Genetic and Expression Analysis of HER-2 and EGFR Genes in Salivary Duct Carcinoma: Empirical and Therapeutic Significance

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Abstract

**Purpose:** Salivary duct carcinoma overexpresses epidermal growth factor receptor (EGFR) and HER-2, although the underlying mechanisms remain undefined. Because of the potential utilization of these markers as treatment targets, we evaluated protein and gene status by several techniques to determine complementary value.

**Experimental Design:** A tissue microarray of 66 salivary duct carcinomas was used for immunohistochemical analysis of HER-2 and EGFR expression (semiquantitatively evaluated into a three-tiered system), and fluorescence in situ hybridization for gene copy number, and chromosomes 7 and 17 ploidy status. Sequencing of exons 18, 19, and 21 of the EGFR gene for mutations was carried out.

**Result:** For EGFR, 46 (69.7%) of the 66 tumors showed some form of EGFR expression (17 at 3+, 17 at 2+, 12 at 1+) but none gene amplification. Five (9.4%) of 53 tumors showed mutations in exon 18 (n = 3) and exon 19 (n = 2). Polysomy of chromosome 7 (average >2.5 copies/cell) was detected in 15 (25.0%) of 60 tumors (6 at 3+, 5 at 2+, 2 at 1+, 2 at 0+ expression) and correlated with poor 3-year survival (P = 0.015). For HER-2, 17 (25.8%) of 66 tumors expressed HER-2 (10 at 3+, 3 at 2+, 4 at 1+). Eight tumors showed HER-2 gene amplification (6 at 3+, 1 at 1+, 1 at 0+ protein expression). Chromosome 7 polysomy was found in 8 (15.7%) of 51 tumors; two had HER-2 expression (3+, 1+).

**Conclusion:** Our study shows that salivary duct carcinomas (a) harbor EGFR gene mutations in a subset of tumors that may guide therapy, (b) pursue an aggressive clinical course in cases with chromosome 7 polysomy and high EGFR expression, and (c) with HER-2 gene amplification and protein high expression, may be selected for targeted therapy.

Salivary duct carcinoma is a high-grade, clinically aggressive adenocarcinoma variant with remarkable phenotypic and biological resemblance to mammary high-grade ductal carcinoma (Figs. 1A and 1C; refs. 1, 2). Patients with locally advanced, recurrent, and/or metastatic disease have limited therapeutic options and generally succumb to their disease. In the past two decades several individual genes, markers of different cellular pathways, chromosome alterations, and hormonal and growth factor receptors have been evaluated in an effort to define the underlying molecular and biological pathways associated with their development and progression (1, 3–7). Several of these studies have led to the characterization of certain hormonal and growth factors that are known to play a central role in the biology of breast cancers, including HER2 and epidermal growth factor receptor (EGFR; refs. 5, 8). However, the optimum methodology that best reflects their functional level for therapeutic purposes remains a subject of debate.

Growth factors and their receptors play a central role in mammary ductal tumorigenesis and in other adenocarcinomas, but the expression levels alone fail to predict responders to therapies (9). Several studies have shown that HER2 gene amplification status has improved the identification of possible responders to targeted therapy with trastuzumab (10–14). However, concurrent expression and gene amplification studies have identified a subset of low and intermediate HER2 gene expressions with high gene amplification, suggesting that immunohistochemical analysis alone leads to underestimation of patients eligible for therapy (14, 15). Central to addressing this issue is whether protein expression and gene amplification are mutually exclusive or complementary events in assessing the biological role of these factors for therapeutic stratification of patients with these tumors. Accordingly, initial immunohistochemical screening followed by gene amplification analysis by fluorescence in situ hybridization...
Translational Relevance

Our concurrent analysis, the most comprehensive to date, of the expression and the genomic alterations of the epidermal growth factor receptor (EGFR) and HER-2 receptor, defines the molecular alterations of these genes in salivary duct carcinomas for the biological stratification of patients with these tumors. Both activating mutations (10%) in the EGFR gene and chromosome 7 polysomy (25%) were identified in a subset of salivary duct carcinomas. These alterations have correlated with tyrosine kinase inhibitor treatment response in different adenocarcinomas and may potentially be applicable in salivary duct carcinoma patients. Additionally, chromosome 7 polysomy portends a poorer clinical outcome that trends with high EGFR expression. The finding of both overexpression of HER-2 and gene amplification defines a group of patients who may benefit from targeted therapy with trastuzumab. The results of this study highlight the spectrum of alterations in the EGFR family of receptors as potential targets for therapy in salivary duct carcinomas.

