HER-2/*neu* Gene Amplification in Esophageal Adenocarcinoma and Its Influence on Survival

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SYNOPSIS

The incidence and significance of HER-2/*neu* gene amplification in esophageal adenocarcinoma is unknown. Only 16% of esophageal adenocarcinoma patients had HER2 gene amplification, and its presence did not effect survival in this study.

ABSTRACT

Introduction: HER-2/*neu* (c-*erb*B-2, HER2) gene amplification and protein overexpression have been associated with poor prognosis in several solid tumors, including breast and gastric cancer. Its incidence and significance in esophageal adenocarcinoma is unknown. **Methods**: Tissue microarrays were successfully constructed from 89 paraffin-embedded archival specimens of esophageal adenocarcinomas for HER2 gene amplification by silver-enhanced *in situ* hybridization (SISH). No patients had undergone neoadjuvant therapy. Protein overexpression was tested with immunohistochemistry (IHC) using automated immunostaining (Ventana Benchmark). Incidence of HER2 positivity, correlation to clinicopathological variables in esophageal cancer patients, and concordance between SISH and IHC were determined. **Results**: True HER2 gene amplification was detected in 14 (16%) esophageal cancer specimens, and 92% of those with high-level HER2 amplification showed positive HER2 protein overexpression. No significant associations were found among gene amplification and clinicopathological factors. Five-year survival rates were 57% for esophageal cancer patients with HER2 amplification compared to 32% without, but the difference in overall survival was not significant (P=0.37). The correlation between SISH and IHC was statistically significant (P<0.0001). **Conclusion**: While molecular targeting may be possible for approximately 16% of esophageal adenocarcinoma patients, HER2 oncogene amplification did not influence survival in this study.

INTRODUCTION

Targeted molecular therapy in upper gastrointestinal cancer has become an increasingly popular topic over the past few years. In part, this is due to rapid advances in our capability to characterize tumor biology. Another consideration is our less-than-satisfactory ability to predict a particular tumor's response to neoadjuvant therapy. Esophageal adenocarcinoma is an example of an aggressive cancer in which only one third of patients present with resectable disease. And of this select group, the average 5-year survival is only 35 to 45%.[1] The addition of neoadjuvant therapy has significantly improved 5-year survivals, but much improvement is still needed. Targeted molecular therapy may help in this regard.

Human epidermal growth factor receptor gene HER-2/*neu* (also known as c-*erb*B-2, now HER2) was recognized as an important prognostic factor in breast cancer in 1987.[2,3] However, its role in other solid tumors is controversial.[4-9] The published frequency of HER2 overexpression in esophageal cancer ranges from 11 to 73%.[10] Reports evaluating its significance are also varied in their conclusions. Nevertheless an international randomized Phase III trial, evaluating the survival benefit in gastric or gastro-esophageal junction cancer patients of the humanized anti-HER2 monoclonal antibody (Trastuzumab), **has just been published**.[11]

The aims of our study were 1) to determine the frequency of HER2 gene amplification and overexpression in esophageal adenocarcinoma; 2) to evaluate the association of HER2 gene amplification with patient and tumor characteristics and patient survival; and 3) to examine the correlation between amplification and expression of HER2 using silver-enhanced *in situ* hybridization (SISH) and immunohistochemistry (IHC).

MATERIALS AND METHODS

Patient Selection

All patients who had undergone a surgical resection for invasive upper gastrointestinal adenocarcinoma were identified from an Adelaide-wide Esophageal Cancer Surgery audit database, held at the Royal Adelaide Hospital in Adelaide, Australia. Since July 1997, prospective follow-up data has been collected and stored in this database. Esophagectomy was performed by a 2-surgeon synchronous Ivor-Lewis technique via a right antero-lateral thoracotomy and an upper midline laparotomy, as described previously.[12] A conservative lymph node dissection (removal of all nodes adjacent to the tumor) was performed in all patients, regardless of operative technique.[13] **Patients who underwent neoadjuvant therapy were excluded from this study to obtain a homogeneous cohort of patients in terms of treatment and to circumvent possible stage migration following chemoradiation therapy.** The study was approved by the Research Ethics Committee at the Royal Adelaide Hospital, Adelaide, South Australia.

