsburgh, Cancer Institute, Pittsburgh, Pennsylvania.

Abstract

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common type of cancer worldwide. The increasing amount of genomic information on human tumors and cell lines provides more biological data to design preclinical studies. We and others previously reported whole exome sequencing data of 106 HNSCC primary tumors. In 2012, high throughput genomic data and pharmacological profiling of anticancer drugs of hundreds of cancer cell lines were reported. Here we compared the genomic data of 39 HNSCC cell lines with the genomic findings in 106 HNSCC tumors. Amplification of eight genes (*PIK3CA, EGFR, CCND2, KDM5A, ERBB2, PMS1, FGFR1* and *WHSCIL1*) and deletion of five genes (*CDKN2A, SMAD4, NOTCH2, NRAS* and *TRIM33*) were found in both HNSCC cell lines and tumors. Seventeen genes were only mutated in HNSCC cell lines (>10%) suggesting that these mutations may arise through immortalization in tissue culture. Conversely, 11 genes were only mutated in >10% of human HNSCC tumors. Several mutant genes in the EGFR pathway are shared both in cell lines and in tumors. Pharmacological profiling of eight anticancer agents in six HNSCC cell lines suggested that *PIK3CA* mutation may serve as a predictive biomarker for the drugs targeting the EGFR/PI3K pathway. These findings suggest that a correlation of gene mutations between HNSCC cell lines

*Corresponding Author: Jennifer R. Grandis, University of Pittsburgh School of Medicine, 200 Lothrop Street, Suite 500, Pittsburgh, PA 15213. Phone: 412-647-5979; Fax: 412-383-5409; jgrandis@pitt.edu.

Conflict of Interest: The authors have no financial or other conflict of interests to disclose.

and human tumors may be used to guide the selection of preclinical models for translational research.

Keywords

head and neck squamous cell carcinoma; cell lines; tumors; gene mutations; biomarker

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide arising in the upper aerodigestive tract, including the oral cavity, pharynx, and larynx. HNSCC, with more than 600,000 new cases diagnosed annually, is often lethal with only 40-50% of HNSCC patients surviving five years after their diagnosis (1). Risk factors for HNSCC include tobacco and alcohol use. Infection with the human papilloma virus (HPV) is increasingly identified as a strong contributing factor, especially in oropharyngeal cancer (1,2). Inherited disorders such as Fanconi Anemia are also associated with increased susceptibility to HNSCC (3).

HNSCC cell lines have been developed in many laboratories worldwide (4). Improved understanding of the biologic underpinnings of HNSCC and a more rational approach to therapy can best be accomplished through the judicious use of models that reproduce key features of human tumors. The use of cell lines derived from human tumors dates back to derivation of HeLa cells, a robust cancer cell line developed from a cervical cancer afflicting a patient named Henrietta Lacks in 1951 (5). In 1990, cancer cell lines encompassing nine tumor types were used as preclinical models for drug screening efforts known as the NCI60 collection. To date, over 10,000 different potential therapeutic agents have been tested using these models (6). Although the NCI60 contains a wide variety of cancer types, the collection lacks HNSCC cell lines. In 2010, the genomic profiling and pharmacological data for 311 cancer cell lines was released by GlaxoSmithKline (GSK) via the National Cancer Institute's cancer Bioinformatics Grid™ (caBIG®), including ten HNSCC cell lines (7). However, none of these HNSCC cell lines contained mutations in the six genes analyzed (*HRAS, KRAS, NRAS, BRAF, PIK3CA,PTEN*). While larger cell line panels have been used for compound screening, no study to date has focused on genetically characterized HNSCC cell line panels.

The Cancer Cell Line Encyclopedia (CCLE), a comprehensive database including the mutational status of more than 1600 genes, gene expression, and chromosome copy number data across a panel of 947 human cancer cell lines from 36 different tissue types was recently reported by Barretina *et al* (8). Similar to the NCI60 collection, pharmacological profiling of 24 different anticancer drugs and compounds was carried out in 479 of these cell lines. The profiling focused on identifying preclinical, genetic indicators of sensitivity to specific compounds (8). Thirty two HNSCC cell lines were included in the CCLE and sequencing data were reported for 30 of these models. Drug-sensitivity profiling was carried out in 6 of the HNSCC cell lines. In addition to the CCLE, Garnett *et al* presented their systematic identification of genomic markers of drug sensitivity in cancer cells, which is a

repository of mutation profiles of 639 cell lines including 20 HNSCC cell lines and 130 drugs for screening across the majority of these cell lines. Eleven of these HNSCC cell lines and 25 of the 130 drugs were also studied in the CCLE. In contrast to the high throughput sequencing approach used in the CCLE, the Garnett *et al* database only re-sequenced the full coding exons for 77 genes identified in their Cancer Gene Census (9).

Cancer cell lines are typically used as preclinical models for mechanistic studies. However, the potential for established HNSCC cell lines to reflect the genetic alterations found in human HNSCC tumors has not been thoroughly investigated. Two recent studies have profiled the mutations, using whole exome sequencing, in 106 unique HNSCC tumor samples (10, 11). These efforts revealed a number of oncogenes implicated in the pathogenesis of HNSCC (10-12). The present study was undertaken to compare the gene mutation frequencies between HNSCC human tumors and cell lines to facilitate the rational selection of cell line models for translational HNSCC research.

Materials and Methods

Databases

Databases used in this paper are publically available. The five cohorts are included:

1. HNSCC cell lines genomic and pharmacological profiling from Barretina *et al* database (http://www.nature.com/nature/journal/v483/n7391/full/nature11003.html and http://www.broadinstitute.org/ccle/home).
2. HNSCC cell lines genomic and pharmacological profiling from Garnett *et al* database (http://www.nature.com/nature/journal/v483/n7391/full/nature11005.html).
3. HNSCC tumors genomic profiling from Stransky *et al* database (http://www.sciencemag.org/content/333/6046/1157).
4. HNSCC tumors genomic profiling from Agrawal *et al* database (http://www.sciencemag.org/content/333/6046/1154.full).
5. The Cancer Genome Atlas (TCGA) database (http://www.cbioportal.org/public-portal/).

Statistical Methods

We used logistic regression to estimate the effects of source (cell line or tumor) and site of disease (oral cavity, pharynx, larynx) on mutation frequency. A test of interaction between source and site was conducted. No interaction was detected for any gene thereby prompting tests of main effects. Significant effects were reported for any genes satisfying a maximum 10% expected false discovery rate. To tabulate mutations in common, we utilized a comparison program, created using Microsoft Excel. The mutations (non-synoynomous mutation) and gene copy numbers in HNSCC cell lines were compared to those in HNSCC tumors side by side by this comparison program. The use of this comparison program was reported previously (13). The gene copy number analysis used log 2 ratio as described (8). The log2 ratio of normal (copy-neutral) clones is $log2(2/2) = 0$, single copy losses is

log2(1/2) = −1, and single copy gains is *log2(3/2) = 0.58*. The effects of drug treatment vs DMSO control was repeated three times and compared using an unpaired T-test with Welch's correction ($P<0.05$ as statistically significant).

Cell Cultures

The HNSCC cell lines, CAL-27 and SCC-9 cells were obtained from ATCC, Manassas, VA. All cell lines were genotypically verified. HNSCC cell lines were cultured in the respective culture medium containing 10% fetal calf serum, 1X penicillin/streptomycin solution (Invitrogen, Carlsbad, USA): CAL-27 in DMEM, SCC-9 in DMEM/F12 with 0.4 μg/ml hydrocortisone (Mediatech, Inc, Manassas, VA). All cell lines were maintained in a humidified cell incubator at 37°C, 5% CO_2.

Cloning and Mutagenesis

Cloning and mutagenesis performed as previously described (13).

Cell Transfection and drug treatment

HNSCC cells were plated at 20% confluency in T25 flasks 24h before transfection. Then cells were transfected with either pMXspuro-PIK3CA WT or pMXs-puro-PIK3CA mutants (H1047R) using Lipofectamine® 2000 (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. 24h after transfection, 1×10^4 cells were plated in 48 well culture plates 24h before drug treatment. Cells were treated with either 2.0μM or 8.0 μM Erlotinib for 48h. MTT was performed.

