

# Amplification and Overexpression of *HER2/neu* Gene and *HER2/neu* Protein in Salivary Duct Carcinoma of the Parotid Gland

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**Objectives:** To detect amplification of the *HER2/neu* gene by means of fluorescence in situ hybridization (FISH) in a series of 13 salivary duct carcinomas (SDCs) and to compare the results with immunohistochemical (IHC) assessment of *HER2/neu* protein expression.

**Design:** Retrospective analysis.

**Setting:** Department of Pathology, University of Brescia.

**Patients:** We studied 13 cases of SDC diagnosed between January 1, 1997, and June 30, 2004, all arising from the parotid gland. Twelve patients were treated with surgery and radiotherapy, and 1 patient received only palliative radiotherapy. Seven patients died of disease, 3 patients were alive with disease, and 3 were free of disease.

**Main Outcome Measures:** *HER2/neu* protein expression and *HER2/neu* gene amplification detected by means of IHC assessment and FISH, respectively.

**Results:** With IHC assessment, 10 cases showed overexpression (grade 3+) of *HER2/neu* protein, whereas 3 cases were negative for this protein (grade 0/1+). Using FISH, amplification of the *HER2/neu* gene was found in 8 of the 10 grade 3+ cases, whereas none of the cases negative for the protein according to IHC assessment had amplification of the gene. Because of the small number of patients, it was not possible to statistically correlate *HER2/neu* protein expression or *HER2/neu* gene amplification and survival.

**Conclusion:** Our data demonstrate that *HER2/neu* protein is frequently overexpressed in SDC, and in contrast to previous reports, overexpression of the protein is associated in most cases with *HER2/neu* gene amplification.

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**A**LTHOUGH THE FIRST DESCRIPTION of salivary duct carcinoma (SDC) dates back to 1968,<sup>1</sup> the tumor was not officially recognized as a distinct entity by the World Health Organization until 1991.<sup>2</sup> Salivary duct carcinoma is characterized by peculiar clinical and pathologic features. Most patients are older than 40 years, with an approximate male to female ratio of 3:1. Ninety-five percent of lesions arise in the major salivary glands,<sup>3</sup> especially the parotid,<sup>4</sup> and only occasionally are the minor glands involved. The tumor can develop de novo or in a preexisting pleomorphic adenoma or polymorphous low-grade adenocarcinoma. Salivary duct carcinoma has an aggressive clinical behavior with a tendency for early facial nerve infiltration, extraparotid growth, and regional and distant spreading.<sup>4</sup> The survival rate is poor, with more than 60% of patients dying of the tumor within 3 years despite aggressive treatment, generally consisting of surgical resection and radiotherapy.<sup>4-6</sup> The most

peculiar histologic feature of this neoplasm is its resemblance to ductal carcinoma of the breast, with both intraductal (or in situ) and invasive components.

The resemblance of SDC to breast ductal carcinoma led to the study of hormonal receptor status and the *HER2/neu* gene (human epithelial growth factor receptor), also known as *c-erbB-2*, along with its gene product, a tyrosine kinase growth factor receptor. This gene is amplified in more than 20% of invasive breast cancers and is considered an adverse prognostic factor,<sup>7,8</sup> whereas overexpression of the protein seems to predict resistance to chemotherapy.<sup>9</sup>

*HER2/neu*, which is located at chromosome 17q12-21.32, is involved in many cell activities, including growth, development, and differentiation.<sup>10</sup> According to immunohistochemical (IHC) assessment, overexpression of *HER2/neu* protein has been identified in a high proportion of SDCs,<sup>8,10-12</sup> whereas fewer cases revealed amplification of the *HER2/neu* gene by means of fluorescence in situ hybridization (FISH).<sup>10</sup> Salivary duct carcinomas with

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**Table 1. Scoring System for Assessment of HER2 Protein Overexpression**

Staining Pattern	Score	HER2 Expression
No staining or membrane staining in < 10% of tumor cells	0	Negative
Faint membrane staining detected in > 10% of tumor cells (only part of the membrane is positive)	1+	Negative
Weak to moderate staining of the entire membrane is noted in > 10% of tumor cells	2+	Weakly positive
Strong staining of the entire membrane is noted in > 10% of tumor cells	3+	Strongly positive

either an amplified or nonamplified *HER2/neu* gene with strong IHC staining for HER2/neu protein are associated with a poor prognosis.<sup>10</sup>

