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ORIGINAL ARTICLE

ErbB receptors and fatty acid synthase expression in aggressive head and neck squamous cell carcinomas

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SUMMARY: Overexpression of ErbB receptors is frequent in head and neck squamous cell carcinomas (HNSCC) and seems to be correlated with tumor progression and metastasis. Fatty acid synthase (FASN), the key lipogenic enzyme responsible for the endogenous synthesis of fatty acids, is regulated by ErbB2 and overexpressed in several human malignancies.

METHODS: This study was performed to examine the immunohistochemical expression patterns of ErbB1, ErbB2, ErbB3, ErbB4, and FASN in a tissue microarray, containing 33 representative areas from aggressive primary HNSCC (whose patients had distant metastasis), and 21 matched lung metastasis.

RESULTS: Strong correlation among the expression of ErbB family receptors was found (ErbB1–ErbB2 *P* = 0.008, ErbB1-ErbB4 *P* = 0.018, EbB2-ErbB3 *P* = 0.001, ErbB2-ErbB4 *P* = 0.006, ErbB3-ErbB4 *P* = 0.012) in the HNSCC. FASN expression was significantly associated with ErbB2 (*P* = 0.024). Lymphatic permeation was correlated with ErbB3 (*P* = 0.033) and histological grade with ErbB4 staining (*P* = 0.050). ErbB1 and ErbB2 were found mainly in patients with smoking habit (*P* = 0.011 and *P* = 0.027), and ErbB2 was associated with alcohol consumption and clinical stage (*P* = 0.014 and *P* = 0.031). Finally, FASN was overexpressed in lung metastasis, in comparison with matched HNSCC samples (*P* = 0.006).

CONCLUSIONS: The results showed that high FASN immunohistochemical expression is a feature of HNSCC lung metastasis, and ErbB1-ErbB2, ErbB1-ErbB4, ErbB2-ErbB3, ErbB2-ErbB4, and ErbB3-ErbB4 expression levels are correlated in the respective primary tumors, being ErbB2 the preferred coexpression partner of all the other ErbB receptors.

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Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most frequent cancer worldwide and associated with alcohol and tobacco abuse (Hardisson, 2003; Zhou *et al*, 2006; Curado and Hashibe, 2009). Despite of improvements in surgery, radiotherapy, and chemotherapy over the last decade, the survival rates have improved marginally, and the overall 5-year survival rate for patients with HNSCC is among the lowest of the major cancers (Hardisson, 2003). The high mortality and poor prognosis have been associated with metastatic disease at regional and distant sites (Zhou *et al*, 2006; Curado and Hashibe, 2009).

Receptors of the ErbB or epidermal growth factor (EGF) family have low expression levels on the surface of normal adult cells, except for hematopoietic cell (O-charoenrat *et al*, 2002). Aberrant activation of the ErbB family members has been implicated in many human cancers, including HNSCC (Silva *et al*, 2004, 2008, 2009; Kassouf *et al*, 2008; Lafky *et al*, 2008; Syrigos *et al*, 2008; Wei *et al*, 2008), and it has been significantly correlated with increased tumor progression and metastasis (Pu *et al*, 2007; Zhang *et al*, 2007; Kassouf *et al*, 2008; Zeren *et al*, 2008; Silva *et al*, 2009). This family comprises four members (ErbB1/EGFR/-HER1, ErbB2/neu/HER2, ErbB3/HER3, and ErbB4/-HER4) ubiquitously distributed throughout the animal kingdom (Penuel *et al*, 2001; O-charoenrat *et al*, 2002; Holbro *et al*, 2003). Under normal physiologic conditions, the ErbB receptors play crucial roles in cell proliferation, differentiation, adhesion or migration as well as tumor invasion and apoptosis (O-charoenrat *et al*, 2002; Casalini *et al*, 2004; Roskoski, 2004; Zhang *et al*, 2007).