Tissue Microarrays

In a previous study[1], we re-examined 240 esophageal cancer pathology specimens to determine which variables could improve the accuracy of the TNM staging system. During this project, we also selected appropriate paraffin blocks for construction of tissue microarrays which were used in this study. **To increase our sample size, additional esophageal adenocarcinoma patients after January 2007 (up until December 2009) were included and appropriate paraffin**

blocks were selected for review. Specimen identification numbers were obtained from our database, and the designated paraffin blocks were then retrieved from one of 3 pathology laboratories: ClinPath Laboratories, Institute for Medical and Veterinary Science, and Adelaide Pathology Partners. Tissue microarrays were constructed with 2 cores, each 1.0 mm in diameter, from 2 paraffin blocks (i.e. 4 cores/patient). Representative cores of tumor were selected by A.R.R. based on each block's corresponding hematoxylin and eosin (H&E) stained sections. Other studies have demonstrated the reliability of tissue microarrays in the evaluation of HER2 gene amplification in solid tumors including breast carcinomas.[14]

Double-Staining for HER2 Amplification and AE1/AE3 Cytokeratin Expression

Tissue microarray sections (4 μm) were cut, mounted on Superfrost Plus coated slides, labeled and then placed on a fully automated immunohistochemistry (IHC) staining and In Situ Hybridization (ISH) Ventana Benchmark XT (Roche Diagnostics) instrument. The sections were incubated with ISH-protease 3 (Roche Diagnostics) for 8 min, washed with reaction buffer (Roche Diagnostics) followed by denaturation of tissue DNA at 95 °C. The DNA probe for Human Epidermal Growth Factor Receptor 2 (HER2) (Roche Diagnostics), labeled with Dinitrophenol (DNP), was then added and hybridization occurred for 6 hours. Rabbit anti-DNP (Roche Diagnostics) was used to detect the labeled probe followed by visualization with *ultra*View silver *in situ* hybridization (SISH) detection kit (Roche Diagnostics) in accordance with the manufacturer's standard procedures.[15]

The section was then washed in reaction buffer followed by addition of Cell Conditioning 1 (CC1) solution (Roche Diagnostics) for 30 minutes. CC1 was removed, washed, and the primary

mouse monoclonal epithelial antibody AE1/AE3 (Dako, Carpinteria, CA) for IHC was then added for 36 min whilst the slide was heated to 37°C. The monoclonal antibody AE1/AE3 is widely used because it recognizes a broad range of keratin subtypes expressed in esophageal carcinomas.[16] The *ultra*View™ Universal Alkaline Phosphatase RED kit (Roche Diagnostics), used in accordance with the manufacturer's recommendations, was used to detect the location of the primary antibody AE1/AE3 followed by counterstaining with hematoxylin 11 (Roche Diagnostics).

Evaluation of HER2 Gene Amplification

Evaluation of SISH hybridization was performed with conventional light microscopy by a histopathologist (A.R.R.) and a medical scientist (R.D.). **Both were blinded with respect to patient identification, tumor characteristics on conventional histopathology, and HER2 protein expression.** Gene amplification was assessed as per the Australian HER2 Advisory Board criteria for single HER2 probe testing: diploid = 1 to 2.5 copies/nucleus in more than 50% of tumor cells; polysomy = 2.5 to 4 copies/nucleus in more than 50% of tumor cells; equivocal amplification = >4 to 6 copies/nucleus in more than 50% of tumor cells; low-level amplification = 6 to 10 copies/nucleus in more than 50% of tumor cells; high-level amplification = >10 copies/nucleus in more than 50% of tumor cells. When using the Chromosome 17 probe, the classification of not amplified was when the HER2/Chromosome 17 ratio was <1.8; equivocal >1.8 and <2.2; and amplification was >2.2. HER2 and Chromosome 17 assays were performed on contiguous sections allowing for the identification and exclusion of chromosome 17 polysomy.[2,15]

Staining for HER2 Protein with Immunohistochemistry

Sections (4 µm) of tissue microarrays were cut, mounted on coated slides, labeled, and then placed on the Ventana Benchmark XT (Roche Diagnostics) for detection of the HER2 oncoprotein. The sections were de-waxed then subjected to pre-treatment with CC1 for 30 minutes. Sections were then washed with reaction buffer followed by incubation with the rabbit monoclonal primary antibody HER-2/neu (Clone 4B5, Roche Diagnostics) for 28 minutes. On board detection using *ultra*View™ Universal DAB kit (Roche Diagnostics), used in accordance with the manufacturer's recommendations, was used to detect the location of the primary antibody HER2 followed by counter stain with hematoxylin 11 (Roche Diagnostics).

