The Frequency and Impact of Fibroblast Growth Factor Receptor 1 Amplification and p16 Protein Expression on Clinical Outcomes in Resected Head and Neck Squamous Cell Carcinoma

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Directed by Professor Byoung Chul Cho

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Su Jin Heo

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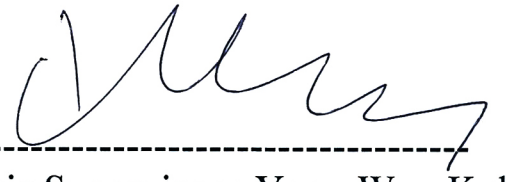
This certifies that the Master's Thesis of
Su Jin Heo is approved.



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ABSTRACT

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(Directed by Professor Byoung Chul Cho)

The aim of this study was to investigate the frequency and the impact of *Fibroblast growth factor receptor1 (FGFR1)* amplification and p16 protein expression on clinical outcomes in curatively resected head and neck squamous cell carcinoma (HNSCC). Tumor tissue from 383 patients with HNSCC from November 2005 and December 2012 were collected and analyzed using an *FGFR1* fluorescent in situ hybridization (FISH) assay. High amplification was defined as percentage of tumor cells containing ≥ 9 signals in ≥ 20% cells, and low amplification was defined as percentage of tumor cells containing 2~8 signals in ≥ 20% cells. High and low amplifications were detected in 1.0% and 41.3%, respectively. In our study, the prognostic impact of *FGFR1* amplification was not observed, probably due to tumor heterogeneity in HNSCC, unstandardized FISH criteria for *FGFR1* amplification, varying adjuvant treatment, and wide variation in *FGFR1* amplification group may contribute to the controversial result. And, as known, p16 protein expression was confirmed as a strong and independent predictor of survival. Further studies are needed to identify the criteria for *FGFR1* amplification and its therapeutic efficacy.

Key words : *FGFR1*, p16, squamous cell carcinoma, head and neck

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I. INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) arises from mucosa lining the paranasal sinuses, nasal cavities, oral cavity, oropharynx, hypopharynx, and larynx. It is the sixth leading cancer by incidence worldwide which affect 600,000 patients per year with 40-50% 5-year survival rate[1]. Risk factors included tobacco use, alcohol consumption, human papilloma virus (HPV) infection, and genetic disorders such as Fanconi Anemia[1-3]. Most of patients with HNSCC are treated with largely uniform approach based on stage and anatomic location, typically using surgery, radiation therapy, and chemotherapy alone or in combination. Despite these multidisciplinary treatments, approximately half of all patients will die of the disease because of local aggressiveness and high rate of early relapse[4]. Cetuximab, anti-epidermal growth factor receptor (EGFR) antibody, is the only approved target agent in treatment of HNSCC since 2006 and yielded modest increases in response rate of 10-13% when used in combination with standard chemotherapy and radiation therapy. Unlike other types of cancers, there have been no validated predictive biomarkers for benefit from cetuximab in HNSCC[5]. Recently, the cancer genome atlas (TCGA) profiled 279 HNSCC to provide a comprehensive landscape of somatic genomic alterations according to HPV status, smoking, and primary tumor sites[6]. Nevertheless, there are no effective targeted

therapies available for HNSCC. The effort to identify the novel therapeutic targets and prognostic markers in HNSCC is under way.

The Fibroblast Growth Factor Receptor (FGFR) tyrosine kinase family comprises four kinases: FGFR1, FGFR2, FGFR3, and FGFR4. These kinases play crucial roles in cancer development and targets for dysregulation by amplification, point mutations, and translocation in many cancers[7]. Amplification or activation of *FGFR1* has been reported in breast adenocarcinoma, lung squamous cell cancer (lung SqCC), esophageal squamous cell carcinomas, ovarian cancer, bladder cancer, rhabdomyosarcoma, and oral squamous carcinoma[8-11]. Kim et al[12] recently reported *FGFR1* amplification in 13% of lung SqCC and its negative prognostic impact. Also, their study showed a positive association between *FGFR1* amplification and smoking dosage. In resected esophageal squamous cell carcinoma, high *FGFR1* amplification had a greater risk of recurrence and death. Like in lung SqCC, high amplification was significantly higher in current smokers than former and never-smokers and increased proportional to smoking dosage[13].

Based on the histomorphological and clinical similarities between HNSCC and other squamous cell carcinoma, many studies have been described a potential role of *FGFR1*. In the first study about *FGFR1* amplification in HNSCC, Freier et al[11] reported *FGFR1* amplifications in 17% oral squamous cell carcinomas in a limited number of patient samples. Recently, Goke et al[14] described *FGFR1* amplification in 15% of patients with HNSCC. It was associated with nicotine and alcohol consumption and parameters of worse outcome, so it represents a potential role of therapeutic biomarker. In vivo studies have demonstrated inhibition of the *FGFR1* pathway with FGFR inhibitors that led to significant tumor shrinkage, suggesting that FGFR inhibitors might be and effective therapeutic option in HNSCC with *FGFR1* amplification[15].

HPV status in tumors can be determined by several assays, including HPV DNA detection by in situ hybridization (ISH) or polymerase chain reaction (PCR), HPVE6/E7 RNA expression detected by quantitative reverse transcriptase–PCR (qRT-PCR), and/or p16 protein expression by immunohistochemistry (IHC) staining as a surrogate marker of oncogenic HPV infection[16-20]. Among these

assays, detection of HPV E6/E7 RNA expression, which indicates active viral oncogene transcription in tumor cells, is considered to be a gold standard[18,19]. However, because RNA isolation for qRT-PCR requires additional sample preparation steps and a larger amount of tumor cells compared with other assays, the most widely used assays are HPV ISH and p16 IHC. The p16 protein is an important tumor suppressor and cell-cycle regulator[21]. In HPV-positive tumors, the viral protein E7 binds to retinoblastoma susceptibility protein (Rb) through cullin 2 ubiquitin ligase complex and rapidly degrades Rb by ubiquitination[22]. Loss of Rb results in upregulation of p16 protein expression by a feedback interaction[23,24]. It is well established that patients with HPV-positive/p16-positive HNSCC have a more favorable prognosis compared with those with HPV-negative/p16-negative HNSCC[16,17,20,25,26].