Materials and Methods

Sixty-six salivary duct carcinomas with archival formalin-fixed paraffin blocks available at the University of Texas, M.D. Anderson Cancer Center formed this study. The current WHO guidelines were used for classifying tumors as salivary duct carcinomas (30). A tissue microarray was created using two 1.0-mm diameter cores consisting of representative tumor from each paraffin block consisting of tumor tissue fixed in 10% buffered formalin. The tissue microarray was used for immunohistochemistry and FISH evaluations. Pathologic findings, which included immunohistochemistry, gene amplification, and mutational status, were compared with clinical factors that included gender, age, and stage along with clinical outcomes and were evaluated by Fisher’s exact test and chi-squared based on the number of comparative groups. A P value of 0.05 was considered significant.

Immunohistochemical analysis

Immunohistochemical analysis for HER-2 and EGFR was done using the automated BOND MAX immunohistochemistry stainer by Vision Biosystems on 4-μm paraffin sections of the tissue microarray material. In brief, following dewaxing, washing, and rehydration of the slides through xylene and graded alcohols, Tris-EDTA buffer was used for antigen retrieval. Slides were subsequently treated with 3% hydrogen peroxide to block endogenous peroxidase. Following incubation with the primary antibodies, HER-2 (clone c2-4001, mouse, 1:300, Labvision) and EGFR (clone 31G7, mouse, 1:50, Zymed), the secondary conjugate antibody was applied. Finally, each specimen-containing slide was developed using the chromogen 3,3’-diaminobenzidine and counterstained with hematoxylin.

Immunohistochemically stained tumor sections for HER-2 were evaluated for membranous expression: 3+, strong complete in >10%; 2+, weak complete in >10%; 1+, partial staining of tumor cells in >10%; 0, negative or <10% staining. EGFR immunohistochemistry in tumor cells was evaluated for membranous expression: 3+, strong membrane staining in >10%; 2+, moderate membranous staining in >10%; 1+, weak membranous staining in >10%, 0, negative or <10% staining.

FISH

A 4-μm paraffin section of the tissue microarray was analyzed by FISH using the manufacturer’s recommended standard methods for HER-2 [PathVysion HER-2 DNA Probe Kit; HER-2 Spectrum Orange/centromere enhancer of position effect (CEP)17 Spectrum Green, Vysis, Abbott] and EGFR (Vysis LSI EGFR Spectrum Orange/CEP 7 Spectrum Green, Vysis, Abbott). A known HER-2 amplified ductal adenocarcinoma was included in the tissue microarray as a positive control as well as normal salivary tissue as a negative control.

Each tumor sample was evaluated for the signals of HER-2 or EGFR signal and amplification pattern (spectrum orange) and the number of centromeric probe signals to chromosome 7 or 17 (spectrum green), respectively, within at least 20 tumor cells evaluated from both cores. Amplification was defined as clusters of probes (>10 copies/tumor cell).
cell) documented in ≥20% of cells analyzed. Tumors were also evaluated for low levels of amplification by comparing the gene to centromeric signals as a ratio, with a ratio <1.8 defined as nonamplified, 1.8 to 2.2 as indeterminate, and >2.2 as amplified (14). Additionally, chromosome copy number for chromosomes 7 and 17 were evaluated. Tumors were classified based on the percentage of cells with increased chromosomal numbers as outlined by Cappuzzo et al. (31), as well as the average number of centromeres per cell with evaluation for correlation with clinical and pathologic parameters. Chromosomal copy numbers per tumor cell were classified as the following: disomy, ≤2 copies in ≥90% of cells; low trisomy, 3 copies in <40% of cells; high trisomy, 3 copies in ≥40% of cells; low polysomy, ≥4 copies in <40% of cells; and high polysomy, ≥4 copies in ≥40% of tumor cells (31).

**EGFR gene sequence mutational analysis**

DNA from paraffin-embedded formalin-fixed tumor was enriched to >70% tumor cells and was suitable for sequencing exons 18, 19, and 21, hotspots of the EGFR gene in 53 of the 66 tumors. PCR was done using previously described primers (27). Amplified target DNA was then sequenced using ABI Big DyeTMv3.1 dye terminator cycle sequencing. Forward and reverse sequencing was carried out. A lung adenocarcinoma with known EGFR gene mutation was used as a positive control.

**Results**

**EGFR Immunohistochemistry.** Forty-six (69.7%) of 66 salivary duct carcinomas showed membranous expression for EGFR. Of the positive tumors, 17 (37.0%) showed strong complete membranous staining (3+), 17 (37.0%) were intermediate (2+), and 12 (26.0%) had weak membranous staining (1+; Table 1).