The objectives of this study were to detect amplification of the *HER2/neu* gene by means of FISH in a series of 13 SDCs and to compare the results with IHC assessment of HER2/neu protein expression. Gene amplification and protein overexpression could potentially identify patients with a good response to treatment using a humanized murine monoclonal antibody (trastuzumab), which binds to the extracellular domain of the tyrosine kinase growth factor receptor, thus preventing its interaction with growth factors.<sup>8</sup>

## METHODS

### TUMOR MATERIAL

A computer search of the surgical pathology files of the Department of Pathology of the University of Brescia was performed for cases of SDC diagnosed between January 1, 1997, and June 30, 2004, and 13 cases were selected.

### IHC ASSESSMENT FOR HER2/NEU PROTEIN EXPRESSION

A 2-step immunohistochemical procedure was used with HercepTest (K5204; Dako A/S, Glostrup, Denmark). Tissue specimens were cut into 2- $\mu$ m sections, mounted on slides (SuperFrost Plus; Bio-Optica, Milan, Italy), deparaffinized in xylene, and rehydrated in descending grades (100%-70%) of ethanol. Specimens were then subjected to heat-induced epitope retrieval by immersion in 0.01M citrate buffer (pH, 8) in a calibrated water bath at 98°C for 40 minutes. Endogenous peroxidase was blocked using a 5-minute treatment with a peroxidase-blocking reagent that contained 3% hydrogen peroxide. The slides were then incubated for 60 minutes at room temperature with a primary rabbit antibody anti-HER2 protein (**Table 1**). Reaction products were visualized using a reagent based on dextran technology and 3-3'-diaminobenzidine chromogen. The slides were counterstained with Mayer hematoxylin. For each run, a composite slide of 3 formalin-fixed human breast carcinoma cell lines representing different HER2/neu protein expression levels (control cell line MDA-231 for score 0, MDA-175 for score 1+, and SKBR for score 3+) was used as a control. In addition, for each case, 1 slide was incubated with normal rabbit serum instead of the primary antibody and used as the negative control.

## INTERPRETATION OF IHC STAINING RESULTS

The IHC preparations were interpreted according to the criteria recommended by Dako for the HercepTest (Table 1). Overexpression of HER2/neu was defined as positive membrane staining in more than 10% of the neoplastic cells. Partial or incomplete, weak to moderate, and moderate to strong membrane staining in more than 10% of the tumor cells was scored as 1+ (negative), 2+ (weakly positive), and 3+ (strongly positive), respectively.

## FISH FOR *HER2/neu* GENE AMPLIFICATION

We performed FISH using 3- $\mu$ m-thick sections. The slides obtained were deparaffinized with a paraffin pretreatment kit (Vysis, Downers Grove, Illinois) before proceeding with the appropriate probe protocol (PathVysion HER-2 DNA Probe Vysis Kit; Vysis), which consisted of locus-specific identifier HER2/neu spectrum orange and chromosome enumeration probe 17 spectrum green probes. We applied 4,6-diamidino-2-phenylindole (DAPI) counterstain and antifade solutions (PathVysion HER-2 DNA Probe Vysis Kit; Vysis).

## FISH INTERPRETATION

The FISH signals, visible as fluorescent spots on interphase nuclei, were counted with an epifluorescent microscope (Nikon Optiphot-2 microscope; Nikon Instruments SpA, Florence, Italy), equipped with selective filters for the fluorochromes used. The FISH images were captured and elaborated with Genikon software (Nikon Instruments SpA) at  $\times 600$  magnification. The number of chromosome 17 and HER2 signals was scored for 60 cells when possible from 3 distinct tumor fields, and the mean HER2 to chromosome 17 copy ratio was calculated. Samples with more than 2.0 copies of HER2 for each chromosome 17 were considered to be amplified.<sup>13</sup>