In this study, we sought to determine the frequency and the impact of *FGFR1* amplification and p16 protein expression on clinical outcomes in curatively resected with surgically resected HNSCC.

II. MATERIALS AND METHODS

1. Patients

This study was conducted in a cohort of patients with HNSCC who underwent curative resection at Severance Hospital, Seoul, Korea, between November 2005 and December 2012. The criteria used for patient selection included (1) surgically resected HNSCC for curative aim, (2) availability of tumor tissue and clinical data on smoking status and survival, (3) no preoperative treatment, and (4) no distant metastasis. The primary tumor sites categorized in four groups as following: oral cavity, oropharynx, hypopharynx, and larynx. Oral cavity included hard palate, tongue, and buccal mucosa. Oropharynx included flour of mouth, base of tongue, and tonsils. Hypopharynx included pyriform sinus and larynx included supraglottis and glottis. We excluded 121 cases which had undergone the process of decalcification and 13 cases which were

not profit to produce tissue microarray. Finally, the tumor samples of 384 patients were available for examination of *FGFR1* amplification. Two pathologists (S.O.Y. and E.K.K.) confirmed the diagnosis of HNSCC by hematoxylin and eosin staining. Paraffin-embedded tumor specimens were used to construct a tissue microarray with 2-mm-diameter cores. Each patient was represented by three tissue cores. Patients' information was collected by reviewing the medical records for evaluation of clinicopathologic characteristics and survival outcomes. Staging was determined using the 7th edition American Joint Committee on Cancer (AJCC) guideline for tumor, node, and metastasis (TNM) classification. Never-smokers were defined as those with a lifetime smoking dose of fewer than 100 cigarettes, former smokers were those who had stopped smoking for more than 1 year, and current smokers were those who currently smoke or quit smoking for less than 1 year[27].

2. *FGFR1* FISH method

Fluorescent in situ hybridization (FISH) assay was performed on the tissue microarrays by using *FGFR1* probes that hybridized to the 8p12–8p11.23 region using the fluorophore, Spectrum Orange (red) and to the centromere region of chromo-some 8 (CEP 8) using the fluorophore, Spectrum Green (Abbott Molecular, Abbott Park, IL) following the manufacturers' instructions. FISH analyses were interpreted by two experienced evaluators (S.O.Y. and E.K.K.) blinded to the clinical data. Cells with sharp borders of nuclei, no signs of overdigestion, non-overlapping nuclei were evaluated. Normal tissue including vessels, fibroblasts, or non-tumor squamous epithelium served as internal positive control. Cases were only further evaluated if control tissue nuclei displayed one or two clearly distinct signals of each color. Tumor tissue was scanned for amplification hot spots by using x 40 or x 63 objectives. If the *FGFR1* signals were homogeneously distributed, then random areas were used for counting the signals. Twenty contiguous tumor cell nuclei

from three hot spots or random areas, resulting in a total of 60 nuclei, were individually evaluated with the x 100 objectives by counting red *FGFR1* and green centromere of chromosome 8 (CEP8) signals. *FGFR1* amplification was defined based on the previous study[14] (Table1).

Table1. Definition of *FGFR1* amplification[14]

High amplification	Nine or more red target signals or clusters of target gene signals as compared with the green reference signals displayed in at least 20% nuclei
Low amplification	Lower than nine but more than two red target signals as compared with the green reference signals displayed in at least 20% nuclei

3. p16 Immunohistochemistry

Tumor p16 expression was evaluated by IHC using a mouse monocoloncal antibody (Clone E0037, Ventana, AZ, USA) and was visualized with the Ventana XT autostainer using the the 1-view secondary detection kit (Ventatn, Tuscon, AZ) for details see manufacturer's recommendations. Tumor p16 expression was scored as positive if strong and diffuse nuclear and cytoplasmic staining was present in at least 75% of the tumor cells, and alternatively >50% staining combined with >25% confluent areas[28,29].

4. Statistical Analyses

Our primary objective was to evaluate the frequency of *FGFR1* amplification and p16 protein expression in patients with HNSCC. Our secondary objectives were to identify the clinical features of patients with *FGFR1* amplified and p16 expressed tumors and to analyze its impact of *FGFR1* on disease-free survival (DFS) and overall survival (OS) in patients. DFS was measured from the time of surgery to initial tumor relapse (local recurrence or distant) or death as a result of any cause. OS, calculated from the time of surgery to death or last follow-up date, and 95% confidence intervals (CIs) were evaluated by survival

analysis using the Kaplan-Meier method. We used Chi-square, Fisher's exact, and Mann-Whitney tests to compare the clinical factors among the patients with level of *FGFR1* amplification and p16 status. Statistical significance was set at $P < .05$ for all analyses. Survival outcomes among the group were compared by using the log-rank test. Multivariate analysis was performed by using Cox regression analysis with the following prespecified variables: sex, smoking, primary tumor site, histologic differentiation, lymphovascular invasion, perineural invasion, resection margin, p16 status, pathologic T stage, pathologic N stage, and *FGFR1* amplification according to both categories. All statistical analyses were performed by using SPSS version 20.0 (SPSS, Chicago, IL).