Although EGFR expression correlated significantly with local recurrence (34.6% versus 65.4%, *P* = 0.046) and was more frequently associated with distant metastases (44.0% versus 56.0%) and poor 3-year survival (59.1% versus 40.9%), it did not reach statistical significance (*P* = 0.6 and 0.5, respectively).

**Gene amplification (FISH).** EGFR gene amplification was not identified in any of the 66 salivary duct carcinomas analyzed by FISH.

**Chromosomal ploidy.** An increase in the chromosome 7 copy number in tumor cells (average >2.5 copies/cell) was found in 15 (25.0%) of the 60 tumors suitable for analysis (Table 2, Fig. 1B). Tumors with an average ploidy of >2.5 copies for chromosome 7 were significantly correlated with a lower 3-year survival (*P* = 0.002) and difference in overall survival by Kaplan-Meier assessment (*P* = 0.015; Fig. 2). Categorical classification showed 13 (21.7%) tumors with disomy for chromosome 7, 25 (41.7%) as low trisomy, 16 (26.7%) as low polysomy, 0 (0.0%) as high trisomy, and 6 (10.0%) as high polysomy (Table 2). Correlative analysis using the Kaplan-Meier method showed a decline in median survival with increased chromosomal alterations from 52.0 months for chromosome 7 disomy to 37.0 months for low trisomy, 30.5 months for low polysomy, and 14.5 months for chromosome 7 high polysomy (Table 2, Fig. 3). All patients alive without disease were noted to be diploid for chromosome 7. Salivary duct carcinomas with increased chromosome 7 copy number (>2.5 average) showed a more rapid clinical course with median survival of 20.0 versus 43.0 months for chromosome 7 <2.5 (*P* = 0.015; Table 2).

Chromosome 7 polysomy was present in 13 (28.3%) of the 46 tumors with EGFR expression (Table 1). Specifically, polysomy was noted in 2 (11.8%) of 0+, 2 (16.7%) of 1+, 5 (31.3%) of 2+, and 6 (40.0%) of 3+ tumors, although this association did not quite reach statistical significance (*P* = 0.06).

**Table 1. Comparative methods for analyzing EGFR and HER-2 in salivary duct carcinoma**

<table>
<thead>
<tr>
<th>Category</th>
<th>Immunohistochemistry expression score</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1+</td>
</tr>
<tr>
<td><strong>EGFR</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IHC</td>
<td>20 (30.3%)</td>
<td>12 (18.2%)</td>
</tr>
<tr>
<td>Gene amp</td>
<td>0 (0.0%)</td>
<td>0 (0.0%)</td>
</tr>
<tr>
<td>Polysomy* chr 7</td>
<td>2/17 (11.8%)</td>
<td>2/12 (16.7%)</td>
</tr>
<tr>
<td><strong>HER-2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IHC</td>
<td>49 (74.2%)</td>
<td>4 (6.1%)</td>
</tr>
<tr>
<td>Gene amp</td>
<td>1† (2.0%)</td>
<td>1 (25.0%)</td>
</tr>
<tr>
<td>Polysomy* chr 17</td>
<td>6/36 (16.7%)</td>
<td>1/4 (25.0%)</td>
</tr>
</tbody>
</table>

Abbreviations: chr, chromosome; Gene amp, gene amplification; IHC, immunohistochemistry.

*Polysomy of corresponding chromosome, average copy number >2.5
†This HER-2–amplified tumor showed 3+ immunohistochemistry on whole tissue section.
Mutational analysis. Our mutational analysis, the first in these tumors, showed that 5 (9.4%) of the 53 tumor specimens suitable for this analysis had mutations in exons 18 and 19, hot spots of the \textit{EGFR} gene: three in exon 18 and two in exon 19 (Supplementary Fig. S1). Point mutations in exon 18 were identified in codons L688P, A698T, and L718P. A 15 bp in-frame deletion, E746_A750del, and a point mutation in codon S752P were identified in exon 19. No mutations were identified in exon 21 (Table 3).

EGFR expression by immunohistochemistry was present in four of the five mutated tumors; two 3+, one 2+, and one 1+ expression. Polysomy of chromosome 7 was present in one of the three cases evaluable by FISH. HER-2 was not expressed or amplified in any of these five tumors. All five patients presented with advanced disease (stage III or IV), and four of the five died from disease from 7 to 42 months following diagnosis; all four developed lymph node or distant metastases.

