Research Paper

Alteration status and prognostic value of MET in head and neck squamous cell carcinoma

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Abstract

The MET pathway plays a key role in various cancers, and its inhibition represents a potential treatment target. However, appropriate biomarkers are needed to facilitate the selection of patients who would benefit from MET inhibiting therapy. We herein conducted a robust confirmatory evaluation of the *MET* copy number alteration status and prognostic significance of c-Met expression in a large series of patients (n = 396) who underwent standard surgical resection and adjuvant chemoradiotherapy for head and neck squamous cell carcinoma (HNSCC). Surgically resected HNSCC samples were subjected to immunohistochemical and H-score analysis of c-Met expression and silver in situ hybridization analysis of *MET* amplification and copy number gains. c-Met expression varied, with mean and median H-scores (scale: 0–300 scale) of 61.2 and 60.0, respectively. The lowest and highest expression levels were observed in SCC of the larynx and oral cavity, respectively. *MET* copy number gains were observed in 16.9% of cases (67/339) and were associated with c-Met protein expression. High c-Met expression, determined according to *MET* gain status, was associated with an inferior overall survival rate, especially among completely resected cases. In conclusion, our robust analysis revealed that c-Met expression in HNSCCs varied according to anatomical site, correlated with *MET* copy number gains, and was associated with poor prognosis. This c-Met expression analysis method, which is based on the *MET* gain status, appears to appropriately predict high-risk HNSCC patients in the context of anti-MET therapeutic decisions.

Key words: Head and neck squamous cell carcinoma; c-Met protein; MET gene; Prognosis

Introduction

Head and neck cancers (HNCs) comprise one of the most common groups of cancers worldwide. Histologically, approximately 95% of all HNCs are head and neck squamous cell carcinoma (HNSCC) [1]; this type of tumor is characterized by frequent recurrence and metastasis, as well as resistance to the conventional cisplatin-based chemoradiotherapy. Accordingly, patients with recurrent or metastatic HNSCC have dismal outcomes [2].

The tyrosine kinase receptor c-Met normally binds with hepatocyte growth factor (HGF), which triggers its involvement in processes such as embryogenesis, cell growth, cell differentiation, and angiogenesis [3, 4]. However, c-Met activation is also involved in processes related to malignant transformation, such as tumor growth, invasion, metastasis, and angiogenesis [5-9]. In addition to paracrine or autocrine signaling via HGF, c-Met may also be activated via protein overexpression or the amplification, mutation, or transcriptional alteration (via microRNA-mediated dysregulation) of *MET* [10-12]. c-Met overexpression and/or *MET*

amplification are known to correlate with a worse prognosis in some types of cancers, including non-small-cell lung cancer and gastric cancer [12]. In HNSCC, c-Met expression has been associated with cisplatin resistance and a strong metastatic ability *in vivo*, [2] as well as a poor patient prognosis [13].

Inhibition of the MET pathway, which plays a key role in various types of cancer, is a potential cancer treatment target. Recently, several types of inhibitors that target various components of the MET signaling pathway have been introduced [12, 14-16]. Of these, an anti-MET monoclonal antibody has yielded promising results against tumors that over express c-Met, as have small molecule inhibitors against tumors harboring *MET* mutation or amplification [12]. However, a biomarker that would facilitate patient selection with respect to MET inhibiting therapy is needed.

The prognostic significance of c-Met expression has not yet been confirmed in studies of MET and HNSCC [13, 17-19], possibly because of variability in detection methods and patient selection and a lack of validation of cut-off values. In addition, *MET* amplification and/or copy number alteration have been rarely studied in HNSCC; to date, a relatively small number of cases of recurrent/metastatic HNSCC at limited anatomical sites have been studied using immunohistochemistry and fluorescence in situ hybridization [13, 17-19]. Accordingly, in this study, we conducted a robust evaluation of c-Met expression and *MET* copy number alteration in a large number of patients with HNSCC at various anatomic sites of the head and neck who had undergone standard surgical resection and adjuvant chemoradiotherapy when indicated. In these patients, we analyzed the association of c-Met expression with *MET* copy alterations, as well as the associations of c-Met/*MET* abnormalities with clinical variables such as anatomical site, tumor-node-metastasis (TNM) stage, human papillomavirus (HPV) status, and survival.

Materials and methods

Patients and clinical data

This study was approved by the Institutional Review Board of Severance Hospital. Formalin-fixed, paraffin-embedded specimens were obtained from consecutive patients who underwent surgical resection with a curative aim for HNSCC at Severance Hospital, Seoul, Korea, between 2005 and 2012 and were archived. The present study excluded HNSCC tissue samples that had been subjected to decalcification for accurate immunohistochemistry and DNA in situ hybridization analyses; as a result, some cases of surgically resected hypopharyngeal/laryngeal and sinonasal SCC were excluded. The inclusion criteria were available tumor tissue, clinical data regarding smoking status, and survival data; lack of preoperative treatment; and no clinicopathologic evidence of distant metastasis at the time of surgery. Ultimately, 396 cases were selected, among which 305 achieved complete R0 resection, defined histologically as tumor-free resection margins. In the present cohort, the affected anatomic sites included the oral cavity (anterior two-thirds of the tongue, mouth floor, hard palate, buccal mucosa, oral cavity; n = 204), hypopharynx (n = 28), larynx (lingual surface of the epiglottis, glottis, supraglottis, subglottis, larynx; n = 42), and oropharynx (tonsils, base of tongue, soft palate, oropharynx; n = 122). Other evaluated parameters included tumor location and size, histologic grade, metastasis to regional lymph nodes, lymphovascular invasion, and perineural invasion.

Tumors were classified according to the Seventh American Joint Committee on Cancer (AJCC) TNM cancer classification system and the World Health Organization system [20, 21]. Two pathologists (S.O.Y. and Y.A.C.) confirmed the histopathologic diagnoses of HNSCC. Clinical data were collected from the patients' medical records and reviewed to evaluate the clinicopathologic characteristics and survival outcomes. The median follow-up period was 37.1 months (range, 0.8–99.6 months). Other clinicopathologic characteristics are described in Table 1.