III. RESULTS

1. Patient Characteristics

A total of 383 patients with surgically resected HNSCC were analyzed of *FGFR1* amplification. The clinical characteristics of the enrolled patients are shown in Table2 and 3, divided by categories A and B. There were 287 (74.9%) male and 96 (25.1%) female with a median age of 58 years (range 22-88). The majority of patients were current (40.5%) or former (20.4%) smokers, and median smoking dosage was 17.0 pack-years (range 0-100). Sites of primary tumor were distributed as follows: 51.7% in oral cavity, 31.1% in oropharynx, 7.3% in hypopharynx, and 9.9% in larynx. The histologic differentiations of squamous cell carcinoma were 36.8% in well differentiation, 50.1% in moderated differentiation, and 13.1% in poor differentiation. In pathologic results, lymphovascular invasion was 19.1%, perineural invasion was 13.3%, and the positive of resection margin was 23.2%. About half of tumors had pathologic T1 or N0 stage. The AJCC stages were I in 27.9%, stage II in 11.2%, stage III in 18.0%, stage IVA in

42.3% and stage IVB in 0.5%. Adjuvant treatment was given in 242 (63.2%) patients and of those, 95 patients were received adjuvant concurrent chemoradiation therapy (CCRT) and 147 patients were received adjuvant radiation therapy.

Table 2. Baseline characteristics of the patients according to *FGFR1* amplification status

Characteristics	All patients n (%)	High amplification n (%)	Low amplification n (%)	No amplification n (%)	*P*-value
Number of patients	383	4 (1.0)	158 (41.3)	221 (57.7)	
Age, year					0.430
Median	58.0	69.5	59.0	58.0	
Range	22-88	42-73	24-87	22-88	
Sex					0.631
Male	287 (74.9)	4 (100.0)	120 (75.9)	163 (73.8)	
Female	96 (25.1)	0 (0.0)	38 (24.1)	58 (26.2)	
Smoking					0.579
Never smoker	150 (39.2)	0 (0.0)	62 (39.2)	88 (39.8)	
Former smoker	78 (20.4)	1 (25.0)	32 (20.3)	45 (20.4)	
Current smoker	155 (40.5)	3 (75.0)	64 (40.5)	88 (39.8)	
Smoking dosage, pack/years					0.061
Median	17.0	45.0	15.0	17.0	
Range	0-100	22-60	0-80	0-100	
Primary sites					0.206
Oral cavity	198 (51.7)	1 (25.0)	78 (49.4)	119 (53.8)	
Oropharynx	119 (31.1)	2 (50.0)	45 (28.5)	72 (32.6)	
Hypopharynx	28 (7.3)	0 (0.0)	16 (10.1)	12 (5.4)	
Larynx	38 (9.9)	1 (25.0)	19 (12.0)	18 (8.1)	
Histologic differentiation					0.106
Well differentiated	141 (36.8)	1 (25.0)	67 (42.4)	73 (33.0)	
Moderated differentiated	192 (50.1)	2 (50.0)	77 (48.7)	113 (51.1)	
Poorly differentiated	50 (13.1)	1 (25.0)	14 (9.9)	35 (15.8)	
Lymphovascular invasion					0.322
Yes	73 (19.1)	1 (25.0)	35 (22.2)	37 (16.7)	
No	310 (80.9)	3 (75.0)	123 (77.8)	184 (83.3)	

Perineural invasion					0.555
Yes	51 (13.3)	1 (25.0)	20 (12.7)	30 (13.6)	
No	332 (86.7)	3 (75.0)	138 (87.3)	191 (86.4)	
Resection margin					0.310
Positive	89 (23.2)	2 (50.0)	34 (21.5)	53 (24.0)	
Negative	294 (76.8)	2 (50.0)	124 (78.5)	168 (76.0)	
T stage					0.946
T1	169 (44.1)	2 (50.0)	69 (43.7)	98 (44.3)	
T2	145 (37.9)	2 (50.0)	60 (38.0)	83 (37.6)	
T3	29 (7.6)	0 (0.0)	10 (6.3)	19 (8.6)	
T4	40 (10.4)	0 (0.0)	19 (12.0)	21 (9.5)	
N stage					0.986
N0	167 (43.6)	2 (50.0)	71 (44.9)	94 (42.5)	
N1	69 (18.0)	0 (0.0)	28 (17.7)	41 (18.6)	
N2	145 (37.9)	2 (50.0)	58 (36.7)	85 (38.5)	
N3	2 (0.5)	0 (0.0)	1 (0.6)	1 (0.5)	
AJCC stage					0.870
Stage I	107 (27.9)	1 (25.0)	44 (27.8)	62 (28.1)	
Stage II	43 (11.2)	1 (25.0)	21 (13.3)	21 (9.5)	
Stage III	69 (18.0)	0 (0.0)	27 (17.1)	42 (19.0)	
Stage IVA	162 (42.3)	2 (50.0)	65 (41.1)	95 (43.0)	
Stage IVB	2 (0.5)	0 (0.0)	1 (0.6)	1 (0.5)	
Adjuvant treatment					0.079
CCRT	95 (24.8)	3 (75.0)	43 (27.2)	49 (22.2)	
Radiotherapy	147 (38.4)	0 (0.0)	47 (29.7)	100 (45.2)	
No	141 (36.8)	1 (25.0)	68 (43.0)	72 (32.6)	
FGFR1 FISH amplification					
Number (median, range)	2.08 (1.55-10.08)	7.30 (6.75-10.08)	2.67 (1.97-6.08)	2.08 (1.55-2.47)	<0.001
FGFR1/CEP8 ratio (median, range)	0.99 (0.43-5.13)	3.40 (2.40-5.13)	0.97 (0.43-2.76)	0.99 (0.48-1.24)	<0.001
p16 status					0.043
Positive	162 (42.3)	0 (0.0)	59 (37.3)	103 (46.6)	
Negative	221 (57.7)	4 (100.0)	99 (62.7)	118 (53.4)	