## RESULTS

### CLINICAL FEATURES

The main clinicopathologic findings are summarized in **Table 2**. Patients were between 40 and 80 years old (10 men and 3 women; mean age, 66 years). All tumors originated in the parotid gland. Nine patients had a preoperative diagnosis of high-grade malignant tumor according to fine-needle aspiration cytologic assessment. The tumors clinically appeared in all cases as a painless mass in the parotid region; swelling had been present for a period ranging from 1 month to 20 years, with evidence of recent enlargement in most cases. At presentation, lymph node metastases and facial nerve paralysis were detected in 10 and 4 patients, respectively. Twelve patients (92%) underwent parotidectomy (total in 8 cases and radical in 4 cases). Neck dissection was performed with a therapeutic and elective intent in 9 patients (69%) and 1 patient (8%), respectively. In only 2 patients a neck dissection was not performed; both had a small primary tumor and no clinical or radiologic evidence of neck metastases. In 1 patient with multiple cervical node and lung metastases, palliative radiotherapy was planned. All surgically treated patients underwent adjuvant radiotherapy on the parotid region and the neck. In 9 patients (69%), extraglandular invasive growth of the tumor was detected. Eleven patients (85%)

**Table 2. Clinicopathologic Features of 13 Patients With Salivary Duct Carcinomas**

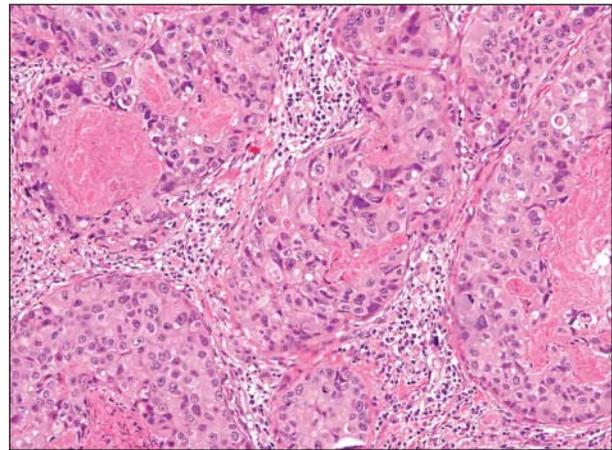
Patient No./ Sex/Age, y	Dimension, mm	Seventh Nerve Infiltration	Treatment	Extraglandular Extension	Nodal Metastasis	TNM	Follow-up (Site of Failure)
1/M/70	170	No	TP, ND, and RT	Yes	Multiple ipsilateral	T4a N2b M0	DOD at 18 mo (lung)
2/M/66	20	No	TP and RT	No	No	T2 N0 M0	DOD at 42 mo (lung)
3/F/70	15 (Multiple)	No	TP, ND, and RT	No	Multiple ipsilateral	T1 N2b M0	DOD at 92 mo (lung)
4/M/63	25	No	TP, ND, and RT	No	Multiple ipsilateral	T2 N2b M0	NED at 72 mo
5/F/40	13 (Multiple)	Yes	RP, ND, and RT	Yes	Multiple ipsilateral	T4a N2b M0	DOD at 25 mo (lung)
6/M/57	20	No	TP, ND, and RT	Yes	Multiple ipsilateral	T4a N2b M0	DOD at 27 mo (brain and lung)
7/M/75	18	Yes	RP, ND, and RT	Yes	Multiple ipsilateral	T4a N2b M0	AWD at 21 mo (locoregional and brain)
8/M/58	13	No	TP and RT	Not evaluable	No	T1 N0 M0	AWD at 50 mo (bone)
9/M/80	80	Yes	RP, ND, and RT	Yes	No	T4a N0 M0	DOD at 26 mo (local)
10/M/58	35	No	TP, ND, and RT	Yes	Multiple ipsilateral	T4a N2b M0	NED at 38 mo
11/M/43	20	No	TP, ND, and RT	Yes	Multiple ipsilateral	T4a N2b M0	AWD at 29 mo (locoregional)
12/F/71	35	Yes	RP, ND, and RT	Yes	Multiple ipsilateral	T4a N2b M0	NED at 28 mo
13/M/67	60	Not evaluable	Palliative RT	Yes	Multiple ipsilateral	T4a N2b M1	DOD at 2 mo (locoregional and lung)

Abbreviations: AWD, alive with disease; DOD, dead of disease; ND, neck dissection; NED, no evidence of disease; RP, radical parotidectomy; RT, radiotherapy; TP, total parotidectomy.

had stage IV disease. Follow-up information was available in all cases. Seven patients (54%) died of the disease in a period ranging from 2 to 92 months (mean survival, 33 months), mainly from metastatic spread of disease. Three patients each (23%) were alive with disease and free of disease (mean follow-up, 39.7 months) (Table 2).