Results

Clinical and pathologic features of HNSCC genotyped cell lines

Thirty nine HNSCC cell lines were included in the reports by Barretina *et al* and Garnett *et al*. Eleven cell lines are common to both data sets while Barretina *et al* included 30 unique HNSCC cell lines and Garnett *et al* reported on 20 additional HNSCC cell lines (Figure 1). Clinical and pathological information from these 39 HNSCC cell lines is summarized in Table 1 and Supplemental Table 1. The mean age of the 28 patients with known age from whom the cell lines were derived was 58.2. Thirty (83% of known gender) patients were male, six (17% of known gender) were female, and three patients have no reported information for sex. The oral cavity is the most highly represented anatomic tumor site, accounting for 68% of the cell lines with known primary site. Pharyngeal tumors account for four (12%) of the cell lines, six (18%) of the cell lines were derived from the larynx, one (3%) cell line was from the nasal septum and the anatomic site of the remaining five are unknown. Only three cell lines are reported to be HPV-negative and the remaining cell lines have no reported HPV status. Given the paucity of confirmed HPV-positive HNSCC cell lines, it is likely that none of the sequenced HNSCC cell lines are derived from HPV-positive HNSCC (14). Although the clinical and pathologic information available for the sequenced HNSCC cell lines is incomplete, the age and male predominance reflects most HNSCC clinical cohorts. Cell lines are generally derived from resected tumor specimens. Given the increasing tendency over the past few decades to treat tumors of the pharynx and

larynx primarily with non-operative therapy, it is not surprising that the majority of cell lines are derived from oral cavity tumors where the primary treatment strategy is surgical resection. Similarly, since the HPV-HNSCC tumors are largely pharyngeal (especially tonsil) cancers (14), the small number of cell lines derived from tumors arising in the pharynx likely contributes to the limited availability of HPV-HNSCC cell line models.

Comparison of gene mutations and alterations between HNSCC cell lines and human tumors

Cancer is thought to arise, at least in part, by the accumulation of genetic mutations, including activating oncogenes and inactivating tumor suppressor genes (15-17). However, the dynamic evolution of mutation calling algorithms and varying approaches between institutions can limit the ability to compare sequencing results. We first compared the methods to generate the genetic data including HNSCC cell line data in these two cancer cell line databases and HNSCC primary tumor data in the two reported human cohorts (8-11). All of the studies used Affymetrix SNP6.0 arrays to elucidate genotypes. For sequencing, the cell line samples in the CCLE and the 74 tumor samples from our prior study were both performed at the Broad Institute on Illumina instruments and generated gene profiles using similar strategies (7,9). Seventeen tumor samples in the other human HNSCC cohort were also sequenced using Illumina while 15 tumor samples were performed by Solid (Table 2) (10). Barretina *et al* provide genomic data for 947 cell lines including gene mutation status of 1651 cancer related genes for 30 HNSCC cell lines (Table 2). These cell lines harbored 1637 mutations in 654 genes. Garnett *et al* reported markedly fewer mutations for each cell line with 49 mutations in seven genes since only selected 77 genes were sequenced in their project. A total of thirteen genes were mutated in more than 50% of the HNSCC cell lines evaluated (Supplemental Table 2). Human HNSCC tumors from the Stranksy, *et al*. cohort harbored 7165 mutations in 4897 genes, whereas the human HNSCC tumors in the Agrawal, *et al*. cohort contained 609 mutations in 561 genes.

In an attempt to further determine the potential of immortalized HNSCC cell lines to reflect the underlying biology of human HNSCC tumors by gene mutation status, the mutation rates of specific genes in HNSCC tumors and cell lines were compared. Here, we analyzed the HNSCC cell line mutation data from Barretina *et al* and Garnett *et al* databases as a cell line panel and the two human HNSCC tumor cohorts as a human tumor panel. Three hundred and thirty four genes were found in both HNSCC cell lines and human tumors representing 51% of the mutated genes in HNSCC cell lines (Figure 2A). The fifteen most commonly mutated genes in HNSCC cell lines are depicted in Table 3. *TP53* is the most commonly mutated gene both in HNSCC tumors and cell lines [64.2% (68/106) of human tumors and 84.6% (33/39) of cell lines cell lines tested] (Supplemental Table 3). We noted that almost all patient tumors (103 of 106) and most cell lines (33 of 39) are from oral cavity, pharynx or larynx (Supplemental Table 4). We analyzed the mutant genes with frequencies over 5% in both human HNSCC cell lines and human tumors from these anatomic sites. The mutation frequencies were computed and tested for differences with logistic regression adjusting for disease site. The mutation rates of nineteen genes were not different in HNSCC cell lines and primary tumors while four genes had greater frequency of mutation among the tested cell lines compared to tumor specimens with an expected false discovery to 10% or less

including *CDKN2A* (42% in cell lines vs 11% in tumors), *FMN2* (30% in cell lines vs 6% in tumors), *MLL3* (30% in cell lines vs 6% in tumors) and *TTN* (61% in cell lines vs 28% in tumors) (Figure 2B and Supplemental table 5). We further compared the gene mutation status between the HNSCC cell lines and tumors according to the primary anatomic tumor site. There were 7 genes with different mutation rates by site including *CSMD3, CUBN, NAV3, NSD1, PKHD1, TP53* and *TTN*. *TTN* was the only gene with a mutation rate differing by both source (cell line versus human tumor and anatomic site (Supplemental Figure 1). There was no interaction between source and site indicating that differences in mutation frequency by site are consistent across source and vice-versa.

Some genes, however, were only mutated in HNSCC cell lines, indicating a discordance between HNSCC human tumors and cell line models. Seventeen genes mutated in more than 10% of HNSCC cell lines were never mutated in the human tumors (Table 4) while 11 genes were only mutated in human HNSCC tumors with frequencies over 10% but never reported in cell lines (Supplemental table 6). It is noteworthy that six genes were mutated in more than 50% of cell lines but never mutated in tumors including *VEGFC, PRKDC, GRIA3, MAML3, GPR112,* and *NEK3*.

Analysis of DNA copy numbers in HNSCC cell lines in the two cell lines database revealed 194 amplified genes (log2>0.58) including 40 genes amplified in over 50% of HNSCC cell lines while 60 genes were deleted (log2<−1) in these cell lines. Since the human HNSCC tumor studies only reported gene copy alterations of 14 genes including 13 amplified genes and one deleted gene, we also compared the gene copy number alterations in HNSCC cell lines and in tumor samples in TCGA (The Cancer Genome Atlas) cohort. Only eight genes were amplified (*PIK3CA, EGFR, CCND2, KDM5A, ERBB2, PMS1, FGFR1* and *WHSCIL1*) and five genes were deleted (*CDKN2A, SMAD4, NOTCH2, NRAS* and *TRIM33*) in both cell lines and tumors (Figure 2C). All of these genes had higher alteration frequencies in HNSCC cell lines compared with primary tumors. These cumulative findings suggest that mutations and gene copy number alterations exist in HNSCC cell lines, but not in human tumors, and may result from selection and/or propagation in tissue culture that does not reflect critical biologic properties of this cancer. In addition, tumor cells containing selected HNSCC mutations may not survive the process of cell line selection and can only be modeled by cell line engineering.

Mutation of genes in the EGFR signaling pathway

The epidermal growth factor receptor (EGFR) is a member of the type 1 tyrosine kinase family and is recognized as a key regulator of cellular differentiation and proliferation by activating downstream signal transduction pathways, including the Ras-Raf-MAPK pathway and phosphatidylinositol-3 kinases (PI3K). Increased EGFR expression, by gene amplification and transcriptional activation, is one of the most frequent alterations found in HNSCC where *EGFR* has been identified as a prognostic biomarker and molecular therapeutic target (18). Although *EGFR* over-expression is one of the most common alterations found in HNSCC, the incidence of *EGFR* mutations in HNSCC is negligible (19, 20). We defined the EGFR pathway and found ten genes in the pathway mutated in both HNSCC cell lines and human tumors (Figure 3). Genes in the Ras-Raf-MAPK pathway,

including *HRAS, KRAS, RAF1* and *MAPK1 (ERK1)* were mutated both in HNSCC cell lines and tumors, whereas *N-RAS* and *MAP2K1* (*MEK1*) were only mutated in HNSCC cell lines and *B-RAF* was only mutated in HNSCC tumors. In the PI3K pathway, *GAB1*, PI3K p110 subunits (*PIK3CA, PIK3CD* and *PIK3CG*), three MAP3Ks (*MAP3K3, MAP3K4* and *MAP3K6*) and *MAP2K4 (MEK4)* were mutated in both cell lines and tumors while *MAPK8 (JNK1)* was only mutated in HNSCC cell lines and *MAPK9 (JNK2)* mutations were restricted to HNSCC tumors. *STAT1* was mutated both in HNSCC tumors and cell lines while *STAT3* mutation was only found in HNSCC tumors (Supplemental Table 7). These results suggest that mutations of genes in the EGFR signaling pathway may contribute to HNSCC either in tumorigenesis or tumor growth despite of the paucity of *EGFR* mutations.

