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Fluorescence in situ hybridization gene amplification analysis of EGFR and HER2 in patients with malignant salivary gland tumors treated with lapatinib

Laura Vidal[1], **Ming S Tsao**[1], **Gregory R Pond**[1], **Ezra EW Cohen**[2], **Roger B Cohen**[1], **Eric X Chen**[1], **Mark Agulnik**[1], **Sebastien Hotte**[1], **Eric Winquist**[1], **Scott Laurie**[1], **D Neil Hayes**[3], **James Ho**[1], **Janet Dancey**[4], and **Lillian L. Siu**[1]

[1]Princess Margaret Hospital Phase II Consortium, Toronto, Canada

[2]Section of Hematology/Oncology, Department of Medicine, University of Chicago, Chicago, USA

[3]The University of North Carolina, Chapel Hill, USA

[4]National Cancer Institute, Bethesda, USA

Abstract

Aim—Gene amplification status of the epidermal growth factor receptor (EGFR) and the human epidermal growth factor receptor 2 (HER2) were analyzed and correlated with clinical outcome in patients with progressive malignant salivary glands tumors (MSGT) treated with the dual EGFR/Her2 tyrosine kinase inhibitor lapatinib

Methods—Fluorescence in situ hybridization (FISH) analysis for both EGFR and HER2 gene amplification was performed successfully in the archival tumor specimens of 20 patients with adenoid cystic carcinomas (ACC) and 17 patients with non-ACC, all treated with lapatinib.

Results—For ACC, no EGFR or HER2 amplifications were detected. For non-ACC, no EGFR gene amplifications were detected but 3 patients (18%) were HER2 amplified and all had stained 3+ for both EGFR and HER2 by immunohistochemistry (IHC) in their archival specimens. Two of these patients had time-to-progression (TTP) durations of 8.3 months and 18.4 months respectively. Interestingly, patients with low and high HER2/chromosome-specific centromeric enumeration probe (CEP) 17 ratio had a prolonged TTP than those with moderate ratios for both ACC and non-ACC subtypes.

Conclusions—HER2 to CEP17 FISH ratio may predict which patients with MSGT have an increased likelihood to benefit from lapatinib. The finding of HER2:CEP17 ratio as a predictive marker of efficacy to lapatinib warrants further investigation.

Keywords

MSGT; lapatinib; EGFR and HER2 gene amplification; FISH

Corresponding author: Lillian L. Siu, MD, FRCPC, Department of Medical Oncology and Hematology, Princess Margaret Hospital, University Health Network, 610 University Avenue, Suite 5-718, Toronto, Ontario, M5G 2M9, Canada, Tel: 416-946-2911, Fax: 416-946-4467, Email: lillian.siu@uhn.on.ca.

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Introduction

Malignant salivary gland tumors (MSGT) only represent approximately 7% of all head and neck tumors. Surgery and/or radiotherapy are the primary treatment options when disease is localized. Adenoid cystic carcinomas (ACC) comprise between the 30% and 40% of the major and minor salivary gland cancers respectively. These tumors are characterized by both late local recurrences and distant metastases and their poor response to conventional cytotoxic chemotherapy (1,2,3,4). Contrarily, the non-adenoid cystic carcinomas (non-ACC) encompass a more heterogeneous group with different histologies, clinical presentation and biological behavior. Although non-ACC tend to have better response rates to chemotherapy agents, these are usually short-lasting (5,6).

The development of new treatment strategies for MSGT remains a challenge, especially in patients who have relapsed after definitive local therapy or those with metastatic disease. Previous reports indicate that MSGT present variable degree of EGFR or/and HER2 protein overexpression (7,8,9,10,11). While the prognostic significance of EGFR overexpression has not been well defined, overexpression of the HER2 oncoprotein has been associated with biological aggressiveness and poor prognosis in most studies (12,13). Therefore, there is a strong rationale to investigate the antitumor effect of agents targeting these receptors in patients with MSGT. Based on the clinical experience with targeted agents to EGFR and HER2 in other tumor types, the identification of biological markers is important and may help to select MSGT subgroups most likely to respond to these agents. Recent reports demonstrated that increased EGFR gene copy number as detected by fluorescence in situ hybridization (FISH) appears to correlate with improved clinical outcomes in non-small-cell lung and head and neck cancer patients receiving treatment with small molecule EGFR tyrosine kinase inhibitors (14,15). Furthermore, HER2 amplification status measured by FISH has been shown to correlate well with HER2/Neu protein overexpression by immunohistochemistry (IHC) and has similar predictive and prognostic significance in breast cancer (16,17).