Tissue microarray preparation

Sections of formalin-fixed, paraffin-embedded tissues were prepared and stained with hematoxylin and eosin (H&E). Representative tumor areas were confirmed microscopically, selected, and used for tissue microarray (TMA) construction. Two or three different representative areas per case were selected; core tissues (3 mm in diameter) were taken from the individual tissue blocks (donor blocks) and arranged in recipient paraffin blocks (tissue array blocks) using a trephine apparatus.

Immunohistochemistry and analysis

Immunohistochemistry was performed on 4-µm TMA tissue sections with a Ventana Bench Mark XT Autostainer (Ventana Medical Systems, Tucson, AZ, USA) as described in previous reports [22]. The following primary antibodies were tested: c-Met (pre-dilution; clone SP44; Ventana) and p16 (RTU; Ventana). c-Met protein expression was analyzed according to the semiquantitative H-score method; this method yields a total score range of 0–300 [23] by multiplying the dominant nuclear staining intensity

score (0, no staining; 1, weak or barely detectable membranous staining; 2, distinct brown membranous staining; 3, strong dark brown membranous staining) by the percentage (0–100%) of positive cell nuclei (Figs. 1A–D). Conventionally accepted criteria were used for p16 immunohistochemistry, and positivity was defined as the presence of strong and diffuse nuclear and cytoplasmic staining in >70% of the HNSCC cells. All other staining patterns were scored as negative [24].

Table 1. Clinicopathological characteristics of 396 HNSCC patients.

Category	Variables	No. of cases (n = 396)	Percentage (%)
Sex	Male	298	75.3
	Female	98	24.7
Age	Median (IQR), yrs	58 (22-88)	
Smoking	Never smoker	154	38.9
	Ex-smoker	80	20.2
	Current smoker	162	40.9
Anatomical site	Oral cavity	204	51.5
	Oropharynx	122	30.8
	Hypopharynx	28	7.1
	Larynx	42	10.6
p16 IHC status	Negative	236	59.6
	Positive	160	40.4
Lymphovascular invasion	Absent	320	80.8
	Present	76	19.2
Perineural invasion	Absent	342	86.4
	Present	54	13.6
Resection margin status	R0*	305	77.0
	Non-R0	91	23.0
Pathologic T stage	pT1-2	326	82.3
	pT3-4	70	17.7
Pathologic N stage	pN0-1	248	62.6
	pN2-3	148	37.4
Postoperative therapy	No treatment	145	36.6
	RTx alone	150	37.9
	CCRT	99	25.0
	CTx alone or other CTx alone or incomplete CCRT	2	0.5
Recurrence/metastasis	Absent	293	74.0
	Present	103	26.0
Survival	Alive	324	81.8
	Expired	72	18.2

HNSCC: head and neck squamous cell carcinoma; IQR: interquartile range; IHC: immunohistochemistry.

* R0 resection status was defined as complete tumor resection.

Dual-color silver in situ hybridization and interpretation (SISH)

Dual-color SISH was performed on a Ventana BenchMark XT apparatus (Ventana Medical Systems) using the MET DNA (Roche-Ventana) and Chromosome 7 enumeration (CEP7) probes (Roche-Ventana). Signals were enumerated in 100 tumor cells with intact, non-overlapping nuclei per core under a light microscope with a magnification of 600X. Tumor nuclei with clearly distinct color signals

for *MET* (black signal) and CEP7 (red signal) were evaluated (Figs. 1 E–H). If clusters of dots representing many copies of *MET* gene were noted, small and large clusters of multiple signals were counted as 6 and 12 signals, respectively, according to the interpretive guide provided for INFORM HER2 DNA probe staining of breast carcinoma (Ventana Medical Systems). *MET* SISH results were interpreted by two experienced evaluators (S.O.Y. and Y.A.C.) blinded to the clinical data.

To define *MET* copy number gain or amplification, several criteria were considered with reference to previous fluorescence in situ hybridization-based studies of tyrosine kinase receptor genes, including the University of Colorado Cancer Center (UCCC) criteria for the epidermal growth factor receptor gene [25] and criteria for the fibroblast growth factor receptor gene in HNSCC [26] and *MET* in several tumor types [18, 19, 27-29]. To address tyrosine kinase receptor gene heterogeneity in tumors [30], the proportion of tumor cells harboring copy number alterations was also considered. Previous studies that used the SISH method to detect *MET* copy number changes in several types of cancer (e.g., colorectal, gastric, non-small-cell lung, HNSCC) used different criteria for *MET* amplification, high *MET* copy numbers, and copy number gains. For example, *MET* amplification/copy number gain/polysomy was defined as a copy number ≥ 4 or ≥ 5, and the proportion of tumor cells (10%, 40%, or 90%, according to polysomy status) exhibiting copy number alterations was also considered in some definitions of *MET* gene amplification/copy number gain/polysomy [19, 31-33].

In our study, we used the following definitions of *MET* copy number gain and amplification: 2–4 *MET* signals per nucleus in ≥20% of tumor cells, and ≥5 *MET* signals per nucleus in ≥20% of tumor cells, respectively.