Pharmacological profiling of HNSCC cell lines

Six agents have been FDA-approved for the treatment of HNSCC including methotrexate (approved in 1956), 5-FU (approved in 1957), bleomycin (approved in 1973 and rarely used in modern oncology), cisplatin (approved in 1978), and docetaxel and cetuximab (both approved in 2006). Six HNSCC cell lines were included in the pharmacologic profiling in the Barretina *et al* database including Cal 27, Detroit 562, FaDu, HSC-2, SCC9 and SCC25 using 24 chemotherapy and molecular targeting agents (8). We analyzed the responses of these HNSCC cell lines to the 24 compounds by the activity area (Defined as the area between the pharmacologic dose-response curve and a fixed reference $A_{ref} = 0$. Using the fixed reference, *Activity area* = 0 corresponds to an inactive compound) (8). Paclitaxel (targeting TUBB1), panobinostat (targeting HDAC), 17-AAG (targeting HSP90), and irinotecan (targeting TOP2) most potently inhibited the growth of HNSCC cell lines. However, HNSCC cell lines were resistant to PLX4720 (targeting BRAF), PF2341066 (targeting c-MET), PD-0332991 (targeting CDK4), sorafenib (a multi-kinase inhibitor), nultin-3 (targeting MDM2), and L-685458 (targeting GS) (Figure 4A). Compared to cell lines derived from other organ sites, HNSCC cell lines were significantly more sensitive to the EGFR TKI erlotinib (p=0.002). The pharmacologic data in Garnett *et al* cohort also confirmed this finding. We then further analyzed the drug responses of these 6 HNSCC cell lines to EGFR inhibitors. Among the 6 HNSCC cell lines tested, HSC-2 and Detroit 562, derived from tumors of the oral cavity and pharynx, respectively, were resistant to the five compounds including AZD0530 (targeting Src family kinases), erlotinib (targeting EGFR), lapatinib (targeting HER2 and EGFR), TKI 258 (targeting EGFR) and ZD-6474 (targeting EGFR and VEGFR) (Figure 4B). To determine the correlation between gene mutations and drug sensitivity, we compared the genomic profile of HSC-2 and Detroit 562 with the other four HNSCC cell lines profiled. Interestingly, *PIK3CA* was only mutated in HSC-2 and Detroit 562 cell lines, whereas the other four cell lines harbored WT *PIK3CA*. To determine if *PIK3CA* mutation contributes to sensitivity to EGFR targeting agents, we introduced a *PIK3CA* mutation (H1047R) or WT *PIK3CA* into Cal 27 and SCC9 cells that harbor WT *PIK3CA* and assessed the impact of *PIK3CA* mutation on drug sensitivity. Cal 27 and SCC9 expressing mutant (H1047R) or WT *PIK3CA* were treated with erlotinib. Both Cal 27 and SCC 9 expressing mutant *PIK3CA* were significantly more resistant to erlotinib treatment at both concentrations tested comparing with isogenic cells expressing WT *PIK3CA* (Figure 4C). These results indicate that *PIK3CA* mutation might serve as a negative predictive

biomarker for erlotinib in HNSCC indicating that the mutational status of *PIK3CA* may be used to select preclinical models for response to EGFR TKI.

Discussion

Human tumor-derived cell lines serve as important preclinical models to identify therapeutic targets and mechanisms of anticancer agents for translational studies. The first high throughput cancer cell line screening program was the NCI60 platform which led to the development of many new technologies for drug screening from 1984 to 2005 (18). However, this platform is limited by the lack of representation of cell lines derived from a number of human tumors, including HNSCC. The reduced cost of gene sequencing in conjunction with the efforts of The Cancer Genome Atlas (TCGA) and other groups has led to the increased availability of mutation data linked to human cancer cell lines and patient tumors. In addition, the large number of molecular targeting agents in clinical development underscores the need to link baseline tumor cell characteristics to drug responses to improve treatment selection. The Cancer Cell line Project at the Wellcome Trust's Sanger Institute is well known for re-sequencing the most common cancer-associated genes in human tumor-derived cell lines and 77 genes in 770 cancer cell lines have been re-sequenced in this project to date. The Cancer Cell Line Encyclopedia (CCLE) project at the Broad Institute generated genomic profiles including a compilation of gene expression, chromosomal copy number, and massively parallel sequencing data from 947 human cancer cell lines with pharmacologic profiles for 24 anticancer drugs across 479 of the lines (8). Matching normal genomic DNA is rarely, if ever, available for cell lines since most established cell lines have been in culture for many years and the paired normal material is not available. None of the sequencing results to date on HNSCC cell lines have utilized matched normal DNA. In the Cancer Cell Line Encyclopedia (CCLE) project, gene mutations were evaluated in conjunction with dbSNP134 or allele frequency in the NHLBI Exome Sequencing Project or 1000 Genomes Project to exclude common germline variants (8). There were 1147 gene mutation sites found in HNSCC cell lines in CCLE, only 72 of which were reported SNPs according to dbSNP134 and the 1000 Genomes Project. Although not all of the mutations found in HNSCC cells represent somatic changes, such large annotated cell line collections can still be used to facilitate preclinical stratification for anticancer agent testing. To date, the genomic data from HNSCC cell lines and human tumors has not be linked to allow investigators to rationally select preclinical models for translational studies. The present study was undertaken in an attempt to address this gap in knowledge.

Genetic profiling of large cell line panels has been used to determine correlations between tumor and cell lines in other cancers. Neve *et al*. showed that 51 breast cancer cell lines display the same heterogeneity in copy number and gene expression as 145 primary subtype tumors. These breast cancer cell lines harbored most of the recurrent genomic abnormalities associated with clinical outcome in primary tumors (21). Lin *et al* reported that cultured melanoma cells encompass the spectrum of significant genomic alterations present in primary tumors (22). Although both of these prior reports suggested that cell lines could mirror primary tumors from the corresponding tumor type by genomic copy number and transcriptional profiles for the cell lines with primary tumors, gene mutations were not compared. In the present study we analyzed the correlation of mutations in 39 HNSCC cell

lines and 106 human tumors. Our results suggest that the mutated genes in HNSCC cell lines can reflect many, but not all, of the mutated genes in HNSCC primary tumors. 51% (334) of mutated genes were detected in both HNSCC cell lines and primary human HNSCC tumors and 83% (19 of the 23 genes) of genes with mutation frequencies over 5% in cell lines have similar mutation frequencies in primary tumors. Even in a specific signaling pathway such as the EGFR pathway, the mutations in cell lines mirror most of the mutations in human tumors. These findings suggest that genes commonly mutated in HNSCC tumors are generally reflected in cell line models.

Our results indicate some discordance between mutations in HNSCC cell lines and tumors including genes, which had higher mutation frequencies in cell lines and genes that were mutated only in cell lines, but not in tumors. In breast cancer, cell lines were reported to harbor more genetic aberrations and high-level amplifications than primary tumors (23). Ross *et al* analyzed the variation in expression of approximately 8,000 unique genes among the NCI60 cell lines and found that genes involved in proliferation were generally up-regulated in cell lines (24). Others have reported that expression of $p16^{INK4a}$ steadily increases in culture epithelial cells immortalized by telomerase while loss of $p16^{INK4a}$ expression is common in human cancers that are derived from epithelia (25, 26). It has been postulated that high-level gene amplification may provide a selective advantage for growth *in vitro* (22). Our analysis showed that many genes have higher mutation frequencies in HNSCC cell lines and 17 genes that are mutated only in HNSCC cell lines over 10%. We further extended our analysis to check these genes' mutation status in The Cancer Genome Atlas. (TCGA). There are 308 HNSCC tumor samples contained in the recently published TCGA, much more than the Stransky *et al* and Agrawal *et al* cohorts. Overall, the comparison yielded results consistent with our previous comparisons. Although the genes that had previously believed to be mutated exclusively in cell lines were also found to be mutated in TCGA tumor samples, they were found at frequencies far below their incidence in cell lines. For example, *VEGFC* is mutated in the majority of HNSCC cell lines (79.5%) but never in human HNSCC tumors in the Stransky *et al* and Agrawal *et al* cohorts. Although *VEGFC* is found to be mutated in TCGA, the mutation frequency is still less than 1% of the 308 tumors contained in this database. *VEGFC* is a member of the VEGF family and it plays an important role in lymphangiogenesis and angiogenesis in embryos and tumors. It enhances cancer cell mobility and invasiveness and contributes to the promotion of cancer cell metastasis by activating *Fit-4*. *VEGFC* overexpression in the lung cancer cell line H928 induces cell migration and invasion through the p38 MAPK pathway (27). It was reported by Benke *et al* that forced expression of *VEGFC* in HNSCC HN4 cells with low endogenous *CXCL5* levels increased cell growth and suppression of *VEGFC* inhibited migration of HNSCC HN12 cells (28). Although the functional consequences of *VEGFC* mutations are incompletely understood, the mutations of this gene, found uniquely in HNSCC cell lines, may represent an artifact of the culture process. It suggested that these genes, which just found mutated or have much higher mutation frequencies in HNSCC cell lines might be acquired through the immortalization process.