The signal transduction is mediated through binding of the ligand with receptor, except for ErbB2 that has no

ligand, and it is strongly activated by interactions with other ErbB family receptors (Penuel *et al*, 2001; O-charoenrat *et al*, 2002). The ligands are more numerous and varied than ErbB receptors and include, epidermal growth factor, transforming growth factor α, heparin-binding epidermal growth factor, amphiregulin, betacellulin, epiregulin, epigen, neuregulin 1–4, tomoregulin/TMEFF 1–2, and neuroglycan-C (O-charoenrat *et al*, 2002; Stein and Staros, 2006). The differential activation and coupling of ErbB family sustain intense activation of intracellular signaling pathways like mitogen-activated protein kinase (MAPK) or phosphatidylinositol 3-kinase (PI3K)-AKT (O-charoenrat *et al*, 2000, 2002; Penuel *et al*, 2001; Holbro *et al*, 2003; Roskoski, 2004; Stein and Staros, 2006).

Transcriptome analysis revealed a molecular connection between ErbB2 and the enzyme fatty acid synthase (FASN) through the MAPK and PI3K-AKT pathways (Kumar-Sinha *et al*, 2003; Menendez *et al*, 2004). FASN is a major lipogenic enzyme responsible for the endogenous production of saturated long-chain fatty acids from acetyl-CoA and malonyl-CoA (Jayakumar *et al*, 1995; Baron *et al*, 2004). In normal human tissues, FASN is down-regulated because of ingestion of sufficient level of dietary fatty acids. However, this enzyme is differently hyperactivated and overexpressed in some aggressive tumors and it has been associated with a poor prognosis (Alo *et al*, 1996, 2000; Gansler *et al*, 1997; Baron *et al*, 2004; Silva *et al*, 2009). The differential expression of FASN between normal and cancer tissues has led to the consideration of this enzyme as a potential target for anticancer therapy (Gabrielson *et al*, 2001; Pizer *et al*, 2001; Baron *et al*, 2004).

In view of these recent findings, the aim of this study was to evaluate the expression patterns of ErbB family receptors, FASN and their correlation with demographic and clinicopathologic factors in primary aggressive HNSCC whose patients had metastasis to the lungs during the follow-up. In addition, these molecular makers were compared in HNSCC and in the matched lung metastasis.

Materials and methods

Study population

A retrospective study was performed by analyzing 33 patients with primary HNSCC diagnosed and treated at the Department of Head and Neck Surgery and Otorhinolaryngology, A.C. Camargo Hospital, São Paulo, Brazil. The eligibility criteria included previously untreated patients with diagnosis of SCC exclusively localized in the head and neck and submitted to treatment in this institution. All included patients had lung metastasis from 3 to 93 months (median 23 months) after initiated the treatment and were submitted to resection of pulmonary nodules. Twenty-one out of 33 patients had paraffin embedded tissue samples from the lung metastasis. Demographic (age, gender, and race), lifestyle (smoking habit and alcohol consumption), clinical (macroscopy, tumor site, and clinical stage), and pathological factors (histological grade, vascular embolization, perineural infiltration, lymphatic permeation, and surgical margins) were analyzed. The tumors were staged according to the 2002 version of the International Union Against Cancer (TNM) classification and grouped as early (T1 + T2) or advanced clinical stage (T3 + T4); all cases were followed-up after treatment, being the disease recurrence microscopically confirmed. The histological grade was determined on the basis of the classification proposed by the World Health Organization (Wahi *et al*, 1971). Vascular embolization was classified according to the presence or absence of neoplasic cells, located both in the wall and in lumen of the blood or lymphatic vessels; perineural infiltration considered present when the tissue adjacent to the peritumoral and/or intra-tumoral nerves was involved by the neoplasic cells; surgical margins considered involved when invasive and/or '*in situ*' carcinoma was observed in the margins of the surgical specimens (margins with less than 5mm were classified as exiguous). This study was carried out with the approval of the Human Research Ethics Committee of A.C. Camargo Hospital.

TMA construction

From the previous defined areas, core biopsies were taken using a *Tissue Microarrayer* (Beecher Instruments, Silver Springs, MD, USA). Tissue cores with a dimension of 1.0 mm from each specimen were punched and arrayed in duplicate on a recipient paraffin block. Each core was spaced 0.2 mm apart. After cutting on the recipient block and transferring with an adhesive tape to coated slides for subsequent UV cross-linkage (Instrumedics Inc, Hackensack, NJ, USA), the slides were dipped in a layer of paraffin to prevent oxidation and were kept at −20°C.