Therefore, the aim of this study is to determine the gene amplification status of EGFR and HER2 by FISH in the archival tumor specimens of MSGT patients participating in a phase II clinical trial with the reversible dual EGFR and HER2 tyrosine kinase inhibitor lapainib, and to correlate such findings with clinical outcome (18).

Methods

Clinical study

Eligible patients with ACC and non-ACC were treated in two separate cohorts in a multi-center phase II trial with lapatinib given as 1,500 mg orally once daily and continuously until disease progression, unacceptable toxicity, patient refusal, or physician's decision to withdraw the patient (18). The primary objective of this study was to determine the objective response rate of lapatinib in MSGT, as classified by RECIST criteria. Secondary objectives were to evaluate the duration of response; rate and duration of stable disease; progression-free, median and overall survival rates, as well as safety and tolerability of lapatinib in this population. Institutional review board approval was obtained at all participating centers.

Tumor material

All patients enrolled in the study gave written informed consent for the collection, storage, and analysis of formalin-fixed paraffin-embedded primary tumor diagnostic specimen. All patient histologies were previously reviewed and confirmed by a blinded pathologist. For a patient to be eligible for the trial, tumor samples must stain at least 1+ for EGFR by IHC and/or at least 2+ for HER2 by IHC.

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IHC for EGFR and erbB2 expression

For EGFR immunohistichemical analysis, the mouse monoclonal EGFR antibody (clone 31G7, Zymed Lab, South San Francisco, CA) was incubated during 1 hour (1:50 dilution) with tissue sections and detected using routine avidin-biotin technique. For HER2 analysis, the sections were subjected to antigen retrieval by boiling in citrate buffer followed by incubation with the primary antibody (rabbit antip185/Her-2, Herceptest, DAKO AS, Copenhagen, Denmark). After sections were washed, they were incubated for 20 minutes each with biotinylated secondary antibody, followed by streptavidin–horseradish peroxidase using the Multi-Species Ultra Streptavidin Kit (Signet Laboratories, Dedham, MA).

The slides were developed for 5 minutes using the NovaRed substrate kit (Vector Laboratories, Burlingame, CA), and then counterstained with Mayer's hematoxylin. Slides were scored on a 0 to 3+ scale: 0, staining in less than 10% of tumor cells or no staining; 1+, faint and partial membrane staining in ≥10% of tumor cells; 2+, weak to moderate complete membrane staining in ≥10% of tumor cells; or 3+, moderate to strong complete membrane staining in ≥10% of tumor cells.

FISH for EGFR and HER2 amplification

For EGFR gene amplification, FISH assay was performed according to a previously published protocol (19). Enumeration of the number of locus-specific identifier (LSI) EGFR and CEP7 signals per nucleus was done according to the guidelines set forth by Varella-Garcia (20). Disomy, low trisomy, high trisomy and low polysomy were classified as FISH negative, while high polysomy and gene amplification were considered FISH positive.

For HER2, FISH was performed with the use of the FDA approved assay PathVysion HER2/*neu* DNA probe kit from Vysis (Abbott Molecular, Des Plaines, IL) according to the manufacturer's directions.

Enumeration of the number of LSI HER2/neu and CEP17 signals per nucleus for 25 tumor cells was done by two independent readers. A specimen was considered amplified for the HER-2/neu gene with a ratio of ≥2.0 and non-amplified with a ratio of <2.0.

Statistical analysis

Statistical analysis was carried out using S-plus 2000 for Windows (Insightful Corporation, Seattle, WA). Time to progression (TTP) and overall survival (OS) estimates were calculated using the Kaplan-Meier method and 95% confidence intervals constructed. Exploratory analysis was performed to investigate whether EGFR, EGFR:CEP7 ratio, HER2 or HER2:CEP17 ratio was predictive of TTP or OS using the log-rank test. Patients were grouped by the median EGFR and HER2 score, amplification status and IHC category. All statistical tests were two-sided and performed at the 0.05 level of significance.