Evaluation of HER2 Protein Expression

Evaluation and scoring of HER2-protein expression was performed according to the Dako HercepTest™ scoring system for breast cancer. This scoring system has been validated for use in gastric cancer with minor modifications:[3,17] 0/negative = staining or membranous reactivity in <10% of cells; 1+/negative = faint membranous reactivity in >10% of cells or cells with reactivity only in part of their membrane; 2+/equivocal = weak/moderate complete or basolateral membranous staining in >10% of tumor cells; 3+/positive = strong complete or basolateral membranous staining in >10% of tumor cells.

Statistical Analysis

The presence of HER2 gene amplification and/or protein overexpression was correlated with clinical outcome. Overall survival was calculated from the date of operation to **July 15, 2010** (if alive) or to the date of death (as recorded from the South Australian Cancer Registry) according to the Kaplan-Meier method. **Fisher's exact tests were used to compare variables between the two HER2 amplification groups (present/not present).** Survival was compared between the groups using a log rank test. Differences in survival between the HER2 groups were assessed using a log-rank test. Correlation between SISH and immunohistochemistry was calculated using the Kendall Tau-b correlation coefficient.[18] Statistical significance was set at the 5% level. Calculations were performed using SAS Version 9.2 (SAS Institute Inc., Cary, NC, USA).

RESULTS

Patients

There were **336 patients** who underwent a surgical resection for esophageal cancer between July 1997 and **December 2009** identified from the database. The 30-d mortality rate was 4.8%. Of these, 140 met inclusion criteria of an esophageal adenocarcinoma and no chemoradiotherapy prior to surgical resection. A further 51 patients were excluded for various reasons, and we were left with a study population of **89 patients (Figure 1)**.

Patients' and tumor characteristics are listed in Table 1. The mean age was 63.9 years (95% CI 61.7-66.1 years). There were 74 men (83%) and 15 women (17%). The median time of patient follow-up was 20.6 months (627 days). Complete follow-up was available for all **89** patients with an overall 5-year survival rate of 35%, and a median survival of 22.1 months.

HER2 Amplification or Overexpression

Fourteen esophageal cancer patients had HER2 gene amplification (Figure 2). Similar numbers of patients had weak/moderate or strong membrane staining for HER2 protein overexpression (Table 2). **HER2 amplification was seen more commonly in pT1 (25%) and pT4 tumors (27%) versus pT2 (9%) and pT3 (11%) tumors but this difference was not significant (*P*=0.25).** The presence of low or high HER2 amplification did not influence any other patient or tumor characteristic (Table 3). **Five-year survival rates were 57% (median, 68.9 months) for esophageal cancer patients with HER2 amplification compared to 32% (median, 20.6**

months) without, but the difference in overall survival was not significant (*P*=0.37) (**Figure 3**). Similarly, in the Barrett's cancer subset of patients, there was no significant difference in overall survival between groups (*P*=0.29).

Correlation between HER2 Amplification and Overexpression

When SISH results were compared with HER2 immunohistochemical (IHC) data, eleven of twelve cases (92%) with high-level gene amplification showed positive 3+ protein expression (Table 4). The remaining case was negative for protein expression. One of two low-level gene amplification cases was equivocal (2+) on IHC testing, while the other was negative (1+). None of the diploid nor Polysomy 17 cases showed equivocal or positive protein expression. We did not classify any cases in the equivocal category for HER2 amplification using SISH. Overall, there was a significant correlation between SISH and immunohistochemistry for HER2 gene amplification and expression ($P<0.0001$). The correlation coefficient between SISH and IHC was 0.636 (moderate/strong association) ($P<0.0001$).

DISCUSSION

Close to 16% of our esophageal cancer patients had HER2 gene amplification and overexpression in their primary tumor. The previously quoted range of 11-73% for HER2 overexpression largely originates from studies conducted in the 1990s, and using primarily immunohistochemistry.[19-25] Some of these older studies concluded that HER2 protein overexpression corresponds with poor survival.[19,20] **But more recently, studies have examined the frequency of HER2 gene amplification in esophageal adenocarcinoma at the DNA level using either polymerase chain reaction (PCR) or some form of *in-situ* hybridization (ISH)**.[7,10,26-29] Our results correspond to these latter studies **(except one[26] with a small sample size of 25)** in which frequencies of HER2 amplification are consistently lower and range from **12-24%**.