Immunohistochemistry

The tissue samples were obtained from the archives of the Department of Anatomic Pathology, A.C. Camargo Hospital, São Paulo, Brazil and comprised by 33 aggressive HNSCC whose all patients developed lung metastasis (all diagnosis were confirmed histologically) and 21 matched lung metastasis. The TMA slides were deparaffinized for 30 min at 60°C with xylene rinses, and then rehydrated in graded ethanol solutions and water rinses. Thereafter, sections were treated with the endogenous peroxidase quencher solution (0.3% H_2O_2 for 15 min), and also blocked for avidin/biotin (DAKO Biotin Blocking System, Dako Cytomation, Denmark) and for protein (DAKO Protein Block Serum-Free), 20 min each prior to primary antibody incubation. Pressure cooker antigen retrieval consisted of one period at 125°C for 30 min and 90°C for 10 min in 10 mM citric acid solution (pH 6.0) followed by a washing step with phosphate-buffered saline (PBS). The incubations with the primary antibodies anti-ErbB1/EGFR (Dako – 1:400), anti-ErbB2 (Dako – 1:10000), anti-ErbB3 (Labvision Corporation, Fremont, CA, USA – 1:300), anti-ErbB4 (Neomarkers Corporation, Fremont, CA, USA – 1:300), and anti-FASN (Transduction Laboratories, Lexington, KY, USA – 1:3000) diluted in PBS were

made overnight at 4°C. Sections were washed again and incubated with secondary antibodies (Advanced ™ HRP Link; Dako) for 30 min followed by the polymer detection system (Advanced ™ HRP Link; Dako) for 30 min at room temperature. Reactions were developed with a solution containing 0.6 mg ml^{-1} of 3.3′-diaminobenzidine tetrahydrochloride (DAB; Sigma, St Louis, MO, USA) and 0.01% H_2O_2, counterstained with Mayer's hematoxylin, dehydrated and mounted with glass coverslip and xylene-based mountant. Positive and negative controls were included in all reactions. The negative control consisted of (1) omitting the primary antibody and incubating slides with PBS; (2) replacing the primary antibody with normal (species of antibody) serum. The immunohistochemistry (IHC) reactions were performed in duplicate on different TMA levels, representing 4-fold redundancy for each case. The second slides were 25 sections deeper than the first, resulting in at least 250 μm of distance between the two sections with different cell samples for each tumor.

IHC scoring was blinded to the outcome and clinical aspects of each tumor specimen. Each slide was scanned in low power field to choose the most stained area. The presence of a clearly visible dark brown precipitation was considered immunopositivity. Positivity for ErbB family members was identified as a sharply demarcated cell membrane staining or a diffuse intracytoplasmic labeling. Only cytoplasmatic staining for FASN was considered as positive. The intensities of the immunostaining were classified as: 0, no visible reaction; 1, weak, 2: moderate; and 3, strong reaction intensity, in a blinded analysis performed by two authors (SDS and IWC).

Statistical analysis

For frequency analysis in contingency tables, statistical analyses of associations between variables were performed by the Fisher's exact test (with significance set for $P < 0.05$) and for continuous variables the non-parametric Mann–Whitney *U*-test. The Wilcoxon rank-sum test was used to test group differences in the adjusted mean protein expression between aggressive HNSCC and matched lung metastasis. The intensities of the immunohistochemical reactions were grouped as: 0 (negative reaction), 1 (weak), and 2 (moderate + strong reactions); or negative (0) and positive (1 + 2+3) for the statistical analysis. The overall survival was defined as the interval between the beginning of treatment (surgery) and the date of death or the last information for censored observations, and it was estimated by the Kaplan–Meier method. All analyses were performed using the statistical software package STATA (STATA Corporation, College Station, TX, USA).