### PATHOLOGIC FINDINGS

Macroscopically, the tumors were poorly circumscribed with invasive growth to adjacent tissue. The tumors ranged in size from 1.3 to 17.0 cm, with a mean size of 4.0 cm. The cut surface was yellow-gray and contained necrotic, hemorrhagic, and cystic areas. Microscopically, the tumors were composed of well-defined islands of epithelial cells that exhibited a cribriform, papillary-cystic, and solid pattern. Central comedonecrosis, strongly resembling ductal carcinoma of the breast, was a frequent finding. The infiltrative component consisted of solid, trabecular, and tubular structures often associated with a desmoplastic stromal reaction. The tumor cells had round-to-oval nuclei with prominent nucleoli and abundant, eosinophilic, granular, or vacuolated cytoplasm. Intracytoplasmic vacuoles produced negative results on periodic acid–Schiff stains. Mitotic figures were frequently observed. Perineural and intraneural invasion were frequently seen. An in situ component comprised closely packed, smooth, and discrete expanded salivary glands that lacked a definitive lobular arrangement. In situ SDC was present in most cases. The salivary ducts showed a fenestrated, solid, papillary, or cribriform pattern with foci of comedonecrosis. An attenuated layer of myoepithelial cells that expressed cytokeratin 5/6 could be clearly identified around some of the epithelial units (**Figure 1**). In 3 cases (patients 6, 9, and 10), SDC arose in a pleomorphic adenoma. Another patient (patient 1) had a 20-year history of parotid gland swelling with recent rapid enlargement; no concomitant pleomorphic adenoma was detected. Two tumors (in patients



**Figure 1.** Salivary duct carcinoma of the parotid gland composed of solid and cribriform growth patterns with comedonecrosis (hematoxylin-eosin, original magnification  $\times 40$ ).

2 and 7) showed a combination of SDC and Warthin tumor. A lesion (in patient 4) showed areas of typical SDC, but lakes of epithelial mucin-containing malignant cells were also present (ie, mucinous colloid carcinoma); these features were consistent with the so-called mucin-rich variant of SDC.

### IHC ASSESSMENT FINDINGS AND *HER2/NEU* GENE AMPLIFICATION

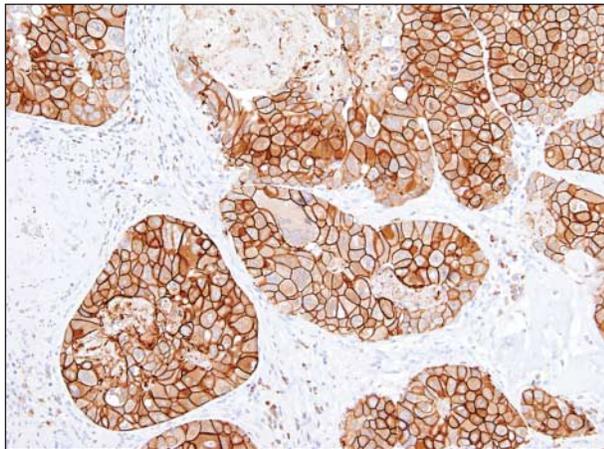
The results of IHC assessment and FISH are summarized in **Table 3**. On IHC assessment, 10 of 13 cases (77%) showed overexpression (grade 3+) of the *HER2/neu* protein (**Figure 2**), whereas 3 cases (23%) were negative for the protein (grade 0/1+). On FISH analysis, amplification of the *HER2/neu* gene was observed in 8 of the 10 overexpressed cases (80%) (**Figure 3**). Two cases were not amplified despite grade 3+ IHC assessment results.