Unlike one study in esophageal adenocarcinoma[10], upon which current Herceptin-based trials seem to be based, we found no correlation between the presence of HER2 amplification and patient survival. Nor was there any correlation between HER2 amplification and clinicopathological factors. Brien *et al* evaluated HER2 amplification with FISH in 63 Barrett's adenocarcinoma patients, and although they found no significant association between HER2 amplification and clinicopathological factors, they reported a significant association between its presence and poorer survival.[10] However, in this study, a low threshold of 4 or more signals (rather than the currently accepted threshold of 6 or more signals[15]) per nucleus was used to determine the presence of HER2 amplification.[10] In addition, patients with chromosome 17 polysomy were not excluded. Aneuploidy of chromosome 17, usually involving an increase in the number of chromosomal copies (i.e. polysomy), has been reported in approximately one third of breast cancers. However, increased protein expression at the significant 3+ level does not

seem to result from this mechanism because HER2 appears to remain normally regulated.[26] Some investigators have suggested that controversy regarding the role of HER2 amplification and its affect on survival might be explained by the failure to distinguish between true HER2 gene amplification and chromosome 17 polysomy.[26,30]

In esophageal adenocarcinoma at least, our results seem to be the norm rather than the exception.[22-25,27,29] The lack of any apparent effect of HER2 amplification on patient survival is supported by the absence of any association between HER2 amplification and known poor prognostic pathological factors (i.e. pT-stage, pN-stage). Results of the ToGA (Trastuzumab for Gastric Cancer) trial suggested that HER2-positive patients with junctional gastro-esophageal cancers were potential responders to anti-HER2 monoclonal antibody-based therapy.[11] However, even the authors of this trial point out that the survival benefit seen in the HER2-positive group may have been due to the presence of HER2 overexpression alone rather than the result of HER2-targeted therapy.

Support for HER2 amplification as a prognostic and predictive factor in gastric adenocarcinoma is also controversial with several studies showing a significant association[31], and others not.[32] The most recent of these encompassed 924 gastric cancer cases and is the largest study to date showing that HER2 expression is not related to patient prognosis.[33] Unfortunately, the authors did not confirm their results with *in situ* hybridization techniques. Similarly, the importance of HER2 amplification and expression in esophageal squamous cell carcinoma remains unclear. Soares *et al* found that 37% of patients were HER2-positive with immunohistochemistry, while only 19% of these were HER2-positive by FISH criteria. Those positive on FISH were shown to have significantly poorer survival.[5] However, Gibault *et al* reported overexpression of HER2 in

only 2.8% of patients with esophageal squamous cell cancer, and they concluded that HER2 "appears to be of poor interest" as a potential therapeutic target in this type of esophageal cancer.[34]

Aside from methodological factors (discussed in greater detail below), we may not have found a survival advantage in HER2-negative cases due to the clonal divergence of primary tumors and disseminated tumor cells (DTCs). Klein *et al* recently reported that HER2 gene amplification was not conserved between primary tumors and DTCs (i.e. neither the presence nor absence of HER2 amplification in the primary tumor was predictive for the HER2 status in DTCs of the same patient). More importantly, they found that HER2 amplification in the primary tumor did not affect survival, while HER2 amplification in DTCs led to significantly shorter survival suggesting an increased dependence on HER2 signaling in the latter group.[35] **This too is controversial however with Reichelt *et al* reporting the opposite finding.[27] They found perfect correlation of HER2 amplification using FISH between the primary tumor and lymph node/distant metastases, and no effect on overall survival.**

There are several limitations to our study. Perhaps foremost, our negative findings may relate to sample size (type II statistical error). **Our initial submission to the journal described the results of 70 esophageal adenocarcinoma patients. We reported a *P* value of 0.06 when comparing survival rates between those with HER2 amplification and those without (67% vs. 28%, respectively). Upon request by the journal, we re-analyzed failed SISH specimens in an attempt to increase our sample size. With a new total of 89 patients, we found similar differences in survival (57% with HER2 amplificaiont vs. 32% without) but a much less**

convincing *P* value of 0.37 suggesting that HER2 amplification has no influence on survival (at least in the negative sense).