### Table 2. Assessment of polysomy using average chromosome number versus categorical classification

<table>
<thead>
<tr>
<th>Chr no.</th>
<th>Average chr no.</th>
<th>Categorical classification*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>≥2.5</td>
<td>≥3.0</td>
</tr>
<tr>
<td>17 (n = 51)</td>
<td>8 (15.7%)</td>
<td>4 (7.8%)</td>
</tr>
<tr>
<td>7 (n = 60)</td>
<td>15 (25%)</td>
<td>10 (16.7%)</td>
</tr>
<tr>
<td>Chr 7</td>
<td>20.0 vs. (43.0 for &lt;2.5) vs. 22.0 vs. (37.0 for &lt;3.0)</td>
<td>52</td>
</tr>
</tbody>
</table>

Abbreviation: na, not applicable.

*Disomy defined as ≤2 chromosome copies in >90% of tumor cells; low trisomy, 3 chromosomes in <40% of cells; high trisomy, 3 chromosomes in ≥40% of cells; low polysomy, ≥4 chromosomes in <40% of cells; high polysomy, ≥4 chromosomes in ≥40% of cells.

†Median survival from Kaplan-Meier analyses (Figs. 2, 3).
HER-2

**Immunohistochemistry.** Seventeen (25.8%) of the 66 salivary duct carcinomas were positive for membranous HER-2 staining (Table 1). Of these, strong continuous membrane expression (3+) was present in 10 (58.8%), weak continuous staining (2+) in 3 (17.6%), and weak partial staining in 4 (23.5%).

No correlation between HER-2 expression levels and clinical factors including age, sex, tumor (T) and node (N) stage, or clinical outcome were found (Supplementary Table S1).

**Gene amplification (FISH).** HER-2 gene amplification by FISH was present in 8 (12.1%) of the 66 tumors. Seven of the eight amplified tumors showed protein expression: 6 (66.7%) with strong immunostaining (3+) and 1 with weak staining (1+; Table 1). The gene-amplified tumor with negative immunohistochemistry expression on tissue array showed areas of strong (3+) expression on the
The detection of five tumors with mutations in the EGFR gene in exons 18 and 19 suggests TKIs such as erlotinib may indeed provide targeted therapeutic options for a subset of salivary duct carcinoma patients. Several of these activating mutations were identified at sites that have previously correlated with high response rates to TKIs gefitinib or erlotinib in lung adenocarcinomas (21–23). We noted, however, that cases with mutations manifested variable levels of EGFR expression. This finding as previously noted in lung carcinomas suggests that immunohistochemistry is a poor surrogate for mutational status/clinical response to TKIs (9, 36–38). Therefore, molecular testing of the EGFR gene sequence is recommended to identify the subset of patients likely to benefit from these targeted TKIs like erlotinib. Moreover, we identified chromosome 7 polysomy in a quarter of salivary duct carcinomas. Recent studies in lung and colonic carcinomas have suggested that high polysomy

corresponding whole tissue section (heterogeneous distribution). HER-2 gene amplification was characterized by an excess of 5-fold (10+ copy number) per cell showing clusters of probe signal (Fig. 1D). There was a significantly higher proportion of gene amplified cases in the 3+ immunohistochemistry expression group than in the tumors with low expression (no immunohistochemistry expression group than in the tumors with low expression (P = 0.0004). Chromosomal polysomy. Chromosome 17 polysomy, an average of 2.5 copies/cell, was present in 8 (15.7%) of 51 tumors; of these eight, two were HER-2 positive by immunohistochemistry (one 3+ and one 1+) and one had HER-2 gene amplification. Ploidy classification showed 23 (45.1%) tumors with chromosome 17 disomy, 10 (19.6%) low trisomy, 1 (2.0%) high trisomy, 12 (23.5%) low polysomy, and 5 (9.8%) high polysomy (Table 2). No correlations between chromosome 17 polysomy and immunohistochemical expression, gene amplification, age, sex, T and N stage, or clinical outcome were noted regardless of polysomy categorization (Supplementary Table S1).

Correlations between HER-2 and EGFR

Similar expression profiles of EGFR and HER-2 were present in 22 (33.3%) of tumors (5 at 3+, 2 at 2+, 0 at 1+, and 17 at 0+). Three of the eight HER-2–amplified tumors also had chromosome 7 (EGFR) polysomy (>2.5 average copy number). Polysomy of chromosomes 7 and 17 was found in tumors with different levels of EGFR and HER-2 protein expression (Supplementary Table S2). No positive correlation between EGFR and HER-2 alterations was found (P = 0.008), suggesting independent roles for these markers in salivary duct carcinoma.
for chromosome 7 may also predict responsiveness to TKIs perhaps by reducing the rate of resistance to such therapies (25, 38). Thus, as more correlative information becomes available, molecular assessment through FISH analysis may provide a predictive assessment for therapeutic response.