**Table 3. Results of Immunohistochemical and FISH Analyses**

Case No.	HER2/neu	FISH CEP 17	FISH HER2/neu	Ratio <sup>a</sup>	Clinical Outcome
1	Positive	Polysomy	Amplified	2.73	DOD
2	Negative	Disomy	Nonamplified	0.77	DOD
3	Positive	Polysomy	Amplified	4.28	DOD
4	Positive	Polysomy	Nonamplified	1.19	NED
5	Positive	Disomy	Amplified	5.06	DOD
6	Positive	Disomy	Amplified	8.69	DOD
7	Positive	Disomy	Amplified	9.41	AWD
8	Positive	Disomy	Amplified	5.29	AWD
9	Positive	Disomy	Amplified	7.79	DOD
10	Positive	Disomy	Amplified	5.25	NED
11	Positive	Disomy	Nonamplified	1.03	AWD
12	Negative	Polysomy	Nonamplified	1.07	NED
13	Negative	Polysomy	Nonamplified	1.02	DOD

Abbreviations: AWD, alive with disease; CEP, chromosome enumeration probe; DOD, dead of disease; FISH, fluorescence in situ hybridization; NED, no evidence of disease.

<sup>a</sup>The number of chromosome 17 and HER2 signals was scored for 60 cells, when possible from 3 distinct tumor fields, and the mean HER2 to chromosome 17 copy ratio was calculated. Samples with more than 2.0 copies of HER2 for each chromosome 17 were considered to be amplified.



**Figure 2.** Strong membrane immunostaining in salivary duct carcinomas for HER2/neu protein (score 3+) (HerceptTest; Dako A/S, Glostrup, Denmark) (original magnification  $\times 40$ ).

All cases negative for the protein on IHC assessment were also nonamplified (Figure 3). Because of the small number of patients, it was not possible to statistically correlate HER2/neu protein overexpression or *HER2/neu* gene amplification and survival.

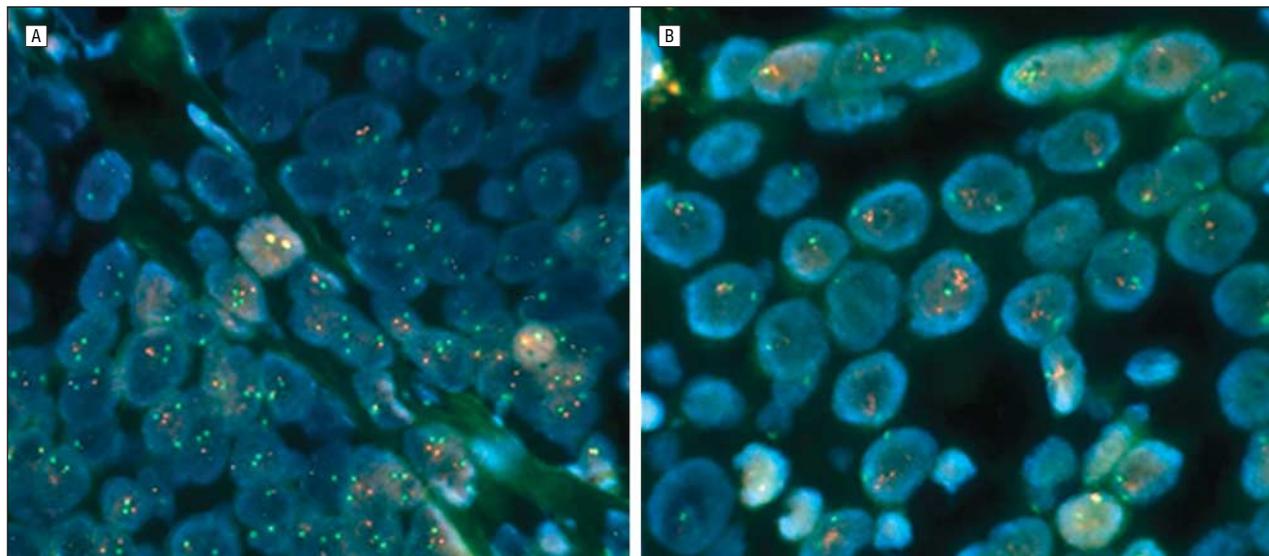
#### COMMENT

Salivary duct carcinoma is a rare neoplasm, with only a few reports focusing on large series.<sup>6,12</sup> Similarly to ductal breast carcinoma, SDC displays infiltrating and intraductal components. The former can include small ducts, cribriform structures, and small nests of cells and trabeculae, all accompanied by stromal desmoplasia.<sup>4,5,14</sup> Cells predominantly exhibit an eosinophilic cytoplasm and often vesicular nuclei that contain prominent central nucleoli.<sup>4</sup> Marked nuclear pleomorphism is seen, and mitotic figures are frequent. Perineural and vascular invasion are also frequent. The intraductal component com-