Results

EGFR and HER2 gene and protein expression

Thirty-seven of the 39 (94.8%) archival specimens from the primary tumor of the patients enrolled onto the phase II clinical trial were successfully analyzed by FISH (Fig 1). Twenty samples were from patients with ACC and 17 were from non-ACC patients. Table 1 provides details on the histologies, EGFR and HER2 expression by IHC, EGFR and HER2 gene amplification status and CEP ratios by FISH.

No EGFR amplifications were found in ACC or non-ACC patients. HER2 gene amplification using conventional criteria (HER2:CEP17 ratio ≥2.0) were not found in ACC,

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but were found in 3 of the 17 (18 %) non-ACC patients. The tumor specimens of these 3 patients also stained 3+ by IHC for both EGFR and HER2. Overall, 75% (15/20) and 84% (16/19) of AAC and non-ACC patients had ≥2+ EGFR staining by IHC, respectively. However, the frequency of HER2 protein overexpression was in general low with staining ≥2+ in 5% (1/20) of ACC and in 42% (8/19) of non-ACC patients. Only 5% (1/20) of the ACC patients and 40% (8/19) of the non-ACC had any positive staining for both EGFR/HER2.

Association with clinical outcome

The clinical outcome of the phase II trial has previously been reported (18). No objective responses were seen but 9/20 ACC patients and 4/19 non-ACC patients (total 36%) had stabilization of disease for more than 6 months. For these patients, the mean TTP before initiating therapy was 3 months (range 2-6) (18). These patients with better clinical outcome had greater frequencies of 3+ EGFR (46%) or 3+ HER2 (75%) expression by IHC. As mentioned, 3 of non-ACC patients were HER2 amplified. Only 2 of them were evaluable for efficacy. These 2 patients who had poorly differentiated histologies (1 adenocarcinoma, 1 undifferentiated carcinoma), achieved two of the longest durations of time to progression (TTP) with 18.4 months and 8.3 months, respectively. The median follow–up duration for all patients was 15.8 months.

Exploratory analyses were performed using the EGFR:CEP7 and HER2:CEP17 ratios as predictors for TTP and OS in 20 ACC patients and 16 non-ACC patients whose FISH and efficacy outcome data were both available. No significant correlations were found between the EGFR:CEP7 ratio and clinical outcome. In an exploratory attempt to correlate the HER2:CEP17 ratio with clinical outcome, different cut-off values of the ratio were utilized beyond the conventional amplification definition. For instance, when ACC and non-ACC patients were classified based the cut-off value for the HER2:CEP17 ratio of less than 1 versus 1 or greater, no statistically significant differences were found between the subgroups (Fig 2). However, using an arbitrary set of cut-off values to define the HER2:CEP17 ratio as low (≤0.91), moderate (0.92-1.09) or high (≥1.10), a more striking separation in outcome was observed between the subgroups. Of note, 0.91 was selected since it is equivalent to a ratio of 1:1.10. Significant differences in the clinical outcome of these patients was observed (Table 2). For ACC patients, the TTP of patients with low/high ratios was 9.5 months versus 3.1 months for those with moderate ratios (p=0.03). For non-ACC patients, the TTP of patients with low/high ratios was 8.3 months versus 1.6 months for those with moderate ratios (p=0.015) (Fig 3). Differences were also detected in the OS of these subgroups although not statistically significant (Table 2).

Discussion

The identification of predictive markers remains a challenge in this era of novel molecularly targeted therapeutics. Correlative studies have become an integral part of clinical trials to establish relevant predictors of response to targeted therapies. Our study evaluated the value of EGFR and HER2 gene amplification in predicting outcome for patients with MSGT treated with lapatinib. This is the first study to analyze EGFR and HER2 gene amplification by FISH and to correlate with clinical outcome in this uncommon tumor type. These molecular abnormalities may confer different disease behavior and determine response to targeted therapies.

No EGFR gene amplifications were found in either ACC or non-ACC despite the high proportion of patients with ≥2+ EGFR protein expression by IHC. Although significant discrepancies between publications have been observed, the frequency of EGFR protein overexpression found in our patients with MSGT is similar to previous reports (7,8,9,12,21).