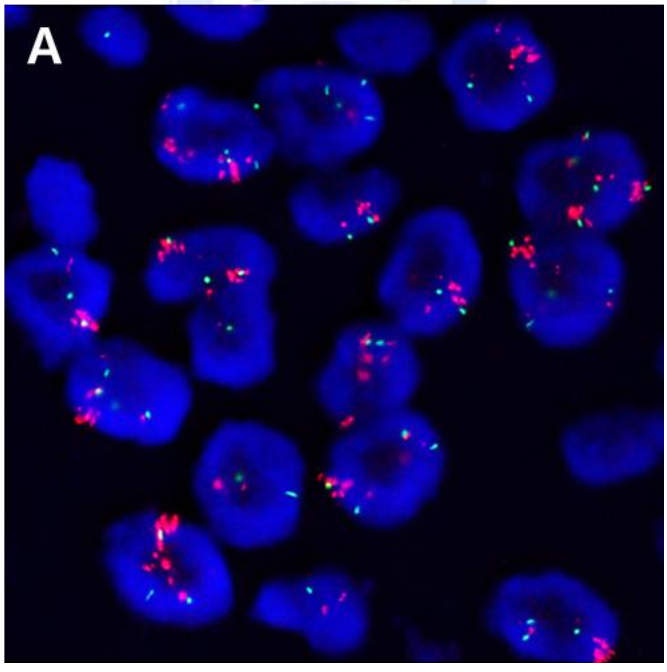
Abbreviations: AJCC, American Joint Committee on Cancer; CCRT, Concurrent chemoradiation therapy *; FGFR1*, *Fibroblast Growth Factor Receptor1*; FISH, Fluorescent in situ hybridization

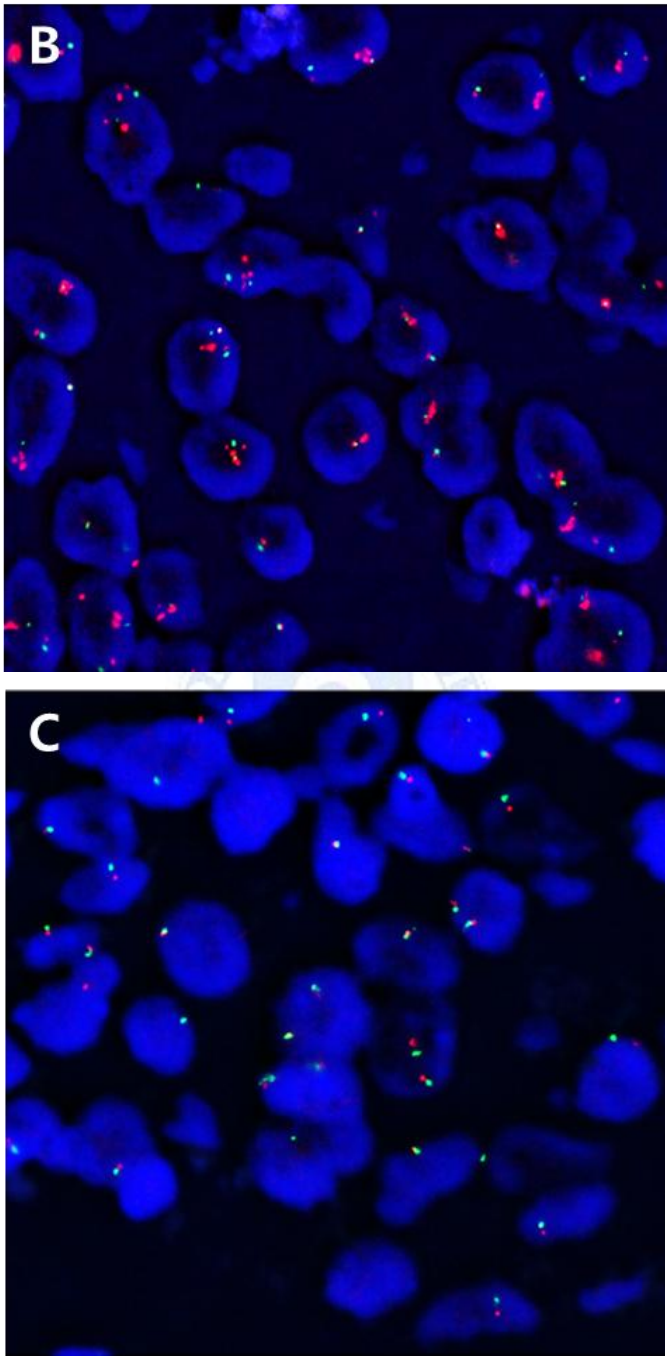
2. *FGFR1* Amplification and p16 status

Among a total of 383 patients, 4 (1.0%) were high *FGFR1* amplification, 158 (41.3%) were low *FGFR1* amplification, and 221

(57.7%) were no amplification (Table2; Figure1). The median *FGFR1* gene copy number per nucleus and the mean *FGFR1*/CEN8 ratio in all patients were 2.08 (range, 1.55 to 10.08 copies per nucleus) and 0.99 (range, 0.43 to 5.13). The median *FGFR1* gene copy number was 7.30 (range, 6.75 to 10.08) in high amplification, 2.67 (range, 1.97 to 6.08) in low amplification, and 2.08 (range 1.55 to 2.47) in no amplification group. The median *FGFR1*/CEN8 ratio was 3.40 (range 2.40 to 5.13), 0.97 (range, 0.43 to 2.76), and 0.99 (range, 0.48 to 1.24) in high, low and no amplification group, respectively. The p16 negative status related to unfavorable prognosis was 100% in high amplification, 62.7% in low amplification, and 53.4% in no amplification. There was statistically significant difference among these three groups.

Figure 1. *Fibroblast growth factor receptor1 (FGFR1)* amplification assessed by fluorescent in situ hybridization (FISH). (A) High *FGFR1* amplification; (B) low *FGFR1* amplification; (C) no amplification.





Based on p16 status, 162 (42.4%) patients were p16 positive and 221

(57.6%) patients were p16 negative in total cohort (Table 3). There were significant difference in smoking status, primary sites, histologic differentiation, lymphovascular invasion, resection margin, pathologic stage, and *FGFR1* amplification between two groups. In primary tumor sites, oropharynx was most common in p16 positive group, and oral cavity, hypopharynx, larynx were relatively common in p16 negative group. Favorable histologic differentiation, no lymphovascular invasion, negative resection margin, early pathologic stage which known to related to good prognosis were common in p16 negative group. In contrast, gene copy number of *FGFR1* amplification was higher in p16 negative group than in p16 positive group.