prises expanded salivary ducts that lack a definitive lobular arrangement with fenestrated (Roman bridge), solid, papillary, cribriform, and comedo patterns.<sup>3</sup> An attenuated layer of myoepithelial cells can be demonstrated around all the neoplastic islands by immunostaining for p63 and actin, indicating the absence of invasion.<sup>3,4</sup> In addition to the usual type of SDC, some rare variants have been described: SDC with papillary areas (and psammoma bodies), sarcomatoid SDC, mucin-rich SDC, and low-grade SDC. Low-grade SDC has a predominantly intraductal growth pattern with low-grade cytologic features<sup>15</sup>; this relatively indolent neoplasm is far less invasive and differs from conventional high-grade SDC<sup>15</sup> in regard to the IHC profile (eg, S100 protein expression and HER2/neu antigen negativity). In conventional SDC, IHC assessment has demonstrated expression of epithelial markers such as cytokeratins, epithelial membrane antigen, carcinoembryonic antigen, and gross cystic disease fluid protein in more than 80% of the cases.<sup>4,5,14</sup> A review of the literature indicates that immunophenotypical SDC also expresses androgen receptor in more than 90% of cases.<sup>16-19</sup> Expression of the estrogen receptor has been demonstrated only occasionally<sup>16,18,20</sup>; progesterone receptor positivity appears to be slightly more common, found in up to 20% of cases.<sup>16,18</sup> Rarely has SDC been found to express prostatic antigen markers.<sup>17,21</sup>

The *HER2/neu* gene proto-oncogene is located at chromosome 17q and is involved in the control of cell growth and development. The gene encodes a 185-kDa transmembrane tyrosine kinase receptor that is 1 of 4 members of the epidermal growth factor receptor family.<sup>22</sup> HER2 is capable of heterodimerization with any of the other 3 HER proteins and can participate in causing a signal transduction cascade with diverse effects that can augment the malignant phenotype.<sup>23</sup>

Amplification of the *HER2/neu* gene leads to marked overexpression of the membrane protein. Laboratory methods for evaluating *HER2/neu* gene amplification and protein overexpression include FISH and IHC assess-



**Figure 3.** Fluorescence in situ hybridization (original magnification  $\times 600$ ) in salivary duct carcinoma. A, Absence of *HER2/neu* gene amplification (red spots) in case 13. B, *HER2/neu* gene amplification (red spots) in case 5. In both A and B, a 17-chromosome disomy (green spots) is shown.

ment, respectively.<sup>10</sup> A wide spectrum of polyclonal and monoclonal antibodies directed against HER2/neu protein are commercially available. However, no clear standardization is available for scoring the degree of HER2/neu testing. The development of the HercepTest (Dako A/S), the only IHC method so far approved by the Food and Drug Administration, was a move toward standardization.<sup>24,25</sup>

Amplification of *HER2/neu* or overexpression of its protein has been identified in several types of human carcinoma, including breast, ovary, endometrial, and thyroid gland neoplasms, and has been associated with a poor prognosis.<sup>7,26</sup> Moreover, also in many salivary gland tumors, overexpression of the HER2/neu protein has been shown to correlate with a dismal outcome.<sup>12,27</sup>