EGFR is a type 1 receptor tyrosine kinase and contributes to cell growth, development, and differentiation. *EGFR* is expressed in most epithelial tissues but is upregulated in many

epithelial malignancies including HNSCC where overexpression compared with corresponding normal tissues has been reported in 80-90% of cases (29). Gandhi *et al* analyzed the alterations of genes in the EGFR signaling pathway in 77 non-small cell lung cancer cell lines and copy number gains were frequent (>10%) for *EGFR*, *HER2*, *HER3* and *KRAS* (30). We found four genes in the EGFR pathway in HNSCC cell lines, which were amplified with the frequencies >10% including *PIK3CA*, *EGFR*, *ERBB2* and *AKT 1*. *PIK3CA* and *EGFR* were also found to be amplified in HNSCC tumors in primary HNSCC tumors. We and others have reported that *EGFR* gene amplification contributes to EGFR overexpression in HNSCC (31). *PIK3CA* amplification has also been found more frequently in HPV-positive HNSCC compared with HPV-negative tumors (13). In addition to gene amplification, twelve genes in the EGFR signaling pathway were mutated in both HNSCC cell lines and human tumors including *PIK3CA*, *RAS*, *RAF*, *STAT1*, *MAP3Ks* and *MAP2K4*. This finding suggests that genetic alteration of the components of the EGFR signaling pathway in HNSCC cell lines may reflect genetic alterations in primary HNSCC tumors.

A new generation of anticancer drugs with molecular targets has emerged as potent therapeutic agents. The EGFR signaling pathway has been implicated in HNSCC biology. Many agents inhibiting EGFR or components of the EGFR pathway have been FDA-approved for cancer treatment. In the CCLE pharmacologic profiling, six HNSCC cell lines were treated by seven agents targeting EGFR. HSC-2 and Detroit 562 cell lines, both of which harbor mutant *PIK3CA* (H1047R), were relatively resistant to seven EGFR pathway inhibitors compared to the other four HNSCC cell lines with wild type *PIK3CA*. *PIK3CA* is the p110α catalytic subunit of the class IA phosphatidylinositol 3-kinases (PI3Ks) and can be activated by growth factor receptor tyrosine kinases, including EGFR (32). We recently reported that genes in the PI3K pathway represent the most common alterations in HNSCC (13). In the present study we analyzed PI3K pathway mutations in HNSCC cell lines and tumors. Eight genes in the PI3K pathway were found to be mutated both in cell lines and tumors while four genes were only mutated in cell lines and four other genes were only reportedly mutated in tumors (Table 5). We also found that *PIK3CA* is the most commonly mutated gene in this pathway in HNSCC tumors (13). All of the *PIK3CA* mutants tested enhanced cell growth and cell lines harboring endogenous *PIK3CA* (H1047R) mutations demonstrated increased sensitivity to PI3K pathway inhibition using a mTOR/PI3K targeted agent (BEZ-235) in *vitro* and in *vivo*. Both HPV-positive and HPV-negative *PIK3CA*-mutated patient tumorgrafts were significantly more sensitive to BEZ-235 *in vivo* compared with tumors containing WT *PIK3CA*. Inhibition of tumor growth was accompanied by decreased PI3K signaling as demonstrated by downregulation of p-AKT (S473), and p-S6(S235/236) in the BEZ-235-treated tumors. *PIK3CA* mutants have been reported to contribute to drug resistance in other cancers but have not been studied to date in HNSCC cell line models. Berns *et al.* reported that the oncogenic *PIK3CA* mutant (H1047R) contributed to trastuzumab resistance in breast cancer cell lines. The presence of *PIK3CA* mutations or low *PTEN* expression was associated with poor prognosis after trastuzumab therapy in breast cancer patients (33). The *PIK3CA* mutant colorectal cancer cell line HCT116 was more resistant to EGFR targeted monoclonal antibodies compared with *PIK3CA* WT controls (34, 35). In *RAS* mutant cancer cells, activating *PIK3CA* mutations could reduce the sensitivity to *MEK* inhibition (36). Two recent studies found that EGFR

TKIs were relatively inactive in unselected HNSCC populations (37). HSC-2 and Detroit 562 cell lines were less sensitive to BRAF inhibitor RAF265 and MEK inhibitors AZD6244 and PD-0325901 in the CCLE report (8). These cumulative findings suggest that identification of individuals whose tumors harbor mutant *PIK3CA* may identify a subgroup of patients who may not be responsive to EGFR targeting and underscore the value of rational cell line model selection to guide the choice of therapies in the clinic.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was funded by grants: NIH P50CA097190, R01CA77308 and R01CA098372 and the American Cancer Society (to JRG)

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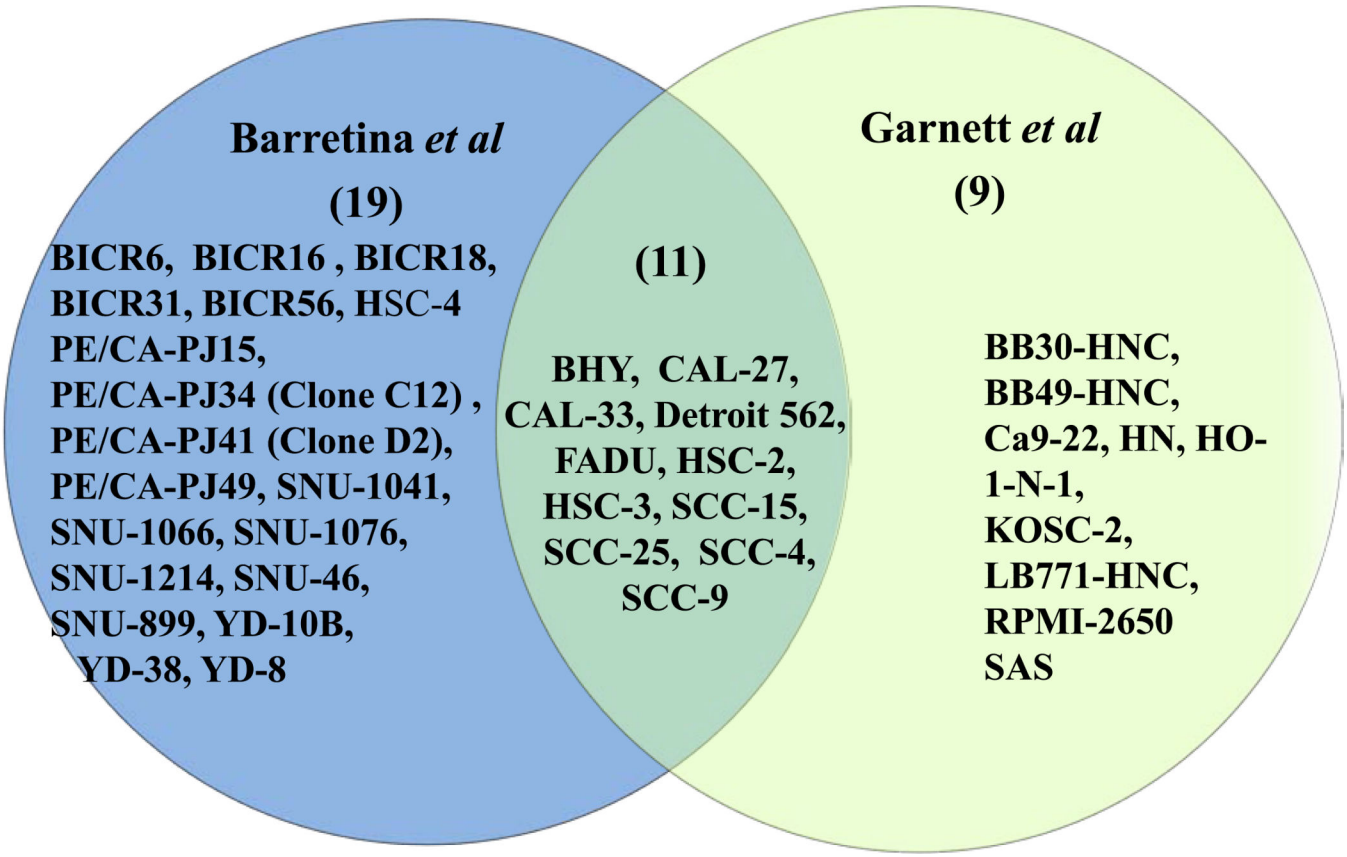
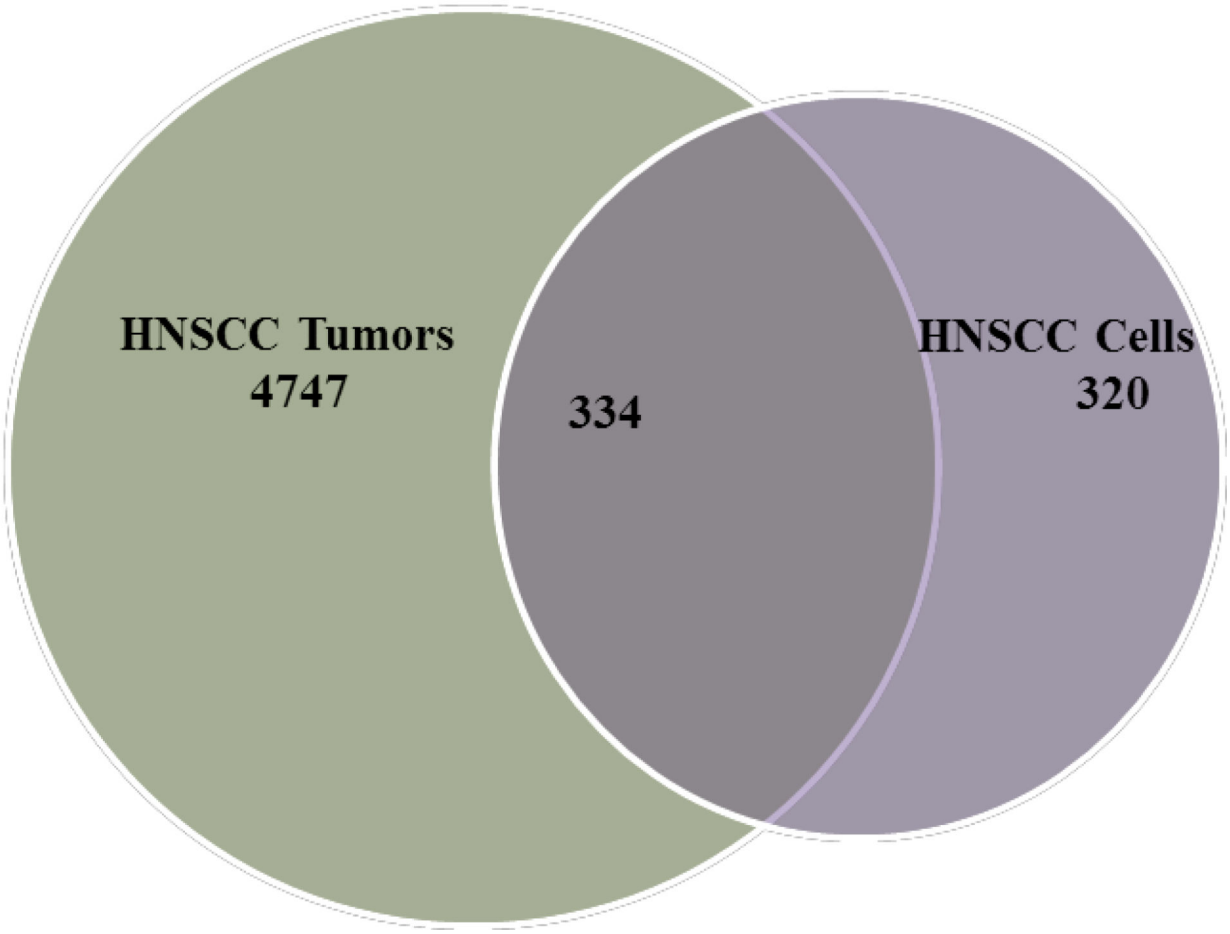