Table 3. Baseline characteristics of the patients according to p16 status

Characteristics	All patients n (%)	p16 positive n (%)	p16 negative n (%)	*P*-value
Number of patients	383	162 (42.4)	221 (57.6)	
Age, year				0.465
Median	58.0	57.0	59.0	
Range	22-88	23-87	22-88	
Sex				0.925
Male	287 (74.9)	121 (74.7)	166 (75.1)	
Female	96 (25.1)	41 (25.3)	55 (24.9)	
Smoking				0.037
Never smoker	150 (39.2)	69 (42.6)	81 (36.7)	
Former smoker	78 (20.4)	23 (14.2)	55 (24.9)	
Current smoker	155 (40.5)	70 (43.2)	85 (38.5)	
Smoking dosage, pack/years				0.979
Median	17.0	15.0	20.0	
Range	0-100	0-100	0-100	
Primary sites				<0.001
Oral cavity	198 (51.7)	51 (31.5)	147 (66.5)	
Oropharynx	119 (31.1)	98 (60.5)	21 (9.5)	
Hypopharynx	28 (7.3)	5 (3.1)	23 (10.4)	
Larynx	38 (9.9)	8 (4.9)	30 (13.6)	
Histologic differentiation				<0.001
Well differentiated	141 (36.8)	32 (19.8)	109 (49.3)	

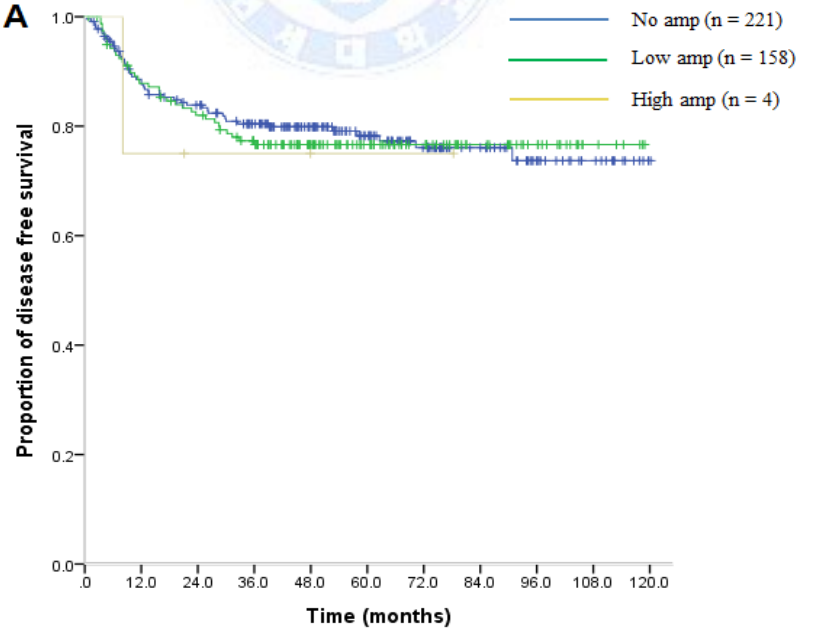
Moderated differentiated	192 (50.1)	96 (59.3)	96 (43.4)	
Poorly differentiated	50 (13.1)	34 (21.0)	16 (7.2)	
Lymphovascular invasion				<0.001
Yes	73 (19.1)	45 (27.8)	28 (12.7)	
No	310 (80.9)	117 (72.2)	193 (87.3)	
Perineural invasion				0.090
Yes	51 (13.3)	16 (9.9)	35 (15.8)	
No	332 (86.7)	146 (90.1)	186 (84.2)	
Resection margin				0.011
Positive	89 (23.2)	48 (29.6)	41 (18.6)	
Negative	294 (76.8)	114 (70.4)	180 (81.4)	
T stage				0.002
T1	169 (44.1)	56 (34.6)	113 (51.1)	
T2	145 (37.9)	78 (48.1)	67 (30.3)	
T3	29 (7.6)	14 (8.6)	15 (6.8)	
T4	40 (10.4)	14 (8.6)	26 (11.8)	
N stage				0.001
N0	167 (43.6)	56 (34.6)	111 (50.2)	
N1	69 (18.0)	26 (16.0)	43 (19.5)	
N2	145 (37.9)	80 (49.4)	65 (29.4)	
N3	2 (0.5)	0 (0.0)	2 (0.9)	
AJCC stage				0.001
Stage I	107 (27.9)	30 (18.5)	77 (34.8)	
Stage II	43 (11.2)	21 (13.0)	22 (10.0)	
Stage III	69 (18.0)	26 (16.0)	43 (19.5)	
Stage IVA	162 (42.3)	85 (52.5)	77 (34.8)	
Stage IVB	2 (0.5)	0 (0.0)	2 (0.9)	
Adjuvant treatment				<0.001
CCRT	95 (24.8)	46 (28.4)	49 (22.2)	
Radiotherapy	147 (38.4)	81 (50.0)	66 (29.9)	
No	141 (36.8)	35 (21.6)	106 (48.0)	
FGFR1 FISH amplification				
Number (median, range)	2.08 (1.55-10.08)	2.06 (1.62-5.43)	2.10 (1.55-10.08)	0.001
FGFR1/CEP8 ratio (median, range)	0.99 (0.43-5.13)	1.00 (0.58-2-23)	0.97 (0.43-5.13)	0.185