Glisson et al<sup>28</sup> studied the overexpression of HER2/neu protein by means of the IHC method (HercepTest) in 137 salivary gland carcinomas subdivided into 2 categories according to the site of tumor origin: excretory duct (mucoepidermoid, squamous, and salivary duct) and intercalated duct (adenoid cystic, acinic cell, adenocarcinoma, malignant mixed, and myoepithelial). Malignant tumors of excretory duct origin showed a higher frequency of HER2/neu positivity: 55% (16 of 31) vs 7% (7 of 106). The frequency of overexpression in the 3 most common subtypes (adenoid cystic, adenocarcinoma, and mucoepidermoid) was only 8%. Salivary duct carcinomas overexpressed (2+ and 3+) HER2/neu in 83% of cases. HER2/neu overexpression in SDC has been reported in the literature, with a wide range of positive results (between 25% and 100%).<sup>10,12,20,29,30</sup>

The antibodies and staining methods vary considerably among different studies, and thus the results are discrepant. Skálová et al<sup>8</sup> published an IHC study of c-erbB-2/HER2/neu in SDC. Using the HercepTest, they showed overexpression in 14 of 15 cases of SDC (93%), whereas Dagrada et al<sup>31</sup> reported that HER2/neu was overexpressed in 11 of 18 cases (61%). In our study, overexpression of HER2/neu was detected in 10 of 13 SDCs

(77%). The reported rate of HER2 overexpression using the HercepTest ranges from 61% to 93%.

Moreover, Skálová et al<sup>10</sup> studied 10 cases of SDCs by means of both IHC assessment and FISH. They found strong IHC positivity (3+ score) in 7 cases, 4 (57%) of which carried *HER2* gene amplification, whereas 3 (43%) were nonamplified. Dagrada et al<sup>31</sup> studied *HER2* gene status in 18 cases of SDC. Eleven cases (61%) showed a score of 3+ on IHC assessment; 8 of 11 (73%) carried *HER2/neu* gene amplification, whereas 3 (27%) were nonamplified. None of the cases negative according to IHC assessment were amplified. On FISH analysis, we found amplification of *HER2/neu* in 8 of 10 (80%) of the 3+ cases by means of IHC assessment, whereas none of the IHC-negative cases showed gene amplification. Two cases (20%) showed overexpression of the HER2/neu protein but were nonamplified. These observations appear to indicate a weak correlation between HER2/neu protein expression and *HER2/neu* gene amplification in SDCs compared with breast cancer.

To explain the discordance between the results of IHC assessment and FISH, it could be hypothesized that antigen retrieval might increase the sensitivity of IHC tests, and consequently nonamplified cases might be interpreted as having false-positive IHC assessment results. However, HER2/neu protein overexpression could be controlled by mechanisms different from gene amplification.<sup>10</sup> Overexpression of a protein is controlled not only by the degree of gene amplification but also by the rate of gene transcription and protein degradation.<sup>10</sup>

The existence of nonamplified *HER2/neu* 3+ cases of SDC might have an important impact on treatment possibilities. According to recent experiences in breast<sup>32</sup> and salivary<sup>33</sup> cancer, both IHC assessment and FISH are necessary to identify the cases of SDC (*HER2* 3+; amplified) amenable to treatment with trastuzumab. However, even though trastuzumab is active in strongly HER2/neu overexpressed cases, the best predictor for response to monoclonal antibody seems to be gene ampli-

fication.<sup>32,33</sup> Supporting this possibility, Dagrada et al<sup>31</sup> concluded that FISH should be preferred over IHC assessment in the selection of patients with SDC considered eligible for trastuzumab treatment.<sup>31</sup>

In conclusion, this study demonstrated that the HER2/neu protein was frequently overexpressed in SDC, and in contrast to previous reports,<sup>12</sup> overexpression of the protein was associated in most cases with *HER2/neu* gene amplification. However, the limited number of patients prevented statistical evaluation of the relationship between HER2/neu protein overexpression or *HER2/neu* gene amplification and survival.

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**Author Contributions:** Dr Lombardi had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. **Study concept and design:** Cornolti, Ungari, and Facchetti. **Acquisition of data:** Cornolti, Rossi, and Lombardi. **Analysis and interpretation of data:** Morassi, Lombardi, and Nicolai. **Drafting of the manuscript:** Rossi and Lombardi. **Critical revision of the manuscript for important intellectual content:** Cornolti, Ungari, Morassi, Facchetti, and Nicolai. **Administrative, technical, and material support:** Rossi and Lombardi. **Study supervision:** Cornolti, Ungari, Morassi, Facchetti, and Nicolai.

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