Figure 1. Venn diagram of unique and common HNSCC cell lines with sequence information

Cell lines derived from the squamous mucosal surfaces of the head and neck (HNSCC) were identified in the Barretina *et al* (blue) and/or in Garnett *et al* (yellow) databases and identified as unique or overlapping.

A



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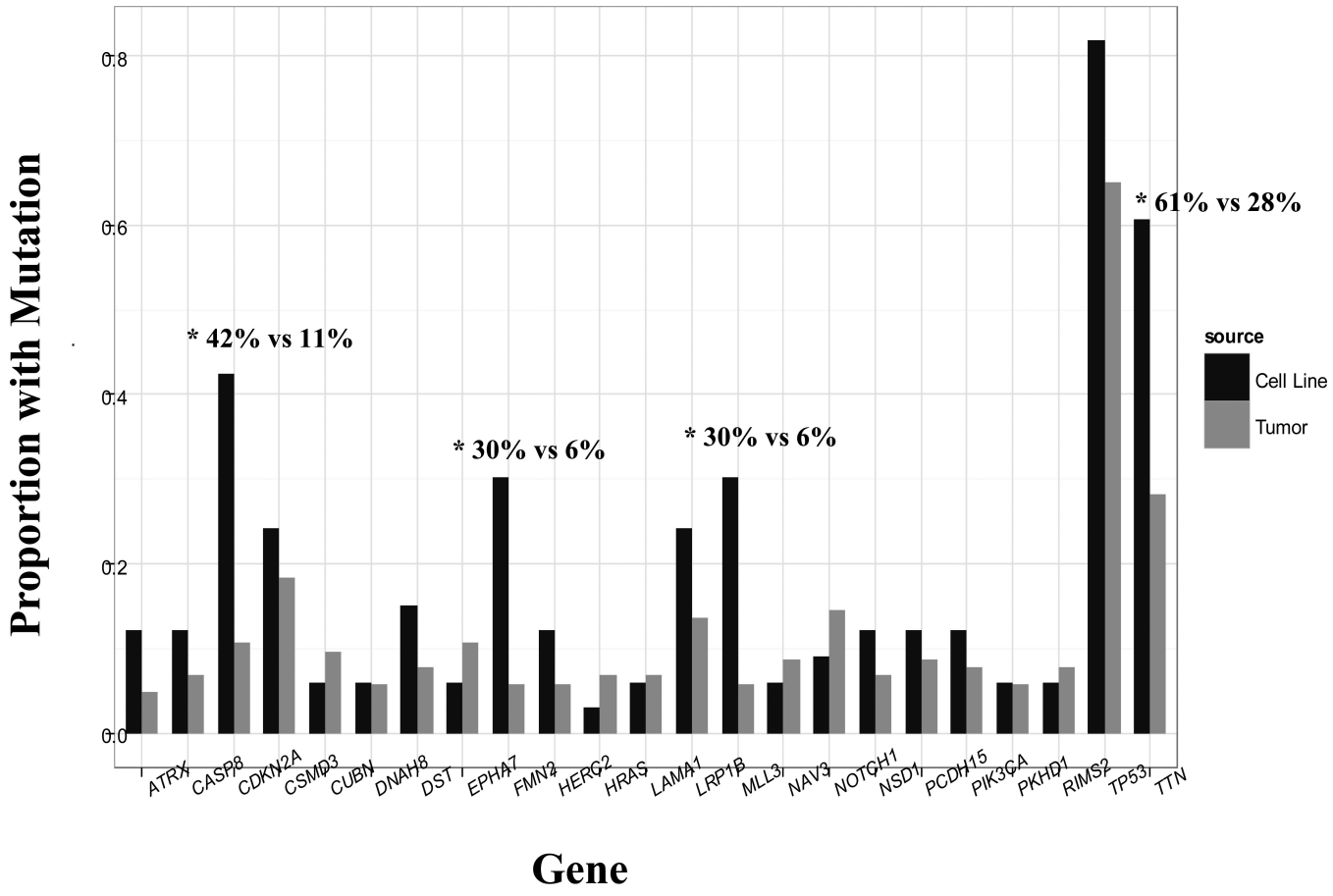


***= Proportion with mutation in cell lines vs tumor with FDR ≤ 10%**

C

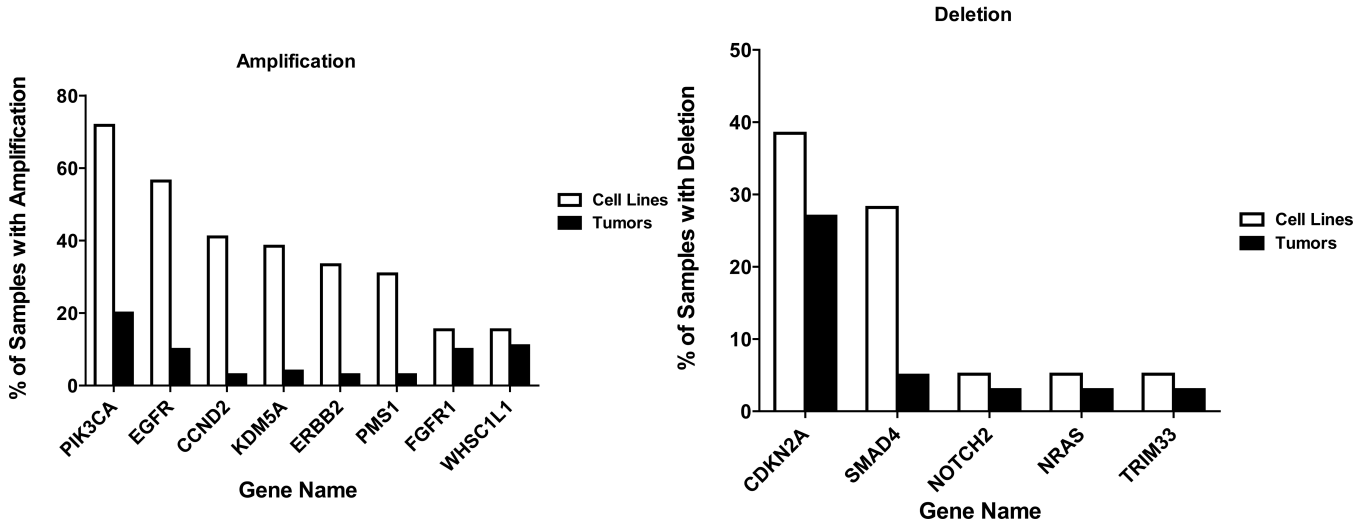


Figure 2. Comparison of gene mutation frequencies and copy numbers between HNSCC cell lines and human tumors