In all 396 cases, immunohistochemistry (IHC) and SISH were performed. Although c-Met IHC was available for all cases, we were not able to properly evaluate several SISH cores due to either disruption of cores or inadequate hybridization. Therefore, after excluding these cases (n=59), only 339 cases of SISH were analyzed properly.
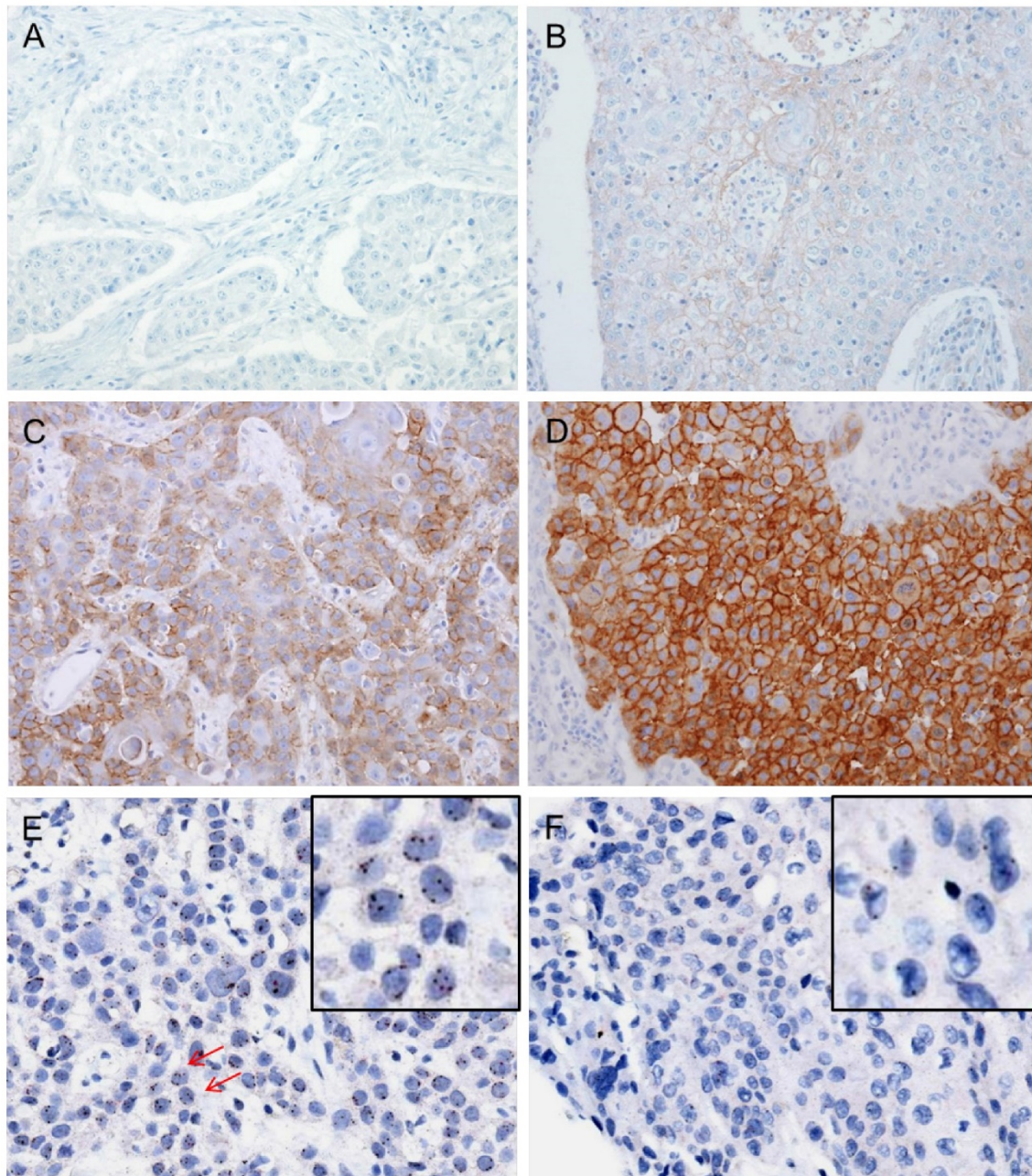
Statistical analysis

The Mann–Whitney (MW) U test, Kruskal–Wallis (KW) test, one-way analysis of variance (ANOVA), two-sample t test, x^2 test, and Pearson's or Spearman's correlation test were used to analyze differences between the evaluated variables.

Overall survival was measured from the date of initial diagnosis to that of death or the last follow-up visit. Progression-free survival was measured from the date of initial diagnosis to that of disease progression, defined as cancer recurrence, continuance of stable disease/partial remission/progressive disease without complete remission, or cancer-related death during the study period. The Kaplan–Meier method was used to analyze survival rates, and differences were compared using the log-rank test. The Cox proportional hazards model was implemented for a multivariate analysis. Two-sided P-values <0.05 were considered statistically significant. Statistical analyses were conducted using IBM SPSS 22 software for Windows (IBM Corp, Armonk, NY, USA).

Figure 1. Immunohistochemical analysis of c-MET protein expression and silver in-situ hybridization (SISH) to determine *MET* copy number alterations. Representative images of immunohistochemical staining for c-Met demonstrate negative staining (intensity score 0, A), weak or barely detectable membranous staining (intensity score 1, B), distinct brown membranous staining (intensity score 2, C), and strong dark brown membranous staining (intensity score 3, D). Each intensity score was multiplied by the percentage of positive nuclear cell staining (0-100%) to calculate the H-score (possible scores: 0–300). In a SISH dataset, the black signals indicate *MET* copies and red signals indicate *CEP7*. The representative case harbored 3–4 MET signals per nucleus in ≥20% of counted tumor cells, indicating a *MET* copy number gain. Arrows point to cells that show 4 MET signals in the nucleus (E). Another representative case exhibits normal disomy of the *MET* and *CEP7* signals (F). Selective magnification of cells is shown inside the black box (E and F).
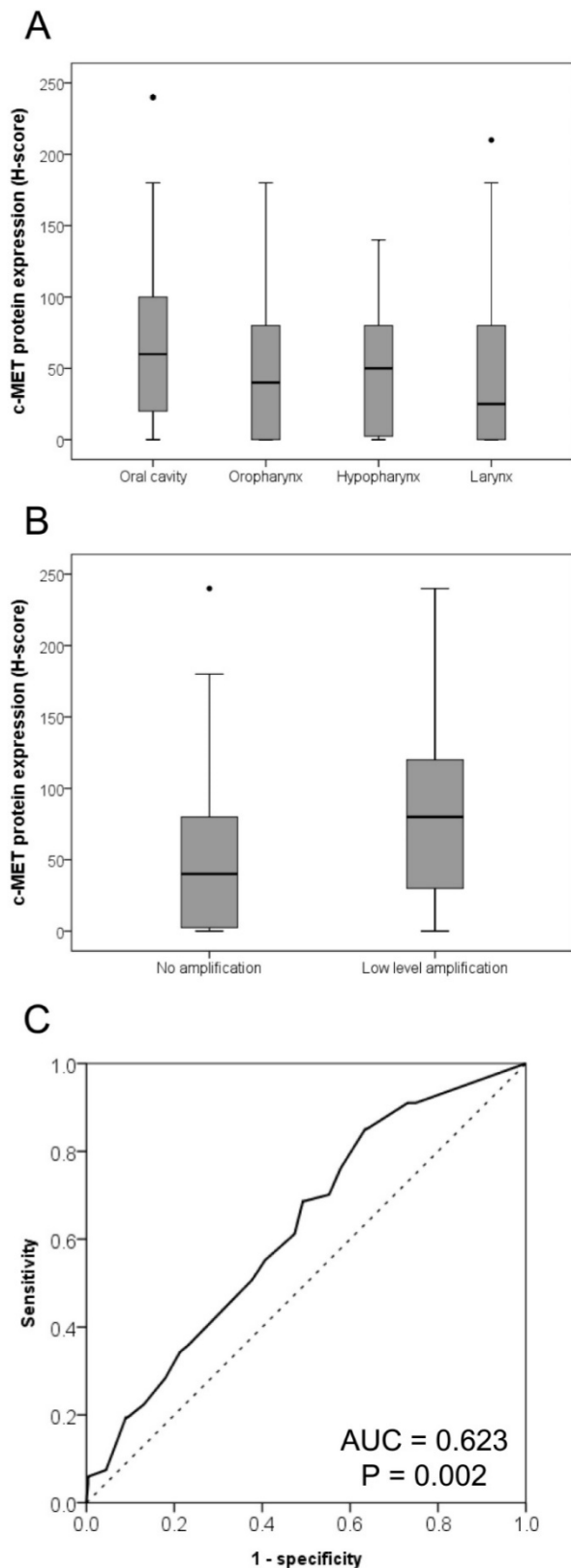
Figure 2. Relationships of c-Met expression with affected anatomic site and *MET* copy number. The c-MET expression levels were lowest and highest in laryngeal squamous cell carcinoma (SCC) and SCC of the oral cavity, respectively (A). Intermediate c-Met levels were observed in oropharyngeal and hypopharyngeal SCC (P = 0.003). The c-MET protein expression level was higher in cases exhibiting *MET* copy number gains than in those without *MET* gains (B, P = 0.002). A receiver operating characteristic curve analysis based on the *MET* copy number gain was conducted to determine the c-Met protein expression cut-off value (C).