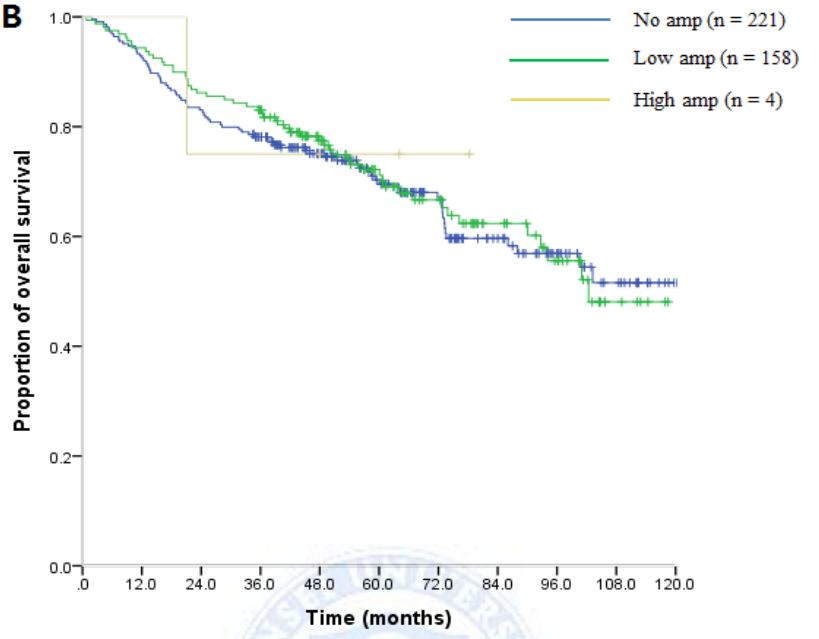
Abbreviations: AJCC, American Joint Committee on Cancer; CCRT, Concurrent chemoradiation therapy *; FGFR1*, *Fibroblast Growth Factor Receptor1*; FISH, Fluorescent in situ hybridization

3. Survival Outcomes According to *FGFR1* Amplification and p16 status

With a median follow-up time of 53.1 months, the 5-year DFS and OS rates for all patients were 150 (39.2%) and 167 (43.6%), respectively. The median DFS for each of the three *FGFR1* groups were not reached (Figure 2A). In comparison of mean survival, patients with high *FGFR1* amplification showed shorter DFS than those with low and no amplification (60.8 vs 95.2 months in low amplification and 95.9 months in no amplification, *P*=0.955). Figure 2B showed OS in Kaplan-Meier method, the median OS for one of the three *FGFR1* groups were not reached. Regarding the mean survival, patients with high *FGFR1* amplification showed shorter OS than those with low and no amplification (64.0 vs 85.9 months in low amplification and 85.8 months in no amplification, *P*=0.933).

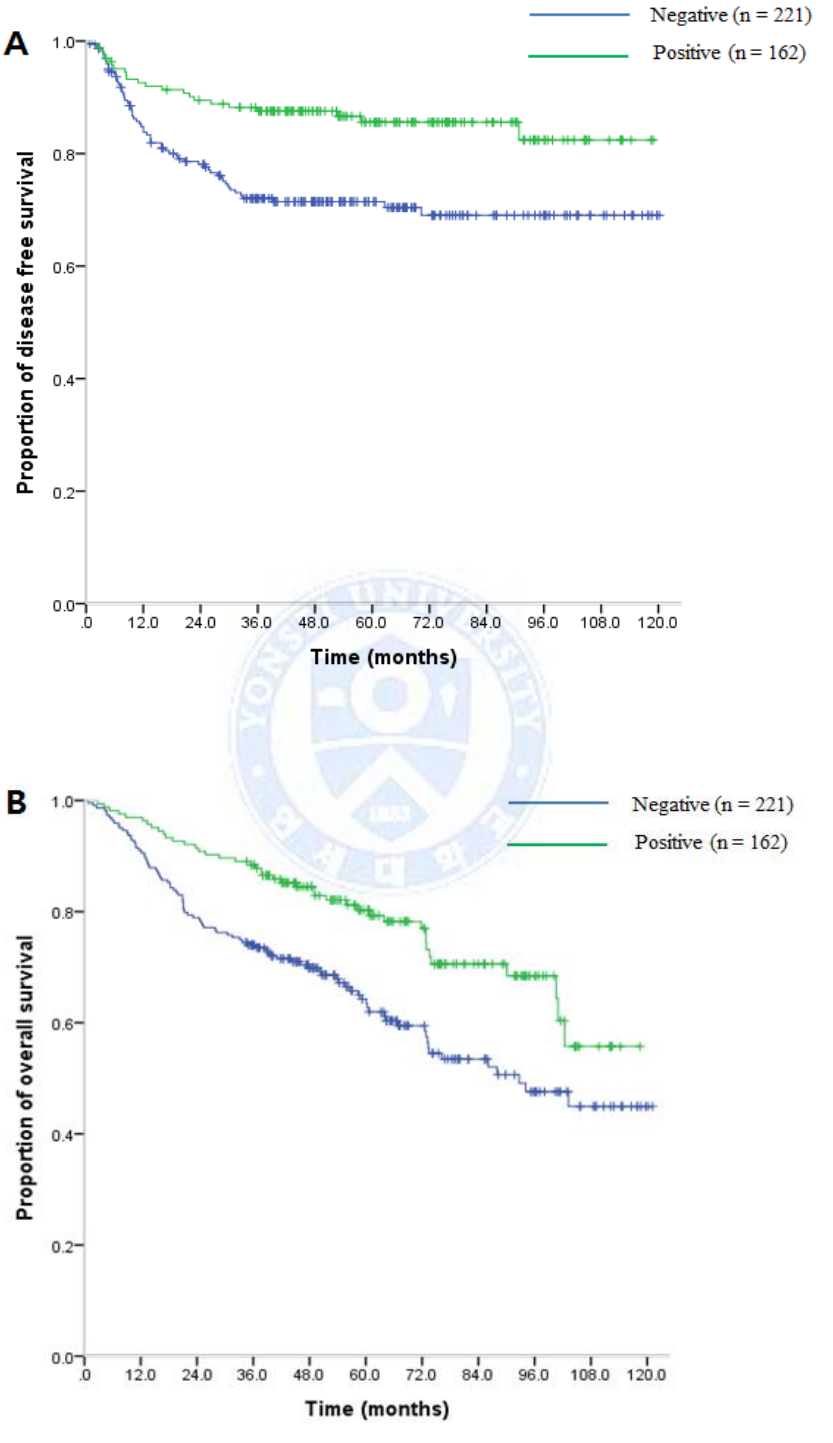
Figure 2. Survival analysis on the bases of *FGFR1* amplification (high, low, and no amplification). (A) Disease free survival, and (B) overall survival were not showed significant difference.