(A) Venn diagram of overlapping mutated genes in HNSCC cell lines (purple) and human tumors (green). Non-synonymous mutations are defined as all mutations, with the exception of silent mutations and mutations occurring in the 3' and 5' UTRs. (B) Comparison of the incidence of mutations in genes mutated in more than 5% of HNSCC cell lines and tumors derived from Larynx, Oral Cavity and Pharynx sites (* indicates significantly higher incidence of mutation in cell lines compared with tumors with FDR ≤ 10%). (C) Comparison of the incidence of gene copy number alterations in HNSCC cell lines and tumors. Left panel shows the incidence of the eight genes amplified both in HNSCC cell lines and tumors. Amplification is defined as having a log2 value of greater than or equal to 0.58. Right panel shows the incidence of the five genes deleted both in HNSCC cell lines and tumors. Deletion is defined as having a log2 value of less than or equal to −1.

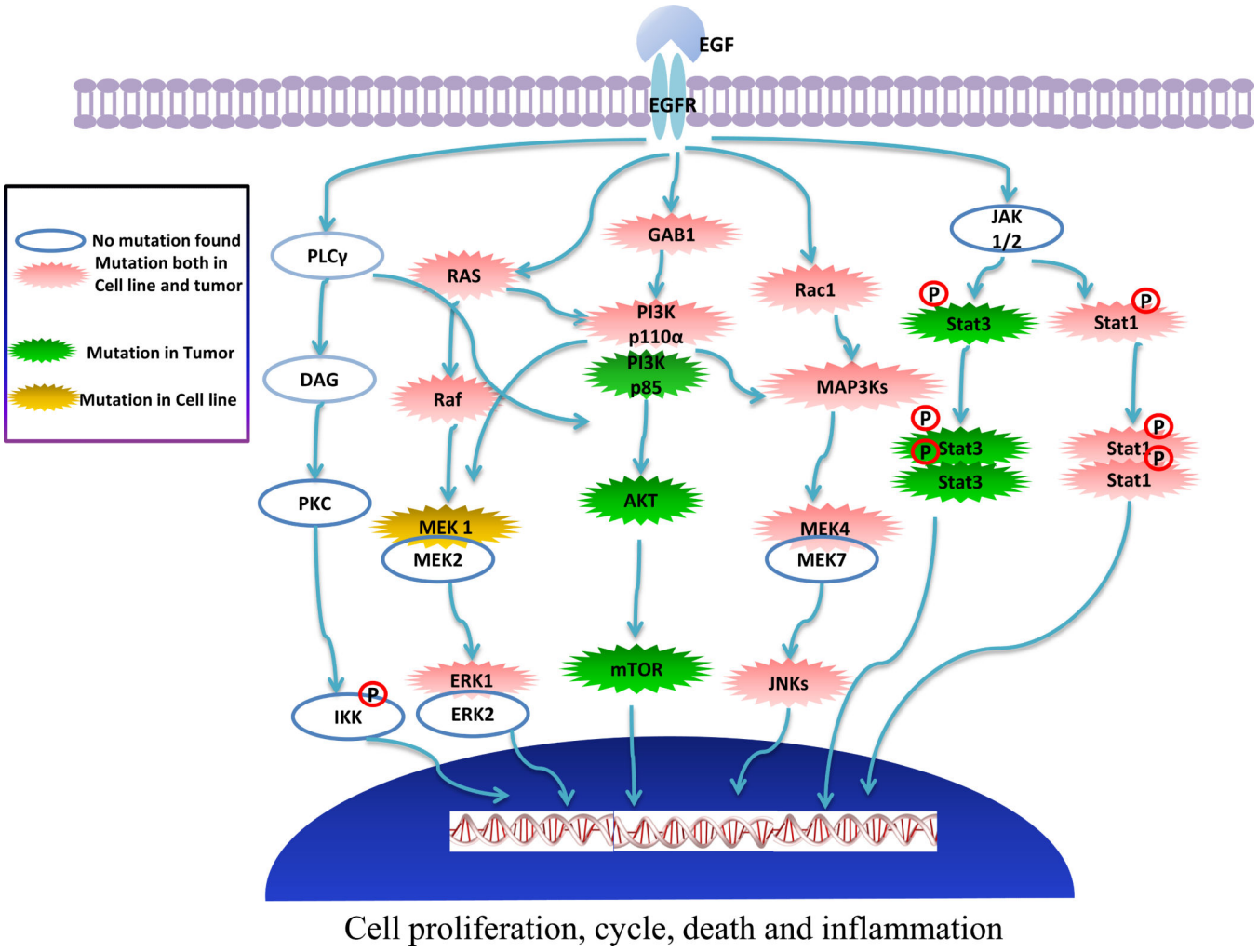
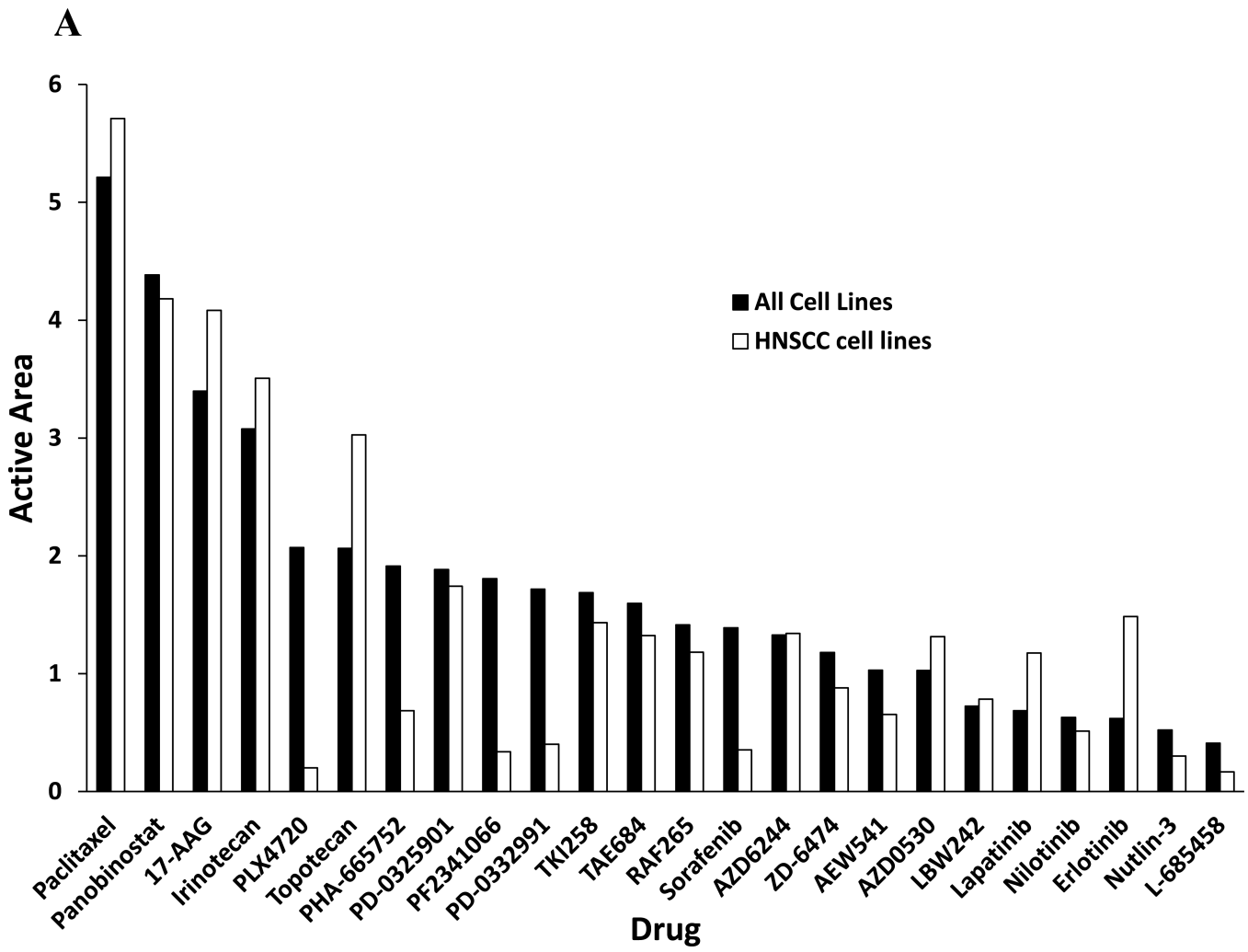


Figure 3. Genes mutated in EGFR signaling pathways in HNSCC cell lines and/or tumors

Unfilled circle indicates that the gene is not mutated in either HNSCC cell lines or human tumors. Red represents a gene that is mutated both in tumors and cell lines. Green indicates a gene that is mutated only in tumors and yellow denotes a gene that is only mutated in cell lines.