Results

The studied population consisted of 33 patients, from which 27 (81.8%) were men and six (18.2%) women, with the mean age of 53 years, ranging from 31 to 68 years. History of alcohol consumption was observed in 26 (83.9%) of the patients, tobacco smoking reported by 28 (90.3%) and alcohol plus tobacco in 26 (83.9%) of the cases. With regard to the ethnic group, 26 (78.8%) were Caucasians and seven (21.2%) non-Caucasians. A total of five cases (17.2%) were at early clinical stages (T1 + T2) and 24 (82.8%) at advanced clinical stages (T3 + T4). Most of the cases (74.2%) had clinically positive lymph nodes (N+). Of the eligible cases, vascular embolization was found in four patients (14.3%), perineural infiltration in 16 (57.1%), and lymphatic permeation in 14 samples (50.0%). Twelve cases (37.5%) were histologically well-differentiated (Grade I), 15 cases (46.9%) moderately differentiated (Grade II), and 5 cases (15.6%) poorly differentiated (Grade III). Of the patients who underwent elective neck dissection, microscopic lymph node capsular rupture was found in 13 cases (39.4%).

The immunohistochemical results are summarized in Table 1. The positive expression rates of ErbB1, ErbB2, ErbB3, and ErbB4 were 79.3%, 31.1%, 12.9%, and 54.8%, respectively. As depicted in Figure 1a–d, distinct patterns for the distribution of ErbB receptors were detected. The intracytoplasmic and nuclear expression of ErbB1, ErbB3, and ErbB4 did not correlate with clinicopathological data. On the other hand, ErbB2 membrane positivity, found in the stratum spinosum and granulosum of the adjacent morphologically normal epithelium as well as in well-differentiated tumors (Figure 1b), was more intense in early clinical stage (T1 + T2) ($P = 0.031$) (Table 1). Its intracytoplasmic staining was observed in undifferentiated tumor cells and more frequently in patients with history of tobacco and alcohol consumption ($P = 0.027$ and $P = 0.048$, respectively). Smoking habit was positively correlated with ErbB1 ($P = 0.011$) and ErbB2 ($P = 0.027$) membrane expression, and the alcohol consumption was also correlated with the presence ErbB2 in the cell membranes ($P = 0.014$) (Table 1). Microscopic characteristic such as lymphatic permeation was correlated with ErbB3 ($P = 0.033$) and histological grade with ErbB4 ($P = 0.050$) (Table 1). A strong positive correlation among the positivity of ErbB family receptors was found in HNSCC samples (ErbB1-ErbB2 $P = 0.008$, ErbB1-ErbB4 $P = 0.018$, ErbB2-ErbB3 $P = 0.001$, ErbB2-ErbB4 $P = 0.006$, ErbB3-ErbB4 $P = 0.012$) (Table 2).

The FASN positivity was intracytoplasmic and weak in the morphologically normal epithelium, where it was restricted to the lower epithelial cell layers. The strongest reactions were found in the HNSCC samples (Figure 1e). Nineteen cases (59.4%) were positive for FASN and this positivity was more intense in well-differentiated (Grade I) tumors ($P = 0.054$). A similar percentage of the cases were strongly positive for FASN and ErbB2 at the cell membrane ($P = 0.024$). Importantly, an inverse correlation of the FASN positivity was observed between primary HNSCC and the lung metastases. From the 21 matched samples, when HNSCC were compared with its lung metastases, 11 patients (52.4%) with negative or weak FASN expression in the oral primary tumors presented strong immunopositivity in the lung metastases ($P = 0.006$) (Table 3). Differences in the expression of ErbB family receptors in HNSCC and lung metastases were not observed.

Table 1 Correlation of ErbB family members and FASN protein expression with clinicopathological variables in 33 aggressive HNSCC primary cases