Results

Expression of c-Met protein in HNSCC

Overall, c-Met protein expression varied among HNSCC cases, with H-scores ranging from 0–240 (mean and median H-scores: 61.2 and 60.0, respectively). Regarding anatomical sites, the lowest and highest expression levels were observed in laryngeal SCC and SCC of the oral cavity, respectively; oropharyngeal and hypopharyngeal SCCs exhibited intermediate levels of expression (P = 0.003; Fig. 2A). Other clinicopathologic variables such as age, sex, smoking, lymphovascular invasion, perineural invasion, pT stage, and pN stage did not differ significantly with respect to c-Met expression level.

MET copy number variation in HNSCC

The median *MET* copy number per case was 2 (range, 1–7), and the median *MET/CEP7* per case was 1.05 (range, 1.00–1.76). Using our above-defined criteria for *MET* copy number gain and amplification, 16.9% (67/335) of the cases harbored copy number gain, whereas none harbored amplification (Table 2).

Table 2. *MET* gene copy number variation of HNSCC.

Category	Definition	No. of cases	Percentage (%)
Negative	Disomy	268	67.7
Copy number gain	2 < *MET* signals < 5 per nucleus in 20% or more of tumor cells	67	16.9
Amplification	*MET* signals ≥ 5 per nucleus in 20% or more of tumor cells	0	0

Relationship between c-Met protein expression and *MET* copy number variation

Cases harboring copy number gains had significantly higher c-Met protein expression levels than did cases without copy number gains (Fig. 2B; P = 0.001). According to a receiver operating characteristic curve analysis of *MET* copy number gain, an H-score of 65, which was higher than the above-mentioned mean and median c-Met expression H-score scores for overall HNSCC cases, was identified as the cut-off value for high c-Met protein expression (Fig. 2C). Using this cut-off value, 41.9% (166/396) of HNSCC cases exhibited high c-Met expression.

Associations of a high c-Met protein expression level and *MET* copy number gain with clinicopathologic variables

Among the overall cohort of HNSCC cases, a high c-Met protein expression was not found to associate significantly with clinicopathologic

variables such as age, sex, smoking, anatomic site, HPV (p16 immunohistochemistry) status, lymphovascular invasion, perineural invasion, pT category, or pN category. No other significant associations were observed between *MET* copy number gain and other clinicopathologic variables (Table 3).

Associations of a high c-Met protein expression level and MET copy number gain with patient survival

In the overall cohort, high c-Met protein expression tended to associate with inferior overall survival with marginal significance (P = 0.071; Fig. 3A; supplementary Table 1). However, *MET* copy number gain was not related to overall survival (supplementary Table 1). Furthermore, neither c-Met protein expression nor *MET* copy number gain associated significantly with progression-free survival (supplementary Table 2). Other clinicopathologic variables, particularly age, anatomical site, p16 expression, pT category, and pN category, were found to associate significantly with both overall and progression-free survival (supplementary Tables 1 and 2).

Among the 305 cases in which complete tumor resection (R0 resection) was achieved, high c-Met expression was significantly related to inferior overall survival (P = 0.037; Fig. 3B and Table 4). In a multivariate Cox proportional hazards analysis, high c-Met expression tended to correlate independently with inferior OS, albeit with marginal significance (P = 0.061; Table 4). However, no significant association was observed between high c-Met expression and progression-free survival (Table 5). In addition, *MET* copy number gain did not associate significantly with overall or progression-free survival among cases involving R0 resection (Table 4 and 5). In this subgroup, other clinicopathologic variables,