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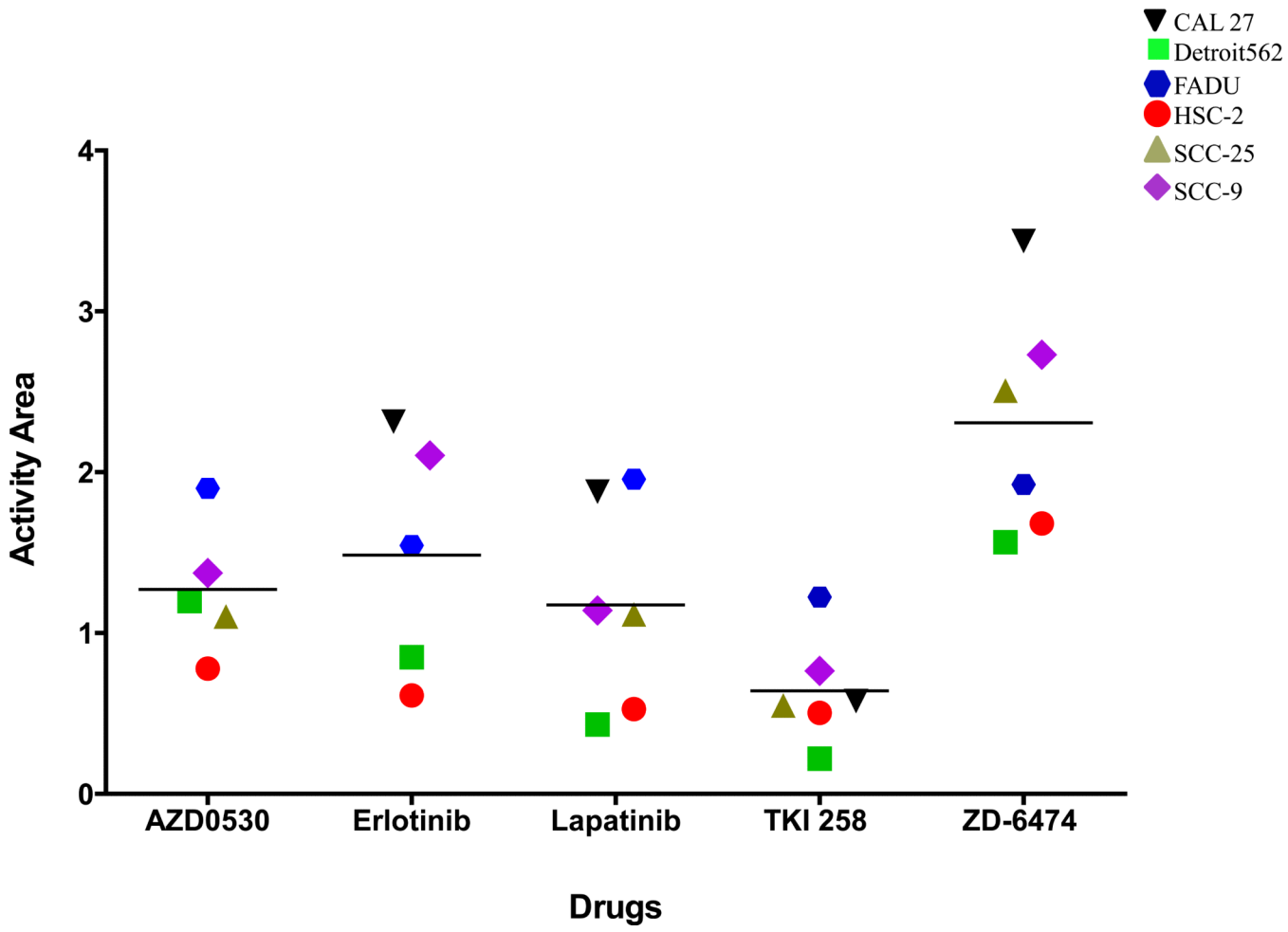


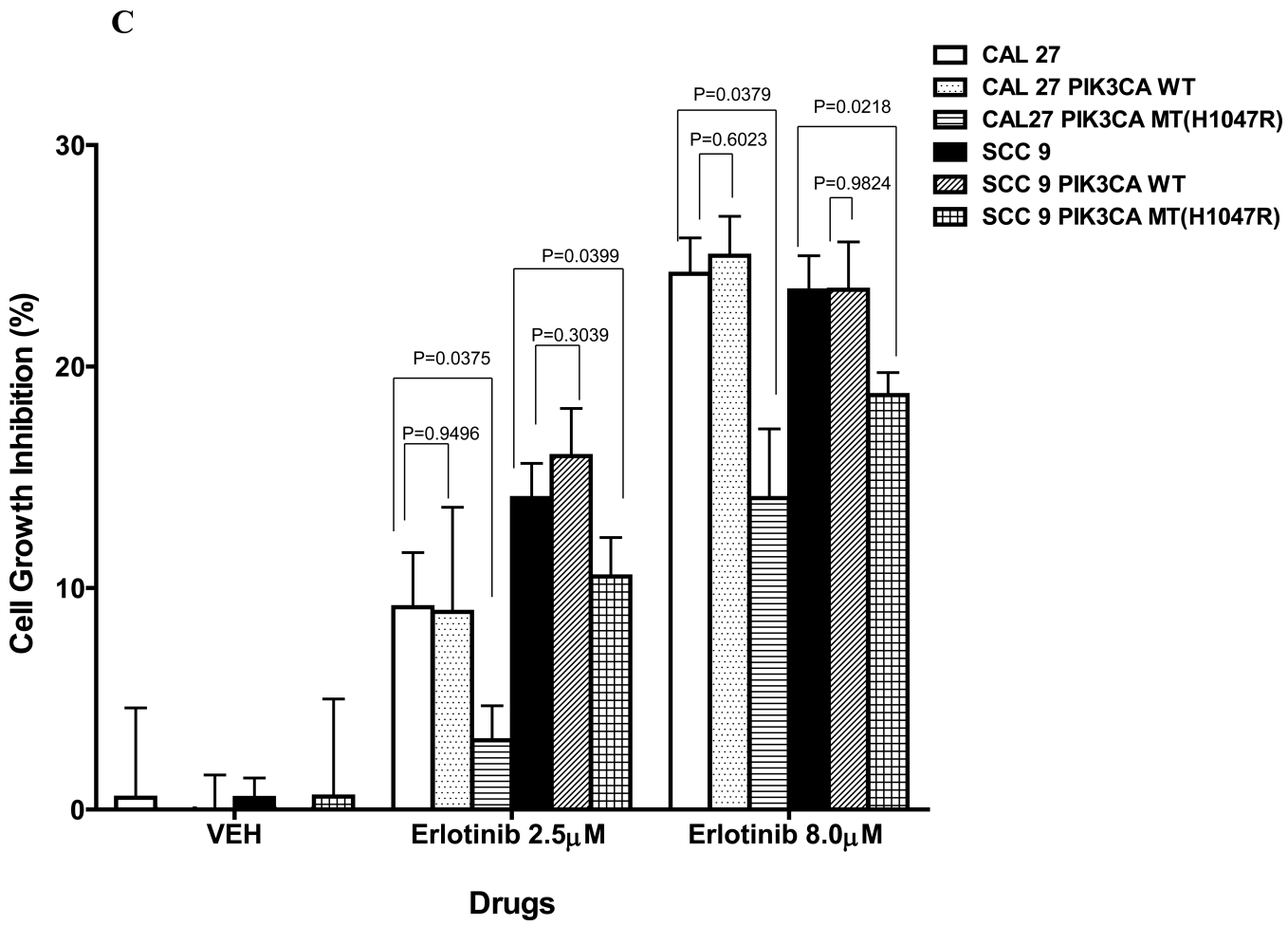


Figure 4. The pharmacological sensitivities of HNSCC cell lines

(A) Drug activity areas were compared between HNSCC cell lines with all cancer cell lines in CCLE. (B) Drug response of HNSCC cell lines for EGFR pathway inhibitors AZD0530, erlotinib, lapatinib, TKI 258 and ZD-6474 as measured by the activity area. The middle bar = median. Black: Cal 27; Green: Detroit 562; Blue: Fadu; Red: HSC-2; Yellow: SCC-25; Purple: SCC-9. (C) Expression of a *PIK3CA* mutation (H1047R) reduces sensitivity to erlotinib in Cal 27 and SCC 9 cells. *PIK3CA* mutant (H1047R) or WT *PIK3CA* were introduced into Cal27 and SCC9 cells followed by treatment with either of two concentrations of erlotinib (2.5 μM or 8.0μM) for 48h. Cell survival was measured by MTT assay. P values were calculated using an unpaired t-test with Welch's correction. The experiments were repeated three times with similar results.