Based on p16 status, patients with p16 positive tumors had significantly better DFS and OS than patients with p16 negative tumors (Figure 3). For DFS, the hazard ratio (HR) was 0.42 (95% CI, 0.26 to 0.70), reflecting a 58% reduction in recurrence for patients with p16 positive tumors (P=0.001). For OS, the HR was 0.52 (95% CI, 0.36 to 0.75), reflecting a 48% reduction in death rate for patients with p16 positive tumors (P<0.001).

Figure 3. Survival analysis on the bases of p16 status. (A) Disease free survival, and (B) overall survival were significantly better in patients with p16 positive tumors.


A
Negative (n = 221)
Positive (n = 162)
Proportion of disease free survival
1.0
0.8
0.6
0.4
0.2
0.0
.0
12.0
24.0
36.0
48.0
60.0
72.0
84.0
96.0
108.0
120.0
Time (months)
B
Negative (n = 221)
Positive (n = 162)
Proportion of overall survival
1.0
0.8
0.6
0.4
0.2
0.0
.0
12.0
24.0
36.0
48.0
60.0
72.0
84.0
96.0
108.0
120.0
Time (months)

In Cox proportional hazard model adjusted for smoking, primary sites (oropharynx, hypopharynx), lymphovascular invasion, perineural invasion, positive resection margin, positive nodal status, p16 positive status and postoperative CCRT, p16 positive status was significantly associated with a longer OS (HR 0.48; 95% CI, 0.30-0.76; P=0.002, Table 4). Lymphovascular invasion, positive resection margin and positive nodal status were significantly related to shorter OS in multivariate analysis. There was no significant difference in OS for sex, smoking status, and *FGFR1* amplification in multivariate analysis.

Table 4. Univariate and multivariate analysis of overall survival

	Univariate analysis			Multivariate analysis		
	HR	95%CI	*P*	HR	95%CI	*P*
Sex	0.77	0.51-1.17	0.222			
Smoking	1.36	0.94-1.95	0.099			
Oral cavity	1.10	0.78-1.55	0.591			
Oropharynx	0.68	0.46-1.00	0.052	0.62	0.37-1.03	0.066
Hypopharynx	1.70	0.96-3.02	0.069			
Larynx	1.26	0.71-2.24	0.438			
Lymphovascular invasion	1.89	1.28-2.80	0.002	1.78	1.17-2.73	0.008
Perineural invasion	1.70	1.07-2.68	0.024			
Positive resection margin	1.57	1.08-2.29	0.019	1.62	1.09-2.41	0.018
Positive nodal status	2.19	1.50-3.19	0.001	2.34	1.56-3.51	0.001
p16 positive	0.52	0.36-0.75	0.001	0.48	0.30-0.76	0.002
FGFR1 amplification	1.00	0.88-1.24	0.625			
CCRT	1.59	1.08-2.35	0.020			
Radiotherapy	0.87	0.61-1.23	0.425			

Abbreviations: HR, hazard ration; 95% CI, 95% confidence intervals; *FGFR1*, *Fibroblast Growth Factor Receptor1;* CCRT, Concurrent chemoradiation therapy *;*

IV. DISCUSSION

In this study, we investigated the frequency and the impact of *FGFR1*

amplification and p16 protein expression on clinical outcomes in patients with resected HNSCC. To our knowledge, this is the first report on the prognostic impact of *FGFR1* amplification in the largest cohort of resected HNSCC patients from East Asians. Our study demonstrated the frequency of *FGFR1* amplification is common 42.3% (1.0% for high amplification and 41.3% for low amplification) and there was no relation to prognostic impact on clinical outcomes. Regarding p16 status, a surrogate marker of oncogenic HPV infection, there were 42.4% of p16 positive tumors and related to favorable prognosis significantly.

The frequency of *FGFR1* amplification has been reported in squamous cell carcinoma of lung, esophagus, SCLC, and HNSCC, for which smoking is known dominant risk factor[10,12,13,30]. Overall, the frequency of *FGFR1* amplification was reported to be 5.6-24.8% in lung SqCC[12], and 6-9.4% in esophageal squamous cell carcinoma[13]. As determined by FISH analysis in our study, *FGFR1* amplification was 42.3%, which was higher compared to a value of 15% reported in a recent study in white patients from Western countries[14]. Of them, low *FGFR1* amplification was observed more commonly in our study, 41.3% vs 14.0%, respectively. There could be ethnic differences in the frequency of *FGFR1* amplification and the prevalence of HPV infection. The other types of carcinoma reported variable ethnic difference in prevalence of *FGFR1* amplification. In SCLC, the frequency of *FGFR1* amplification was reported to be 5.6-6% in western population[31] and 1.9% in East Asian population[30]. In comparison, in esophageal squamous cell carcinoma, there are similar frequency between Western Europe and East Asia, 9.4% and 8.6%, respectively[13].

FGFR1 amplification has been known to be associated with poor prognosis or unfavorable clinicopathologic parameters in squamous cell carcinoma of lung, esophagus, and head and neck with several controversial results. In resected lung SqCC, Kim et al[12] reported *FGFR1* amplification as negative prognostic factor, whereas Heist et al[32] observed no significant difference in OS. In HNSCC, *FGFR1* amplification was significantly associated with poor prognostic factors such as higher T stage, lymphovascular invasion, and higher numbers of visceral metastases[14]. In our study, the prognostic impact of *FGFR1* amplification was

not founded. Tumor heterogeneity in HNSCC, unstandardized FISH criteria for *FGFR1* amplification, excellent surgical skills, varying adjuvant treatment, wide variation for *FGFR1* amplification, and different frequency of *FGFR1* alteration according to HPV status may contribute to the controversial results. In TCGA data[6], *FGFR1* alteration was higher (10%) in HPV negative group, and in HPV positive group, *FGFR3* alteration was more common (11%).