Second, it is possible that by excluding patients who received neoadjuvant therapy, we created a selection bias favoring less advanced tumors. However, 65% of the patients in our study had advanced tumors (pT3 or pT4) due to the more infrequent use of neoadjuvant therapy in the late 1990s. And in our previous study[1], we found no significant difference in survival between 116 patients treated with surgery alone, and 124 patients treated with neoadjuvant therapy and surgery (5-year survival rates of 31% vs. 41%, respectively) (*P*=0.125). Further studies are needed which include patients who have received neoadjuvant therapy as well as those with metastatic disease.

As stated above, many prior studies have used immunohistochemistry (IHC) alone to determine HER2 expression in upper gastrointestinal cancer. However, IHC is susceptible to inter-observer variability and variations in testing protocols (such as insufficient or prolonged formalin fixation).[2,29] As well, a number of studies in breast cancer have indicated that gene amplification is a more accurate predictor of survival than gene expression.[31,36] Fluorescence *in situ* hybridization (FISH) was included in the diagnostic algorithms for HER2 positivity in breast cancer to reduce inter-observer error and confirm cases with equivocal HerceptTest staining (2+). However, FISH is a costly technology requiring both a fluorescence microscope and digital photography, and fluorescent signals will deteriorate over a few weeks.[2,31] In addition, a recent study by Rauser *et al* highlighted the unreliable detection of low-level HER2 amplification in Barrett's cancer using standard FISH in thin (4 μm) tissue sections.[37]

Bright-field *in situ* hybridization such as silver-enhanced *in situ* hybridization (SISH) used in our study is gaining popularity as it requires only a light microscope, and it is fully automated and rapidly performed. Staining remains stable for a long period and it is relatively easy to interpret.[2] **An additional advantage over chromogenic *in situ* hybridization (CISH) is that** HER2 and chromosome 17 assays can be performed on contiguous slides allowing for exclusion of polysomy rather than locus-specific amplification.[2,31] High concordance has been found between FISH and SISH in breast cancer studies (>95%), and high inter-observer concordance exists with SISH (93-95%).[2,36] We found high concordance between IHC and SISH in this study.

CONCLUSION

HER2 gene amplification and overexpression was present in 16% of esophageal adenocarcinomas. It did not appear to influence survival. Although a subset of esophageal adenocarcinoma patients may meet the criteria for anti-HER2 monoclonal antibody therapy, it is too early to suggest that such therapy may decrease disease-free recurrence rates and increase long-term survival. Future studies should employ reproducible methodology using *in situ* hybridization techniques. As well, research into targeted molecular therapies will have to take into account characteristics of both the primary tumor and disseminated tumor cells.

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FIGURE LEGENDS

Figure 1. Study population.

Figure 2. Formalin-fixed, paraffin-embedded esophageal adenocarcinoma tissue microarrays. Representative specimen (a, hematoxylin & eosin stain) showing no HER2 protein expression (b, AE1/AE3 immunohistochemical stain), and no HER2 gene amplification (c, silver-enhanced *in situ* hybridization). Second example (a, hematoxylin & eosin stain) showing 3+/positive HER2 protein expression (b, AE1/AE3 immunohistochemical stain), and high-level HER2 gene amplification (c, silver-enhanced *in situ* hybridization).

Figure 3. Overall 5-year survival according to the presence or absence of HER2 gene amplification for 89 patients who underwent surgical resection of esophageal adenocarcinoma. Although there was a difference in 5-year survival rates between these 2 groups : 57% vs. 32%, it was not significant (P=0.37).

Table 1. Patient and tumor characteristics

Variable	Esophageal Cancer[a] (n=89)
Age, y	
< 70	58 (65)
> 70	31 (35)
Sex	
Male	74 (83)
Female	15 (17)
Tumor location	
Lower 1/3 esophagus	23 (26)
GOJ[b]	66 (74)
Grade of differentiation	
Well/moderate (G1 + G2)	26 (29)
Poor/undifferentiated (G3 + G4)	61 (69)
Unknown	2 (2)
pT-stage	
T1	20 (23)
T2	11 (12)
T3	47 (53)
T4	11 (12)
pN-stage	
N0	37 (42)
N1	52 (58)
pM-stage	
M0	86 (97)
M1	3 (3)
Stage groups (UICC 2002)[c]	
I	19 (21)
IIA	17 (19)
IIB	5 (6)
III	45 (51)
IV	3 (3)
Radial margin	
Negative	43 (48)
Positive	45 (51)
Not assessable	1 (1)
Vascular invasion	
No	24 (27)
Yes	64 (72)
Unknown	1 (1)
Perineural invasion	
No	37 (42)
Yes	43 (48)
Unknown	9 (10)
Barrett's oesophagus	
No	38 (43)
Yes	51 (57)