Table 1

Clinical and Pathological Information for Sequenced HNSCC Cell Lines

Parameters	Class	Number	Proportion of none missing values
	Samples in only CCLE	19	48.72
	Samples in only Sanger	9	23.08
	Samples in both CCLE and Sanger	11	28.21
	Total	39	100
Age			
	<50 y	4	14.29
	>50 and <=60	13	46.43
	>60 and <=70	11	39.29
	Unknown	11	
Gender			
	Male	30	83.33
	Female	6	16.67
	Unknown	3	
Primary Site			
	Oral Cavity	23	67.65
	Pharynx	4	11.76
	Larynx	6	17.64
	Nasal septum	1	2.94
	Unknown	5	
Tumor Grade			
	Well Differentiated	3	21.43
	Moderately Differentiated	10	71.43
	Poorly Differentiated	1	7.14
	Unknown	25	
Tumor Type			
	Primary	7	63.64
	Metastatic	3	27.27
	Recurrent	1	9.09

Parameters	Class	Number	Proportion of none missing values
	Unknown	28	

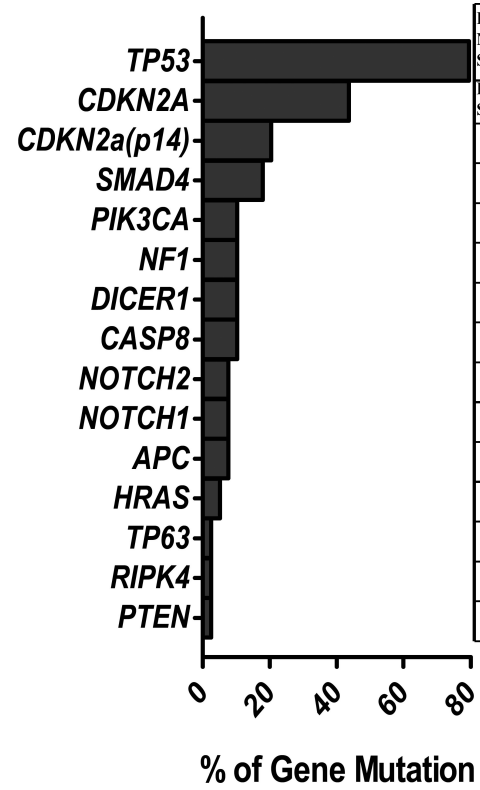
Table 2

Comparison of Sequencing Methods in Reported HNSCC Cell Lines and Tumors

	HNSCC Cell Line		HNSCC Tumor	
Database	**Barretina *et al***	**Garnett *et al***	**Stransky *et al***	**Agrawal *et al***
Sample Number	30	20	74	32
SNP determination	Affymetrix SNP6.0	Affymetrix SNP6.0	Affymetrix SNP6.0	Affymetrix SNP6.0
Sequencing Method	Illumina	Capillary sequencing	Illumina	Illumina/SOLID
Genes for sequencing	1651	77	Whole-exome	Whole-exome
Numbers of mutated genes	654	7	4897	501
Number of mutations	1637	49	7165	609

Table 3

Commonly Mutated Cancer Related Genes in HNSCC Cell Lines



BB30-HNC BHY BICR16 BICR31 BICR56 BICR6 Ca9-22 CAL27 CAL33 DETROIT562 FADU HN HO-1-N-1 HSC2 HSC3 HSC4 KOSC-2 LB771-HNC PECAPJ15 PECAPJ34CLONEC12 PECAPJ41CLONED2 SAS SCC15 SCC25 SCC4 SCC-9 SNU1066 SNU1214 SNU46 YD10B YD38
BB49-HNC BHY CAL-27 CAL-33 Detroit562 FADU HN HSC2 HSC3 HSC4 KOSC-2 LB771-HNC SCC-25 SCC-4 SCC-9 SNU1214 YD10B
BB49-HNC HSC-2 KOSC-2 LB771-HNC Detroit562 HN SCC-9 SCC-25
BB30-HNC BICR31 CAL27 CAL33 FADU HSC3 PECAPJ34CLONEC12
CAL33 DETROIT562 HSC-2 SNU-1076
SCC4 SCC9 YD10B YD8
BICR18 SNU1066 SNU1076 YD38
CAL27 CAL33 HSC2 HSC3
BICR6 HSC4 PECAPJ15
BICR56 PECAPJ49 HSC3
CAL27 CAL33 SNU1066
SCC9 BB49-HNC
HSC2
SNU899
HSC4

Table 4

Genes Mutated in >10% of HNSCC Cell Lines But Not Mutated in Human HNSCC Tumors

Gene Name	Number of Cell Lines with Mutation	Mutation Frequency (%)
VEGFC	31	79.49
PRKDC	30	76.92
GRIA3	30	76.92
MAML3	27	69.23
GPR112	23	58.97
NEK3	22	56.41
MAP3K1	18	46.15
MYST4	15	38.46
AKAP9	10	25.64
HSP90B1	9	23.08
CHD1	8	20.51
CDKN2a(p14)	6	15.38
RBPJ	5	12.82
PCSK7	4	10.26
MLL	4	10.26
TOP2B	4	10.26
KDM6A	4	10.26

Table 5

PI3K Pathway Mutations in HNSCC Cell Lines and Tumors

Gene Name	Cell line /Site	Tumor / Site
MTOR	BICR18 / p.V1692A	HN_62421 / p.L2260H HN_62469 / p.R1161Q
PIK3CA	CAL33 / p.H1047R DETROIT562 / p.H1047R HSC2 / p.H1047R SNU1076 / p.H1047R	HN_62415 / p.E545G HN_62426 / p.E545K HN_62469 / p.H1047R HN_62825 / p.R115L HN_63027 / p.E542K HN_63039 / p.H1047L HN11PT / p.H1047R HN41PT / p.H1047L 325 / p.H1047L
PIK3CD	SNU1076 / p.L558V	HN_62672 / p.T423S
PIK3CG	CAL27 / p.R178C SNU1041 / p.A197T SNU899 / p.R366G p.H577Y	HN_01000 /p.R1021S HN_62532 / p.L843H HN_62854 / p.S446F HN_63021 / p.P526S HN22PT / p.G491E
PIK3C2A	BICR6 / p.M1577V YD38 / p.A1649V	HN_62699 / c.e6 splice site
PIK3C2B	SNU899 / p.R1610H	HN_62739 / p.R564C
PIK3C2G	BHY / p.P129del BICR16 /p.P129del BICR18 /p.P129del BICR6 / p.P129del BICR56 / p.H1304R DETROIT562 /p.P129del PECAPJ15 /p.P129del PECAPJ49 /p.P129del PECAPJ41CLONED2 /p.P129del HSC2 /p.P129del HSC4 /p.P129del SCC15 /p.P129del SNU1041 /p.K714N SNU46 /p.E1301D YD8 /p.P129del YD38 /p.K70R YD38 /p.P129del	HN_00190 / p.V656L
PIK3R1		HN_00361 /p.453_454insN HN_62338 / p.I290V HN_62421 /p.D560H
PIK3R4	BICR56 /p.574_575ND>KY SCC25 / p.E529 splice YD10B / p.A968D	
PIK3R6		HN_62860 / p.R483H
PTEN	HSC4 / p.C136W p.V343 splice	HN_00190 / p.D92E HN_62652 /c.e7_splice_site HN_62741 /p.D252Y HN_62863 / p.P246L HN_63039 / p.R335*
PDK1	BICR18 / p.P283T	
PIK3AP1		HN_62506 /p.A136D HN22PT / p.G313R 91 / p.R478Q 266 / p.T352S
PIK3C3	SNU1066 /p.R162K	
TSC1		HN_00761 / p.R245*
TSC2	PECAPJ49 / p.R1268C	