Characteristics		*Molecular markers*									
		FASN		*ErbB1*		*ErbB2*		*ErbB3*		*ErbB4*	
Variable	*Categories*	*Negative*	*Positive*	*Negative*	*Positive*	*Negative*	*Positive*	*Negative*	*Positive*	*Negative*	*Positive*
Gender	Male	10 (37.0)	17 (63.0)	7 (30.4)	16 (69.6)	18 (75.0)	6 (25.0)	22 (84.6)	4 (15.4)	11 (42.3)	15 (57.7)
	Female	3 (60.0)	2 (40.0)	3 (60.0)	2 (40.0)	2 (40.0)	3 (60.0)	5 (100.0)	0	3 (60.0)	2 (40.0)
Race	Caucasians	10 (40.0)	15 (60.0)	9 (39.1)	14 (60.9)	16 (69.6)	7 (30.4)	21 (87.5)	3 (12.5)	11 (45.8)	13 (54.2)
	Non-caucasians	3 (42.9)	4 (57.1)	1 (20.0)	4 (80.0)	4 (66.7)	2 (33.3)	6 (85.7)	1 (14.3)	3 (42.9)	4 (57.1)
Smoking habit	No	2 (66.7)	1 (33.3)	3 (100.0)	0	0	3 (100.0)	3 (100.0)	0	2 (66.7)	1 (33.3)
	Yes	10 (37.0)	17 (63.0)	6 (29.1)	17 (73.9)*	18 (75.0)	6 (25.0)*	23 (88.5)	3 (11.5)	11 (42.3)	15 (57.7)
Alcohol consumption	No	3 (60.0)	2 (40.0)	3 (60.0)	2 (40.0)	1 (20.0)	4 (80.0)	5 (100.0)	0	3 (60.0)	2 (40.0)
	Yes	9 (36.0)	16 (64.0)	6 (28.6)	15 (71.4)	17 (77.3)	5 (22.7)*	21 (87.5)	3 (12.5)	10 (41.7)	14 (58.3)
T stage	T1 + T2	1 (25.0)	3 (75.0)	0	3 (100.0)	1 (33.3)	2 (66.7)	4 (100.0)	0	2 (50.0)	2 (50.0)
	T3 + T4	10 (41.7)	14 (58.3)	7 (33.3)	14 (66.7)	19 (86.4)	3 (13.6)*	19 (82.6)	4 (17.4)	9 (39.1)	14 (60.9)
Lymph nodes	N0	4 (50.0)	4 (50.0)	2 (40.0)	3 (60.0)	5 (83.3)	1 (16.7)	6 (85.7)	1 (14.3)	3 (42.9)	4 (57.1)
	N+	9 (39.1)	14 (60.9)	7 (31.8)	15 (68.2)	15 (65.2)	7 (31.8)	20 (87.0)	3 (13.0)	11 (47.8)	12 (52.2)
Histological grade	I	6 (54.5)	5 (45.5)	3 (33.3)	6 (66.7)	8 (80.0)	2 (20.0)	9 (90.0)	1 (10.0)	3 (30.0)	7 (70.0)
	II	5 (33.3)	10 (66.7)	4 (30.7)	9 (69.3)	10 (76.9)	3 (23.1)	13 (86.7)	2 (13.3)	10 (66.7)	5 (33.3)
	III	2 (40.0)	3 (60.0)	2 (40.0)	3 (60.0)	2 (40.0)	3 (60.0)	4 (80.0)	1 (20.0)	1 (20.0)	4 (80.0)
Vascular embolization	No	9 (39.1)	14 (60.9)	8 (36.4)	14 (63.6)	15 (68.2)	7 (31.8)	20 (87.0)	3 (13.0)	11 (47.8)	12 (52.2)
	Yes	3 (75.0)	1 (25.0)	1 (50.0)	1 (50.0)	3 (100.0)	0	3 (75.0)	1 (25.0)	1 (25.0)	3 (75.0)
Lymphatic permeation	No	7 (53.8)	6 (46.2)	4 (36.4)	7 (63.6)	8 (66.7)	4 (33.3)	13 (100.0)	0	7 (53.8)	6 (46.2)
	Yes	5 (35.7)	9 (64.3)	5 (38.5)	8 (61.5)	10 (76.9)	3 (23.1)	10 (71.4)	4 (28.6)	5 (35.7)	9 (64.3)
Perineural infiltration	No	4 (33.3)	8 (66.7)	4 (40.0)	6 (60.0)	8 (72.7)	3 (27.3)	11 (91.7)	1 (8.3)	6 (50.0)	6 (50.0)
	Yes	8 (53.3)	7 (46.7)	5 (35.7)	9 (64.3)	10 (71.4)	4 (28.6)	12 (80.0)	3 (20.0)	6 (40.0)	9 (60.0)
Capsular rupture	No	8 (42.1)	11 (57.9)	7 (41.2)	10 (58.8)	12 (66.7)	6 (33.3)	17 (94.4)	1 (5.6)	7 (38.9)	11 (61.1)
	Yes	5 (38.5)	8 (61.5)	3 (27.3)	8 (72.7)	8 (72.7)	3 (27.3)	10 (76.9)	3 (23.1)	7 (53.9)	6 (46.1)

Percentages considering cases with complete information.
*$P < 0.05$.