particularly anatomical site, pT category, and pN category, associated significantly with overall survival and progression-free survival (Table 4 and 5).
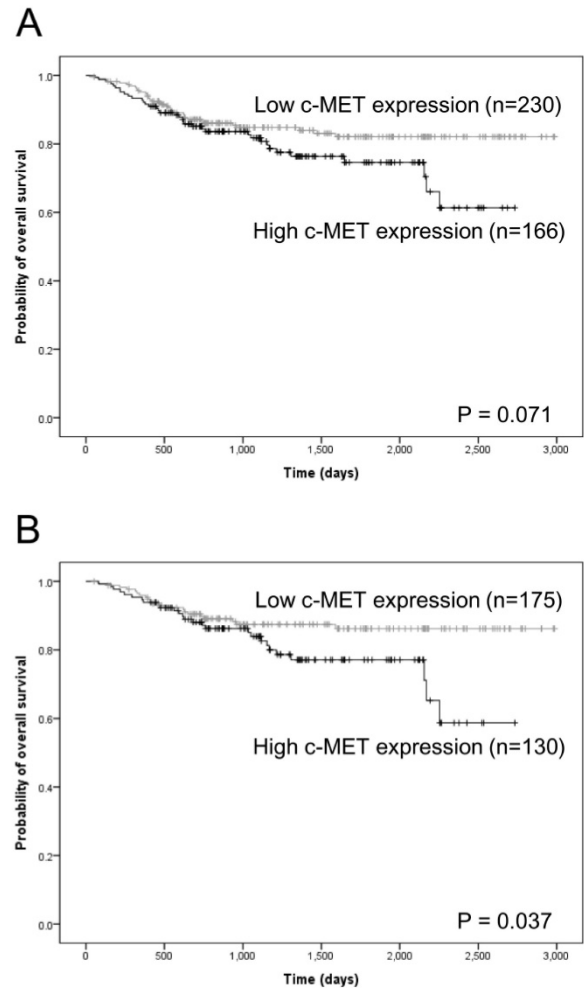
Figure 3. Kaplan–Meier analysis of overall survival according to c-Met expression status. In the overall cohort of head and neck squamous cell carcinoma (HNSCC) cases, a high c-Met protein expression level tended to correlate with inferior overall survival in a marginally significant relationship (A). Among HNSCC cases that achieved complete (R0) resection, a high c-MET protein expression level correlated significantly with inferior overall survival (B).

Table 3. Clinicopathologic characteristics of HNSCC according to the c-MET protein expression and *MET* gene CN gain status.

Category	Variables	No. of cases (n = 396)	c-MET protein			No. of cases (n = 335)	MET copy number variation		
			Low (%) (*n* = 230)	High (%) (*n* = 166)	P-value		No gain (%) (*n* = 268)	CN gain (%) (*n* = 67)	P-value
Sex	Male	298	180 (78.3)	118 (71.1)	0.102	80	65 (24.3)	15 (22.4)	0.749
	Female	98	50 (21.7)	48 (28.9)		255	203 (75.7)	52 (77.6)	
Age (yrs)	< 58	194	111 (48.3)	83 (50.0)	0.733	167	129 (48.1)	38 (56.7)	0.209
	≥ 58	202	119 (51.7)	83 (50.0)		168	139 (51.9)	29 (43.3)	
Smoking	Never smoker	154	88 (38.3)	66 (39.8)	0.924	128	106 (39.6)	22 (32.8)	0.161
	Ex-smoker	80	46 (20.0)	34 (20.5)		66	56 (20.9)	10 (14.9)	
	Current smoker	162	96 (41.7)	66 (39.8)		141	106 (39.6)	35 (52.2)	
Anatomical sites	Oral cavity	204	110 (47.8)	94 (56.6)	0.305	168	129 (48.1)	39 (58.2)	0.164
	Oropharynx	122	76 (33.0)	46 (27.7)		109	94 (35.1)	15 (22.4)	
	Hypopharynx	28	16 (7.0)	12 (7.2)		24	17 (6.3)	7 (10.4)	
	Larynx	42	28 (12.2)	14 (8.4)		34	28 (10.4)	6 (9.0)	
p16 IHC status	Negative	236	134 (58.3)	102 (61.4)	0.524	190	149 (55.6)	41 (61.2)	0.408
	Positive	160	96 (41.7)	64 (38.6)		145	119 (44.4)	26 (38.8)	

Category	Variables	No. of cases (n = 396)	c-MET protein			No. of cases (n = 335)	MET copy number variation		
			Low (%)	High (%)	P-value		No gain (%)	CN gain (%)	P-value
			(*n* = 230)	(*n* = 166)			(*n* = 268)	(*n* = 67)	
Lymphovascular invasion	Negative	320	182 (79.1)	138 (83.1)	0.318	267	208 (77.6)	59 (88.1)	0.057
	Positive	76	48 (20.9)	28 (16.9)		68	60 (22.4)	8 (11.9)	
Perineural invasion	Negative	342	202 (87.8)	140 (84.3)	0.318	285	226 (84.3)	59 (88.1)	0.443
	Positive	54	28 (12.2)	26 (15.7)		50	42 (15.7)	8 (11.9)	
Resection margin status	R0*	305	175 (76.1)	130 (78.3)	0.630	255	202 (75.4)	53 (79.1)	0.522
	Non-R0	91	55 (23.9)	36 (21.7)		80	66 (24.6)	14 (20.9)	
Pathologic T stage	pT1-2	326	188 (81.7)	138 (83.1)	0.720	273	218 (81.3)	55 (82.1)	0.888
	pT3-4	70	42 (18.3)	28 (16.9)		62	50 (18.7)	12 (17.9)	
Pathologic N stage	pN0-1	248	144 (62.6)	104 (62.7)	0.993	204	156 (58.2)	48 (71.6)	0.044
	pN2-3	148	86 (37.4)	62 (37.3)		131	112 (41.8)	19 (28.4)	

HNSCC: head and neck squamous cell carcinoma; CN: copy number; IHC: immunohistochemistry

* R0 resection status was defined as complete tumor resection

Table 4. Cox analysis for overall survival among complete tumor resection (R0 resection; n=305).

Category	Variables	Univariate		Multivariate	
		HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Sex	Female	1		N/A	
	Male	1.242 (0.681-2.265)	0.480	N/A	
Age (years)	< 58	1		1	
	≥ 58	1.881 (1.048-3.493)	0.034	1.752 (0.953-3.220)	0.071
Location	Oropharynx	1		1	
	Oral cavity	2.654 (1.118-6.302)	0.027	3.570 (1.436-8.871)	0.006
	Hypopharynx	3.225 (0.806-12.9)	0.098	2.377 (0.587-9.623)	0.225
	Larynx	1.830 (0.457-7.319)	0.393	2.526 (0.613-10.418)	0.200
Smoking	Never smoker	1		N/A	
	Ex-smoker	0.621 (0.250-1.538)	0.303	N/A	
	Current smoker	1.157 (0.631-2.120)	0.637	N/A	
p16 IHC status		1		N/A	
		1.457 (0.782-2.717)	0.236	N/A	
Lymphovascular invasion	Absent	1		N/A	
	Present	1.795 (0.913-3.528)	0.090	N/A	
Perineural invasion	Absent	1		N/A	
	Present	1.877 (0.907-3.882)	0.090	N/A	
Pathologic T stage	pT1-2	1		1	
	pT3-4	4.089 (2.240-7.464)	< 0.001	2.714 (1.413-5.214)	0.003
Pathologic N stage	pN0-1	1		1	
	pN2-3	2.198 (1.248-3.872)	0.006	2.263 (1.187-4.315)	0.013
c-MET expression	Low	1		1	
	High	1.838 (1.037-3.259)	0.037	1.742 (0.975-3.112)	0.061
MET CN gain	No gain	1		N/A	
	Gain	0.577 (0.244-1.366)	0.211	N/A	

HNSCC: head and neck squamous cell carcinoma; CN: copy number; IHC: immunohistochemistry.