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Not surprisingly, EGFR protein expression did not correlate with tumor response. Most previous studies have shown that expression of EGFR protein in archival tumors by IHC is an unreliable predictor of responsiveness to EGFR inhibitor agents (22,23).

In our study, no HER2 gene amplifications were found in ACC patients. Although only few HER2 gene amplifications were found in non-ACC patients, these patients had better outcome with lapatinib. The HER2 amplified patients all had 3+ EGFR and HER2 staining by IHC, suggesting that HER2 amplification status by FISH based on conventional criteria did not contribute additional predictive or prognostic value in our study. Our findings are consistent with the general observation that EFGR but not HER2 seems to be greater expressed in ACC and vice versa in non-ACC (24). In our study, 9 of 39 (23%) patients with MSGT presented with 2+ and 3+ HER2 protein expression and higher frequency was observed for non-ACC patients (8/19,42%). Among the 8 non-ACC tumors overexpressing HER2, the histologies were as follows: adenocarcinomas (n=4), salivary duct carcinomas (n=2), undifferentiated carcinoma (n=1) and squamous cell carcinoma (n=1). Recent data indicate that some histologies of MSGT, in particular salivary duct carcinomas, present with higher rates of HER2 positivity either by IHC and FISH (25,26), and may be more likely to benefit from agents targeting HER2 such as trastuzumab (27). Furthermore, a parallel can be drawn between our findings in MSGT and that reported with in patients with metastatic breast cancer treated with lapatinib (28,29). In the latter, HER2 overexpression measured qualitatively or quantitatively was more predictive of response with lapatinib than EGFR expression.

From these results, the benefit of adding HER2 gene amplification by FISH as a predictive marker for response to lapatinib seems very limited in MSGT. However, we did observe an interesting relationship between HER2:CEP17 FISH ratio and clinical outcome for both ACC and non-ACC when a non-conventional set of criteria was used. The conventional criteria of HER2:CEP17 ≥ 2 as a cut-off to define amplification status in breast cancer does not necessarily apply to MSGT, as these malignancies likely possess different disease biology. In our exploratory analysis, patients were grouped arbitrarily into moderate versus low/high groups, based on non-conventional cut-off values for the HER2:CEP17 ratio. This subgroup evaluation, intended to be hypothesis generating, may have yielded a relevant marker for MSGT patients treated with agents targeting HER2. Patients with low or high HER2:CEP17 ratios appear to have a longer TTP than those with moderate ratios, reflecting improved clinical outcomes. A low HER2:CEP17 ratio may have prognostic value and reflects patients who have a less aggressive disease biology, whereas a high HER2:CEP17 ratio may have predictive value and reflects patients who benefited most from lapatinib. This finding needs to be confirmed with future larger studies in this disease. Although designing trials to validate these differences is challenging due to the small number of MSGT cases, recent studies have proven that, through collaborative group efforts, accrual can be rapid and optimized. (18,30)