At the end of the follow-up period, 10 (30.3%) patients were alive and 23 (69.7%) were dead because of the HNSCC. The 5-year overall survival rate for patients was of 35.2%. There were no associations among survival probability and membrane expression of ErbB1 (log-rank test, $P = 0.9656$), ErbBb2 (log-rank test, $P = 0.1378$), ErbB3 (log-rank test, $P = 0.9796$), ErbB4 (log-rank test, $P = 0.2802$), and FASN staining (log-rank test, $P = 0.5975$). Interestingly, a significantly lower survival probability for alcohol consumers (log-rank test, $P = 0.048$) was observed among the all studied clinicopathologic variables (data not shown).

Discussion

The improvement in loco-regional control of head and neck carcinomas over the last decades does not appear to modify the 5-year survival rate of these patients, mainly because of the appearance of distant metastases and second primary neoplasms (Hardisson, 2003; Zhou *et al*, 2006; Curado and Hashibe, 2009). HNSCC often spread to the lungs, with a variety of presentations. Resection of lung metastases was shown to significantly influence the mortality and morbidity of these patients (Younes *et al*, 1997). At present, therapeutic decisions are based on clinicopathologic parameters, including age, TNM stage, and histological grade. Although useful, these factors often fail to differentiate between more and less aggressive lesions. Improvement in patient survival requires an increased understanding of tumor invasion and metastatic mechanisms, particularly in aggressive HNSCC.

In this study, we demonstrated that FASN and ErbB family members are highly expressed in aggressive HNSCC samples. In our previous report, we have described both cytoplasmic and membrane staining for ErbB2 (Silva *et al*, 2004, 2008, 2009). Some authors argue that ErbB2 cytoplasmic staining could be non-specific (Press *et al*, 1994); however, ErbB2 cytoplasmic staining correlates with ErbB2 amplification in breast cancer, poor prognosis in lung cancers, and poor outcome in HNSCC samples (Gusterson *et al*, 1987; Cheng *et al*, 2005; Silva *et al*, 2008, 2009). Absence of detectable membrane expression of the ErbB family members by immunohistochemistry can be interpreted as internalized or newly synthesizing molecules within Golgi apparatus (Cheng *et al*, 2005). Apart from membrane staining, ErbB1, ErbB2, and ErbB3 can also be detected in the cell nucleus. However, the significance of cytoplasmic or nuclear staining still remains unclear. In our data, intracytoplasmic and/or nuclear expression of ErbB1, ErbB3, and ErbB4 did not associate with clinicopathologic characteristics. Conversely, in agreement with our previous results, a significant correlation was observed between intracytoplasmic ErbB2 staining and undifferentiated tumor cells (grade III) whose patients presented history of tobacco and alcohol consumption (Silva *et al*, 2008, 2009).

We found a strong positive association among the expression levels of ErbB family receptors, which are involved in a complex array of combinatorial interac-