[a]Esophageal cancer : all adenocarcinoma
[b]GOJ = gastro-esophageal junction
[c]Stage I: T1N0M0; Stage IIA: T2-3N0M0; Stage IIB T1-2N1M0; Stage III: T3N1M0/T4anyNM0; Stage IV: anyTanyNM1

Table 2. Incidence of HER2/neu amplification and immunohistochemical expression in esophageal adenocarcinoma

	Esophageal cancer (n = 89)
Gene amplification[a]	
No amplification	53 (59.5)
Polysomy 17	22 (25)
Low amplification	2 (2)
High amplification	12 (13.5)
Immunohistochemical expression[b]	
0	63 (71)
CY	14 (16)
1+	1 (1)
2+	11 (12)

[a]No amplification = <2.5 signals/nucleus; polysomy 17 = 2.5-5 signals/nucleus; low amplification = 6-10 signals/nucleus; high amplification = >10 signals/nucleus
[b]0 = no staining; CY = cytoplasmic staining; 1+ = weak/moderate complete membrane staining; 2+ = strong complete membrane staining

Table 3. Association between patient and tumor characteristics and HER2/neu amplification in esophageal adenocarcinoma (n = 89)

Variable	No. Patients	HER2/neu + n (%)	*P* value
Age, y			
< 70	58	7 (12)	0.23
> 70	31	7 (23)	
Sex			
Male	74	12 (16)	1.0
Female	15	2 (13)	
Tumor location			
Lower 1/3 esophagus	23	2 (9)	0.51
GOJ[a]	66	12 (18)	
Grade of differentiation			
Well/moderate (G1 + G2)	26	4 (15)	1.0
Poor/undifferentiated (G3 + G4)	61	9 (15)	
Unknown	2	1 (50)	
pT-stage			
T1	20	5 (25)	0.25
T2	11	1 (9)	
T3	47	5 (11)	
T4	11	3 (27)	
pN-stage			
N0	37	5 (14)	0.77
N1	52	9 (17)	
pM-stage			
M0	86	13 (15)	0.41
M1	3	1 (33)	
Stage groups (UICC 2002)[b]			
I	19	4 (21)	0.48
IIA	17	1 (6)	
IIB	5	1 (20)	
III	45	7 (16)	
IV	3	1 (33)	
Radial margin			
Negative	43	7 (16)	1.0
Positive	45	7 (16)	
Not assessable	1	0 (0)	
Vascular invasion			
No	24	5 (21)	0.33
Yes	64	8 (13)	
Unknown	1	1 (100)	
Perineural invasion			
No	37	7 (19)	0.53
Yes	43	5 (12)	
Unknown	9	2 (22)	
Barrett's oesophagus			
No	38	6 (16)	1.0
Yes	51	8 (16)	

[a]GOJ = gastro-esophageal junction
[b]Stage I: T1N0M0; Stage IIA: T2-3N0M0; Stage IIB T1-2N1M0; Stage III: T3N1M0/T4anyNM0; Stage IV: anyTanyNM1

Table 4. Comparative data for SISH HER-2/neu gene copy status and HER-2 IHC (amended HercepTest) in esophageal adenocarcinoma

	IHC[a] 0 (n=63)	IHC 1+ (n=14)	IHC 2+ (n=1)	IHC 3+ (n=11)
Diploid (n = 53)	44	9	0	0
Polysomy 17 (n = 22)	18	4	0	0
Low amplification (n = 2)	0	1	1	0
High amplification (n = 12)	1	0	0	11

SISH = silver *in situ* hybridization; IHC = immunohistochemistry

[a]0 = negative, 1+= faint or incomplete membrane staining; 2+ = weak/moderate membranous staining; 3+ = strong membranous staining