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

IHC and FISH results of ACC and non-ACC patients for EGFR and HER2

EGFR FISH	HER2 FISH	EGFR IHC	HER2 IHC	HER2: CEP17 ratio	HER2 FISH signal	EGFR:CEP7 ratio	EGFR FISH signal	HISTOLOGY	TPP (days)
Non-Amp	Non-Amp	1+	1+	1.00	55	1.2		Undifferentiated carcinoma	162
Non-Amp	Non-Amp	2+	3+	1.00	58	1.1	57	Salivary duct carcinoma	83
Failed	Failed	3+	0					Adenocarcinoma	104
Non-Amp	Non-Amp	2+	2+	0.98	47	0.89	48	Salivary duct carcinoma	45
Non-Amp	Non-Amp	3+	0	0.96	53	1.1	59	Salivary duct carcinoma	NE
Non-Amp	Non-Amp	2+	0	1.10	62	0.90	56	Squamous cell carcinoma	112
Non-Amp	Amplified	3+	3+	4.60	463	1.1	69	Adenocarcinoma	559
Non-Amp	Non-Amp	2+	0	1.10	52	1.0	47	Mucoepidermoid	55
Non-Amp	Amplified	3+	3+	4.00	201	0.96	28	Undifferentiated carcinoma	251
Non-Amp	Amplified	3+	3+	3.00	228	1.1	65	Squamous carcinoma	NE
Failed	Failed	3+	2+					Adenocarcinoma	7
Non-Amp	Non-Amp	3+	0	0.90	57	1.1	53	Squamous carcinoma	56
Non-Amp	Non-Amp	3+	0	1.00	52	1.2	52	Undifferentiated carcinoma	50
Non-Amp	Non-Amp	1+	1+	0.92	59	1.1	53	adenocarcinoma	41
Non-Amp	Non-Amp	1+	1+	0.96	55	0.88	43	Salivary duct carcinoma	50
Failed	Failed	2+	1+					Acinic cell	49
Non-Amp	Non-Amp	3+	0	1.00	58	0.98	43	Mucoepidermoid	28
Non-Amp	Non-Amp	3+	2+	1.00	48	1.1	44	Adenocarcinoma	49
Non-Amp	Non-Amp	?	?	0.96	53	1.0	94	Adenocarcinoma	65
Failed	Non-Amp	3+	3+	0.86	56			Adenocarcinoma	287
ACC									
Non-Amp	Non-Amp	2+	0	1.20	77	0.92	62		650
Non-Amp	Non-Amp	1+	0	1.00	67	1.0	59		112
Non-Amp	Non-Amp	3+	1+	0.95	58	1.1	50		202
Non-Amp	Non-Amp	3+	1+	1.10	76	0.89	55		587
Non-Amp	Non-Amp	2+	0	1.00	59	0.98	57		79
Non-Amp	Non-Amp	1+	0	1.00	54	0.91	43		7

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EGFR FISH	HER2 FISH	EGFR IHC	HER2 IHC	HER2: CEP17 ratio	HER2 FISH signal	EGFR:CEP7 ratio	EGFR FISH signal	HISTOLOGY	TPP (days)
Non-Amp	Non-Amp	1+	0	0.90	64	0.85	47		107
Non-Amp	Non-Amp	2+	1+	1.10	57	1.0	54		109
Non-Amp	Non-Amp	1+	0	0.81	63	0.95	61		531
Non-Amp	Non-Amp	2+	1+	1.10	60	1.0	56		221
Non-Amp	Non-Amp	2+	0	0.83	54	0.82	51		55
Non-Amp	Non-Amp	2+	2+	1.00	53	1.1	57		24
Non-Amp	Non-Amp	2+	0	0.93	52	1.0	49		93
Non-Amp	Non-Amp	2+	0	0.91	58	1.1	46		385
Non-Amp	Non-Amp	2+	0	1.00	58	0.98	43		94
Non-Amp	Non-Amp	2+	1+	0.95	63	0.90	53		12
Non-Amp	Non-Amp	3+	0	0.96	70	1.1	52		111
Non-Amp	Non-Amp	1+	0	1.00	71	1.1	73		104
Non-Amp	Non-Amp	3+	0	0.86	45	1.0	56		356
Non-Amp	Non-Amp	3+	1+	0.90	53	0.85	47		64

Two non-ACC patients not evaluable (NE) due to 1) patient had prior history of second malignancy and 2) patient did not receive study drug.

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Table 2

Median TTP and OS for ACC and non-ACC patients based on HER2 to CEP17 ratio - An exploratory analysis

TTP	Non-ACC	p-value	ACC	p-value
Her2 <1	1.89 (1.5-NR)	0.69	3.59 (2.11-NR)	0.93
Her2 >=1	2.73 (1.64-NR)		3.50 (2.60-NR)	
Moderate	1.64 (1.48-NR)	0.015	3.08 (0.79-NR)	0.032
Low/High Expression	8.26 (3.68-NR)		9.49 (3.52-NR)	
OS				
Her2 <1	7.75 (3.06-NR)	0.11	NR	0.65
Her2 >=1	21.27 (7.34-NR)		NR	
Moderate	9.11 (6.38-NR)	0.17	NR	0.49
Low/High Expression	NR		NR	

Moderate: Her2 to CEP 17 ratio = 0.92-1.09

Low/High: Her2 to CEP 17 ratio ≤ 0.91 OR ≥ 1.10

NR = not reached