Unfortunately, standard definition for *FGFR1* amplification by FISH is not established yet. Indeed, the definition of *FGFR1* amplification by FISH technique has been highly variable in the previous studies[9,10,12,14,32,33]. Unlike breast cancer, lung SqCC exhibits small-clusters and co-amplifications of *FGFR1* and CEN8[33]. Therefore, *FGFR1* FISH assay needs to differentiate between true amplification and polysomy in lung SqCC. In a large cohort study, Schildhause et al[33] proposed a more sophisticated *FGFR1* FISH criteria using average gene copy number per nucleus, *FGFR1*/CEN8 ratio, and percentage of gene clusters at the same time. By the addition of *FGFR1*/CEN8 ratio, 8 out of 47 cases (17.0%) were newly classified as high amplification in that study. In our study, we applied the *FGFR1* FISH criteria in HNSCC previously proposed by Goke et al[14], which not consider co-amplification of CEP8. Applied in our study, if the criteria for *FGFR1*/CEN8 ratio included, 14 patients (3.5%) might have been classified from low amplification group to high amplification group additionally.

In the previous studies[10,12,13], *FGFR1* amplification may be an oncogenic driver mutation in tobacco-associated cancers of the aerodigestive tract. In our study, there was no relation between smoking status and survival outcomes. It's caused by small sample size of high amplification and depending on medical record, underestimated amount of smoking by patients' statement when they had visited to clinic in first time of diagnosis.

In clinicopathologic characteristics of p16 negative group, several factors were known to be related good prognosis: favorable histologic differentiation, no lymphovascular invasion, negative resection margin, early pathologic stage. Nevertheless, p16 negative group had poor disease free and overall survival outcomes. As known in previous studies[26,34,35], p16 protein expression was

confirmed as a strong and independent predictor of survival in oropharyngeal and nonoropharyngeal HNSCC. In p16 negative group, percentage of high/low FGFR1 amplification, and gene copy number of FGFR1 amplification were higher than in p16 positive group, suspiciously in which FGFR1 amplification had tendency for poor prognosis.

Our study had several limitations. The main limitation includes its retrospective nature and selection bias for patient's cohort. This is likely related to selection of surgically resected, earlier stage patients who relatively have favorable prognosis. And during process of manufacturing TMA, we excluded about 170 tissues for hypopharynx and larynx which had been decalcified. Because the sample included only a few high *FGFR1* amplified tumors, we did not have enough statistical power to identify significant differences between the clinical characteristics of patients with *FGFR1* amplification and those without *FGFR1* amplification. To identify such characteristics, a dedicate criteria with HNSCC will be needed.

Several potent selective FGFR tyrosine kinase inhibitors are already in early clinical development. Tyrosine kinase inhibitors targeting multiple receptors including *FGFR1* have been tried in lung SqCC. There are recent reports of promising results with the non-ATP competitive pan-FGFR selective inhibitor LY2874455[36], the FGFR1-3 selective inhibitor AZD4547[37], and the FGFR1-3 selective inhibitor BGJ398[15]. Those agents are reported to have manageable toxicities including hyperphosphatemia, hypercalcemia, and ectopic tissue calcification; correlated with abnormal phosphate and vitamin D homeostasis caused by the blockage of *FGF23* signaling[38]. Further clinical trials could show that *FGFR1* inhibition has a therapeutic effect in *FGFR1* amplified HNSCC. These emerging treatment strategies may shed light on treatment for patients with HNSCC who lack a specific therapeutic target. Further investigation for finding profit biomarkers and promising candidate drugs in clinical trials are currently ongoing and needed to proceed.

V. CONCLUSION

In conclusion, we did not demonstrate the prognostic impact of *FGFR1* amplification in resected HNSCC. As known, p16 protein expression was confirmed as a strong and independent predictor of survival. Further research for finding a dedicate criteria for *FGFR1* amplification and promising candidate drugs in clinical trials will be needed.

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ABSTRACT (IN KOREAN)

수술로 절제된 두경부 편평세포암에서 FGFR1 유전자 증폭과 p16 단백질 발현의 빈도 및 임상적 영향에 관한 연구

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허 수 진

본 연구의 목적은 수술로 절제된 두경부 편평세포암에서 Fibroblast growth factor receptor 1 (*FGFR1*) 유전자 증폭과 p16 단백질 발현의 빈도 및 임상적 영향에 대해 알아보고자 함이다. 2005년 11월부터 2012년 12월까지 383명의 환자로부터 얻은 조직으로 형광동소보합법의 기법을 사용하여 *FGFR1* 유전자 증폭을 분석하였다. 이전 연구를 참고하여, 고증폭의 기준은 9개 이상의 신호를 가진 종양세포가 20% 이상일 경우, 저증폭의 기준은 2개 이상, 9개 미만의 신호를 가진 종양세포가 20% 이상일 경우로 정하였다. 그 결과 *FGFR1* 고증폭은 1.0%, *FGFR1* 저증폭은 41.3% 였고 *FGFR1* 증폭의 정도는 생존율과 관련성을 보이지 않았고 p16 유전자 발현 양성은 42.4% 의 빈도로 생존율 향상과 관련을 보였다. 두경부 종양의 부위별 이질성, 형광동소보합법 기준의 비표준화, 수술 후 치료의 다양성, 넓은 범위의 저증폭 환자비율 등이 이전 연구들의 결과와 차이점을 보이는 것으로 생각된다. 추후 *FGFR1* 증폭 분석 기준의 표준화에 대한 연구와 *FGFR1* 의 치료적 효용성에 대한 추가적인 연구가 필요하겠다.

핵심되는 말 : *FGFR1*, p16, 두경부 편평세포암