Table 5. Cox analysis for progression-free survival among complete tumor resection (R0 resection; n=305).

Category	Variables	Univariate		Multivariate	
		HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Sex	Female	1		N/A	
	Male	1.007 (1.107-1.671)	0.977	N/A	
Age (years)	< 58	1		1	
	≥ 58	1.767 (1.048-2.819)	0.017	1.635 (0.998-2.679)	0.051
Location	Oropharynx	1		1	
	Oral cavity	2.159 (1.126-4.140)	0.020	2.386 (1.100-5.176)	0.028
	Hypopharynx	3.270 (1.135-9.416)	0.028	2.352 (0.755-7.328)	0.140
	Larynx	1.976 (0.730-5.347)	0.180	1.876 (0.620-5.679)	0.265
Smoking	Never smoker	1		N/A	
	Ex-smoker	0.879 (0.436-1.774)	0.720	N/A	
	Current smoker	1.563 (0.948-2.577)	0.080	N/A	
p16 IHC status		1		1	
		1.812 (1.075-3.053)	0.026	0.635 (0.353-1.140)	0.128
Lymphovascular invasion	Absent	1		1	
	Present	1.885 (1.094-3.249)	0.022	1.811 (0.998-3.286)	0.051
Perineural invasion	Absent	1		1	

Category	Variables	Univariate		Multivariate	
		HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
	Present	2.060 (1.168-3.634)	0.013	1.219 (0.639-2.323)	0.548
Pathologic T stage	pT1-2	1		1	
	pT3-4	3.328 (2.003-5.529)	< 0.001	2.290 (1.307-4.014)	0.004
Pathologic N stage	pN0-1	1		1	
	pN2-3	1.969 (1.247-3.110)	0.004	1.842 (1.055-3.215)	0.032
c-MET expression	Low	1		N/A	
	High	1.186 (0.750-1.874)	0.466	N/A	
MET CN gain	No gain	1		N/A	
	Gain	0.494 (0.235-1.037)	0.062	N/A	

HNSCC: head and neck squamous cell carcinoma; CN: copy number; IHC: immunohistochemistry.

Discussion

Although several studies have evaluated c-Met expression levels and *MET* amplification/copy number alteration in several types of cancer, none have determined cut-off values for these parameters with regard to prognostic importance or patient selection for MET inhibitor therapy [13, 17-19, 31-36]. In this study, we aimed to investigate changes in statuses of c-Met protein expression and *MET* copy number, to analyze the association between these factors, and to determine the prognostic value of both factors in patients with HNSCC.

Previous studies of c-Met expression and *MET* copy number alteration in HNSCC have been restricted to specific anatomic sites or stage, or have included relatively small numbers of cases; in addition, the studies were heterogeneous with respect to detection method or treatment modality [13, 17-19]. These differences might explain the lack of confirmation for the prognostic significance of c-Met expression or *MET* copy alteration or cut-off values for these factors. In addition, *MET* amplification or copy number alteration has been rarely studied in HNSCC, particularly in cohorts composed primarily of patients treated with radical surgery. In contrast, the current study presents a robust evaluation of c-Met expression and *MET* copy number alterations in a large number of HNSCC cases that occurred in various anatomic sites of the head and neck; in addition, all involved patients underwent surgical resection with a curative aim and standard adjuvant chemoradiotherapy according to indicated treatment protocols.

In the present cohort of HNSCC cases, overall c-Met protein expression was variable, with an average H-score of approximately 60 (scale: 0–300). The c-Met expression level differed according to anatomical site, with lower and higher levels observed in laryngeal SCC and SCC of the oral cavity, respectively, and intermediate levels in oropharyngeal and hypopharyngeal SCC. These findings agree with a previous study in which high c-Met expression was relatively more frequent in the oral cavity and less frequent in the larynx [18], although the previous study involved a relatively smaller cohort and somewhat different detection criteria. Although little is known about other comparative analyses of c-Met expression according to anatomical site, it appears that SCC of the oral cavity might be associated with high c-Met expression more strongly than HNSCCs at other anatomical sites.

Differences in tumor microenvironment, according to various anatomical sites of HNSCC, might be related to different expression levels of c-Met. Active immune responses against foreign bodies occur in the oral cavity, while HPV infection is predominantly found in oropharyngeal areas. This might suggest a difference in immune responses according to anatomical site, which may be associated with differential c-Met expression. Hepatocyte growth factor (HGF), a ligand of Met, plays a significant role in immune surveillance in HNSCC [37]. In addition to tumor microenvironment, tumor-intrinsic factors, such as invasion, metastasis, and cancer stem cell-like phenotype, may also contribute to differences in c-Met expression. Several studies have shown that HGF/Met signaling is involved in tumor invasion, migration, and metastasis, as well as cancer stem cell-like properties [37]. Therefore, anatomical sites of HNSCC should be considered when choosing candidates in c-Met-associated clinical trials. Further study is needed to identify potential mechanisms of variances in c-Met expression at different anatomical sites.

Regarding *MET* alteration, a copy number gain was observed in 20.0% (67/335) of the tested cases in the present study. A majority (80.0% of tested cases, 268/335) of the remaining HNSCC cases exhibited normal disomy of *MET* and chromosome 7. In addition, no *MET* amplification was observed in the present study. In previous studies, high-level or focal *MET* amplification (clustered *MET* signals) has been reported as a rare event in HNSCC, as well as in other type of cancers (e.g., non-small-cell lung cancer or gastric cancer), with rates ranging 1–8%, despite considerable differences among studies in the detection methods and criteria used to define high

copy number [19, 32, 33, 36]. Although *MET* amplification may be rare, *MET* copy number gain may not be an uncommon event in HNSCC.