Our results also show significant survival differences between patients with and without chromosome 7 polysomy (Fig. 2). Although, the rate-limiting polysomy number of biological significance is not known, our analysis of categorical groupings showed that even low levels of chromosomal duplication affected rate of progression to death (Table 2, Fig. 3). Similar to other carcinomas, polysomy/EGFR copy number in our study correlated with poor prognosis, suggesting DNA aneuploidy and/or relative increase in EGFR copy number plays an adverse biological role (14, 24, 26–28). Interestingly, previous reports have shown that response to cetuximab and panitumumab, two EGF inhibitors (TKIs), correlated with EGFR copy number (polysomy and amplification) in colon adenocarcinoma (25) and not mutational status. Similar correlation was noted in lung carcinomas, with response to gefitinib though EGFR mutations and HER-2 amplification also frequently present in this subgroup of patients (31, 39). The low-level amplification (≥2.5 chromosomal copies/cell) found in this study suggests that even a small increase in gene dose may be clinically significant and needs to be targeted by a therapeutic agent.

Heterodimerization of EGFR and HER-2 growth factor receptors may also cooperate to promote tumor growth and progression and may provide a rationale for the use of therapeutic agents or agents with duel targets (34, 40). As 20% of salivary duct carcinomas express combined EGF and HER-2 receptors, identification of this population may be required for more directed therapy. Moreover, lapatinib, a small-molecule inhibitor of both EGFR and HER-2 kinase activity, has shown response in breast adenocarcinoma, whereas gefitinib, which only blocks EGFR, has not improved response in that patient population (10). These results suggest that blocking multiple members of the growth factor receptor pathway may lead to better response in a subset of these patients. Such complex interactions of the EGFR pathway and targeted agents will require further investigations with careful correlation to uncover the mechanisms leading to therapeutic response.

Our results support those of others that HER-2 amplification is present predominantly in tumors with high (3+) expression (41, 42) and seems to be the dominant mechanism for HER-2 overexpression in this tumor type. This finding suggests that immunohistochemistry evaluation may reflect the functional status of this gene in most salivary duct carcinomas although amplification is notably lower (60–80%) than breast adenocarcinomas (90%) with high (3+) HER-2 expression (7, 41–43). Moreover, the incidence of HER-2 expression by immunohistochemistry in salivary duct carcinomas is similar to breast adenocarcinoma (15). Our findings are consistent with those of Jeahne et al. (8) where the HER-2 high (3+) expression in the 50 salivary duct carcinomas in that cohort was 20.6%, similar to ours and others (3), although was notably lower than in smaller reports (41, 44), which may reflect grouping of 2+ in the high-expression category and tumor selection by immunohistoexpression in some studies. It is worth noting, however, that the incidence of HER-2 expression was slightly lower in this study (15.2%, 3+) than in our previous report that included a majority of these tumors (3). The difference can be attributed to intratumoral heterogeneity (18, 45) of HER-2 and utilization of microarray sections, which underscores that certain markers could be underestimated using tissue array or on small biopsy specimens.

HER-2 gene amplification in a subset of these tumors highlights the potential therapeutic use of trastuzumab (Herceptin) in the management of selected patients similar to the treatment in mammary carcinoma (46). Trastuzumab, a humanized monoclonal antibody, is directed against the extracellular domain of the HER-2 tyrosine kinase receptor. Identifying the subset of patients who may benefit from trastuzumab is important to target the patients who may benefit from treatment (47). This is further supported by initial evidence of response of salivary duct carcinoma to trastuzumab (14, 48, 49).

In conclusion, our results show that molecular alterations of the EGF family of growth receptors, now known to be indicators (possible biomarkers) for potential targeted therapy, are present in a subset of salivary duct carcinomas. Both activating mutations (10%) in the EGFR gene and chromosome 7 polysomy (25%) were identified in a subset of salivary duct carcinomas. These alterations have correlated with tyrosine kinase inhibitor treatment response in different adenocarcinomas and may potentially be applicable in salivary duct carcinoma patients. Additionally, chromosome 7 polysomy portends a poorer clinical outcome that trends with high EGFR expression. The finding of both overexpression of HER-2 and gene amplification defines a group of patients who may benefit from targeted therapy with trastuzumab. The results of this study highlight the spectrum of alterations in the EGF family of receptors as potential targets for therapy in salivary duct carcinomas.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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