HNSCC cases harboring a *MET* copy number gain had significantly higher c-Met protein expression levels than did cases with a normal *MET* status, suggesting a potential association between a *MET* gain and c-Met protein overexpression. This corroborates earlier studies that reported positive correlations of c-Met expression (via immunohistochemistry) with *MET* copy number gain or amplification (via fluorescence in situ hybridization) [32, 33]. To further explore this result, a c-Met expression cut-off value of an H-score of 65, was determined based on the *MET* gain status. As noted previously, this value was slightly higher than the mean and median c-Met expression levels among all tested HNSCC cases, which would generally be used as a cut-off value with which to determine a high value for a quantitative variable.

High c-Met expression, determined through a *MET* gain-based analysis, was significantly related to a poor overall survival rate and tended to be independently related to poor overall survival among cases with R0 resection. In other words, a high tumor c-Met expression level might be associated with high-risk HNSCC even after complete tumor removal. According to the findings of the present study, the devised method for determining high c-Met expression according to a *MET* gain-based analysis appears to be reliable and appropriate for the prediction of patients with high-risk HNSCC.

Recent studies of potential biomarkers to identify patients who would benefit from MET pathway inhibitors suggest a 50% cut-off for c-Met expression. In other words, ≥50% of tumor cells would exhibit moderate (2+) to strong (3+) intensity in a c-Met immunohistochemistry analysis (i.e., H score ≥100) or 1+ or stronger staining (i.e., H score ≥ 50). Although those trials have focused on non-small-cell lung cancer and gastric or esophagogastric junction adenocarcinoma [34-36], the c-Met expression cutoff value determined in the present study could be considered when developing biomarkers to select patients with HNSCC who would benefit from MET inhibitor therapy.

A *MET* gain or increased copy number did not directly associate with the survival prognoses of HNSCC patients in the present study. As in previous studies, the prognostic role of a *MET* gain has not yet been confirmed in HNSCC [19], although other studies have identified a relationship between a *MET* gain and poor prognosis in patients with lung, gastric, or nasopharyngeal carcinoma [27-29, 32]. Regardless

of differences in the detection methods, interpretation criteria, and tumor types between the present study and previous studies, a *MET* gain itself did not seem to affect patient prognosis among patients with HNSCC who underwent curative surgical resection.

In summary, SCC of the oral cavity was associated with higher c-Met protein expression when compared with HNSCCs of other anatomical sites, and the c-Met protein expression level was associated with a *MET* gain. Furthermore, a high c-Met expression level, determined according to the *MET* gain status, was found to correlate with inferior overall survival, especially in patients who had achieved complete resection of HNSCC. The method described herein for determining high c-Met expression based on *MET* gains seemed to be reliable and appropriate for predicting patients with high-risk HNSCC and could facilitate the selection of patients who would benefit from MET inhibitor therapy.

Supplementary Material

Supplementary tables.
http://www.jcancer.org/v07p2197s1.pdf

Acknowledgement

Competing Interests

The authors have declared that no competing interest exists.

References

1. Chaturvedi AK, Anderson WF, Lortet-Tieulent J, Curado MP, Ferlay J, Franceschi S, et al. Worldwide trends in incidence rates for oral cavity and oropharyngeal cancers. Journal of clinical oncology: official journal of the American Society of Clinical Oncology. 2013; 31: 4550-9.
2. Sun S, Wang Z. Head neck squamous cell carcinoma c-Met(+) cells display cancer stem cell properties and are responsible for cisplatin-resistance and metastasis. Int J Cancer. 2011; 129: 2337-48.
3. Joffre C, Barrow R, Menard L, Calleja V, Hart IR, Kermorgant S. A direct role for Met endocytosis in tumorigenesis. Nat Cell Biol. 2011; 13: 827-37.
4. Maina F, Pante G, Helmbacher F, Andres R, Porthin A, Davies AM, et al. Coupling Met to specific pathways results in distinct developmental outcomes. Mol Cell. 2001; 7: 1293-306.
5. Gentile A, Trusolino L, Comoglio PM. The Met tyrosine kinase receptor in development and cancer. Cancer Metastasis Rev. 2008; 27: 85-94.
6. Chu SH, Feng DF, Zhang H, Chen ET, Duan ZX, Li XY, et al. c-Met-targeted RNA interference inhibits growth and metastasis of glioma U251 cells in vitro. J Neurooncol. 2009; 93: 183-9.
7. Merlin S, Pietronave S, Locarno D, Valente G, Follenzi A, Prat M. Deletion of the ectodomain unleashes the transforming, invasive, and tumorigenic potential of the MET oncogene. Cancer Sci. 2009; 100: 633-8.
8. Birchmeier C, Birchmeier W, Gherardi E, Vande Woude GF. Met, metastasis, motility and more. Nat Rev Mol Cell Biol. 2003; 4: 915-25.
9. You WK, McDonald DM. The hepatocyte growth factor/c-Met signaling pathway as a therapeutic target to inhibit angiogenesis. BMB Rep. 2008; 41: 833-9.
10. Kentsis A, Reed C, Rice KL, Sanda T, Rodig SJ, Tholouli E, et al. Autocrine activation of the MET receptor tyrosine kinase in acute myeloid leukemia. Nat Med. 2012; 18: 1118-22.
11. Graziano F, Galluccio N, Lorenzini P, Ruzzo A, Canestrari E, D'Emidio S, et al. Genetic activation of the MET pathway and prognosis of patients with

high-risk, radically resected gastric cancer. Journal of clinical oncology: official journal of the American Society of Clinical Oncology. 2011; 29: 4789-95.

12. Peters S, Adjei AA. MET: a promising anticancer therapeutic target. Nat Rev Clin Oncol. 2012; 9: 314-26.

13. Lo Muzio L, Farina A, Rubini C, Coccia E, Capogreco M, Colella G, et al. Effect of c-Met expression on survival in head and neck squamous cell carcinoma. Tumour biology: the journal of the International Society for Oncodevelopmental Biology and Medicine. 2006; 27: 115-21.

14. Comoglio PM, Giordano S, Trusolino L. Drug development of MET inhibitors: targeting oncogene addiction and expedience. Nat Rev Drug Discov. 2008; 7: 504-16.

15. Blumenschein GR, Jr., Mills GB, Gonzalez-Angulo AM. Targeting the hepatocyte growth factor-cMET axis in cancer therapy. Journal of clinical oncology: official journal of the American Society of Clinical Oncology. 2012; 30: 3287-96.

16. Appleman LJ. MET signaling pathway: a rational target for cancer therapy. Journal of clinical oncology: official journal of the American Society of Clinical Oncology. 2011; 29: 4837-8.

17. Freudlsperger C, Alexander D, Reinert S, Hoffmann J. Prognostic value of c-Met expression in oral squamous cell carcinoma. Exp Ther Med. 2010; 1: 69-72.

18. Choe JY, Yun JY, Nam SJ, Kim JE. Expression of c-Met Is Different along the Location and Associated with Lymph Node Metastasis of Head and Neck Carcinoma. Korean journal of pathology. 2012; 46: 515-22.

19. Kwon MJ, Kim DH, Park HR, Shin HS, Kwon JH, Lee DJ, et al. Frequent hepatocyte growth factor overexpression and low frequency of c-Met gene amplification in human papillomavirus-negative tonsillar squamous cell carcinoma and their prognostic significances. Human pathology. 2014; 45: 1327-38.

20. Edge SB, American Joint Committee on Cancer. AJCC cancer staging manual. 7th ed. New York; London: Springer; 2010.

21. Barnes L, International Academy of Pathology., World Health Organization., International Agency for Research on Cancer. Pathology and genetics of head and neck tumours. Lyon: IARC Press; 2005.

22. Yun S, Koh JM, Lee KS, Seo AN, Nam KH, Choe G. Expression of c-MET in Invasive Meningioma. Journal of pathology and translational medicine. 2015; 49: 44-51.

23. Park E, Park SY, Kim H, Sun PL, Jin Y, Cho SK, et al. Membranous Insulin-like Growth Factor-1 Receptor (IGF1R) Expression Is Predictive of Poor Prognosis in Patients with Epidermal Growth Factor Receptor (EGFR)-Mutant Lung Adenocarcinoma. Journal of pathology and translational medicine. 2015; 49: 382-8.

24. Ang KK, Harris J, Wheeler R, Weber R, Rosenthal DI, Nguyen-Tan PF, et al. Human papillomavirus and survival of patients with oropharyngeal cancer. The New England journal of medicine. 2010; 363: 24-35.

25. Cappuzzo F, Hirsch FR, Rossi E, Bartolini S, Ceresoli GL, Bemis L, et al. Epidermal growth factor receptor gene and protein and gefitinib sensitivity in non-small-cell lung cancer. Journal of the National Cancer Institute. 2005; 97: 643-55.

26. Goke F, Bode M, Franzen A, Kirsten R, Goltz D, Goke A, et al. Fibroblast growth factor receptor 1 amplification is a common event in squamous cell carcinoma of the head and neck. Modern pathology: an official journal of the United States and Canadian Academy of Pathology, Inc. 2013; 26: 1298-306.

27. Cappuzzo F, Marchetti A, Skokan M, Rossi E, Gajapathy S, Felicioni L, et al. Increased MET gene copy number negatively affects survival of surgically resected non-small-cell lung cancer patients. Journal of clinical oncology: official journal of the American Society of Clinical Oncology. 2009; 27: 1667-74.

28. Go H, Jeon YK, Park HJ, Sung SW, Seo JW, Chung DH. High MET gene copy number leads to shorter survival in patients with non-small cell lung cancer. Journal of thoracic oncology: official publication of the International Association for the Study of Lung Cancer. 2010; 5: 305-13.

29. Li Y, Li W, He Q, Xu Y, Ren X, Tang X, et al. Prognostic value of MET protein overexpression and gene amplification in locoregionally advanced nasopharyngeal carcinoma. Oncotarget. 2015; 6: 13309-19.

30. Hanna WM, Ruschoff J, Bilous M, Coudry RA, Dowsett M, Osamura RY, et al. HER2 in situ hybridization in breast cancer: clinical implications of polysomy 17 and genetic heterogeneity. Modern pathology: an official journal of the United States and Canadian Academy of Pathology, Inc. 2014; 27: 4-18.

31. Seo AN, Park KU, Choe G, Kim WH, Kim DW, Kang SB, et al. Clinical and prognostic value of MET gene copy number gain and chromosome 7 polysomy in primary colorectal cancer patients. Tumour biology: the journal of the International Society for Oncodevelopmental Biology and Medicine. 2015; 36: 9813-21.

32. Lee HE, Kim MA, Lee HS, Jung EJ, Yang HK, Lee BL, et al. MET in gastric carcinomas: comparison between protein expression and gene copy number and impact on clinical outcome. British journal of cancer. 2012; 107: 325-33.

33. Weingertner N, Meyer N, Voegeli AC, Guenot D, Renaud S, Massard G, et al. Correlation between MET protein expression and MET gene copy number in a Caucasian cohort of non-small cell lung cancers according to the new IASLC/ATS/ERS classification. Pathology. 2015; 47: 320-8.

34. Iveson T, Donehower RC, Davidenko I, Tjulandin S, Deptala A, Harrison M, et al. Rilotumumab in combination with epirubicin, cisplatin, and capecitabine as first-line treatment for gastric or oesophagogastric junction adenocarcinoma: an open-label, dose de-escalation phase 1b study and a double-blind, randomised phase 2 study. Lancet Oncol. 2014; 15: 1007-18.

35. Spigel DR, Ervin TJ, Ramlau RA, Daniel DB, Goldschmidt JH, Jr., Blumenschein GR, Jr., et al. Randomized phase II trial of Onartuzumab in combination with erlotinib in patients with advanced non-small-cell lung cancer. Journal of clinical oncology: official journal of the American Society of Clinical Oncology. 2013; 31: 4105-14.

36. Koeppen H, Rost S, Yauch RL. Developing biomarkers to predict benefit from HGF/MET pathway inhibitors. J Pathol. 2014; 232: 210-8.

37. Hartmann S, Bhola NE, Grandis JR. HGF/Met Signaling in Head and Neck Cancer: Impact on the Tumor Microenvironment. Clinical cancer research: an official journal of the American Association for Cancer Research. 2016; 22: 4005-13.