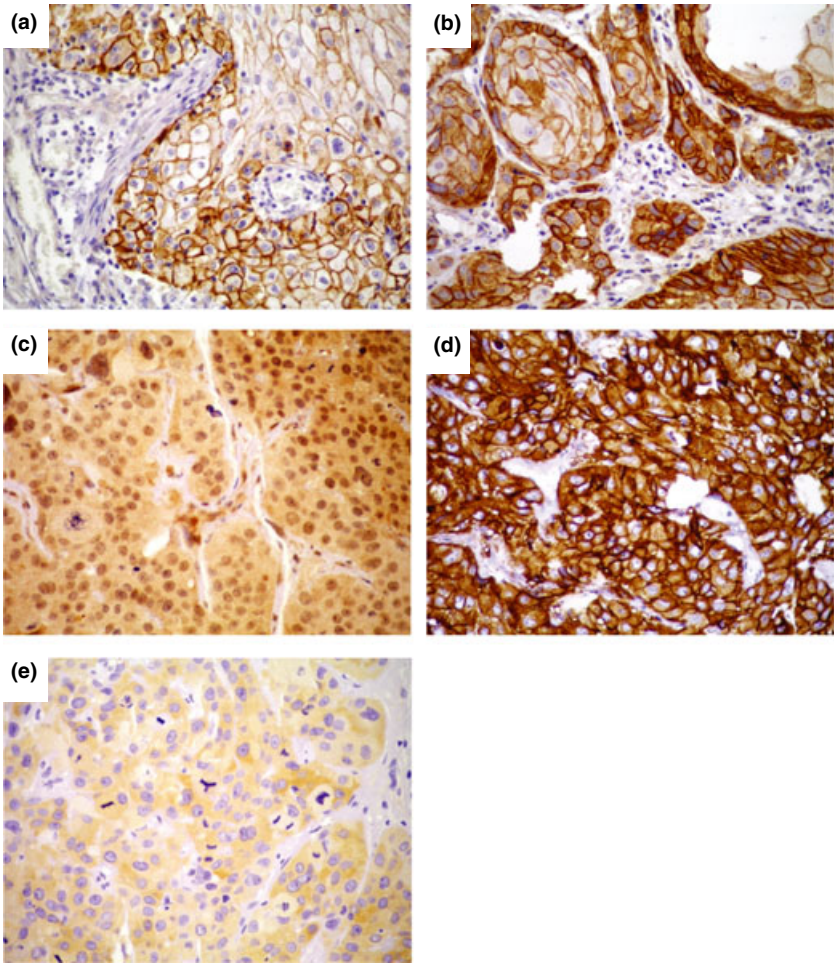

Figure 1 Representative immunohistochemical reactions against ErbB1 (**a**), ErbB2 (**b**), ErbB3 (**c**), ErbB4 (**d**), and FASN in HNSCC samples. Two distinct patterns of ErbB positivity were identified: a sharply demarcated membrane staining and an intracytoplasmic labeling (**a**, **b**, **c**, and **d**). The nuclear positivity was clearly observed for ErbB3 receptor (**c**). The strongest reactions for FAS were found in OSCC samples (**e**). Original magnification: 400×.

Table 2 Correlation among the molecular markers evaluated in HNSCC cases

Molecular markers		*FASN*, n (%)		*ErbB1*, n (%)		*ErbB2*, n (%)		*ErbB3*, n (%)		*ErbB4*, n (%)	
		0 + 1	*2 + 3*	*Negative*	*Positive*	*Negative*	*Positive*	*Negative*	*Positive*	*Negative*	*Positive*
FASN	0 + 1	21 (65.6)	0	6 (35.3)	11 (64.7)	18 (75.0)	2 (25.0)*	18 (90.0)	2 (10.0)	10 (50.0)	10 (50.0)
	2 + 3	0	13 (34.4)	4 (36.4)	7 (63.6)	2 (28.6)	7 (71.4)	9 (81.8)	2 (18.2)	4 (36.8)	7 (63.2)
ErbB1	Negative	6 (60.0)	4 (40.0)	10 (20.7)	0	10 (100)	0*	8 (80.0)	2 (20.0)	7 (70.0)	3 (30.0)*
	Positive	11 (61.1)	7 (38.9)	0	18 (79.3)	9 (50.0)	9 (50.0)	16 (88.9)	2 (11.1)	6 (33.3)	12 (66.6)
ErbB2	Negative	18 (75.0)	2 (25.0)*	10 (52.6)	9 (47.4)*	20 (68.9)	0	20 (100)	0*	13 (68.4)	6 (31.6) *
	Positive	2 (28.6)	7 (71.4)	0	9 (100)	0	9 (31.1)	5 (66.6)	4 (44.4)	1 (22.2)	8 (88.8)
ErbB3	Negative	18 (66.7)	9 (33.3)	8 (33.3)	16 (66.6)	20 (80.0)	5 (20.0)*	27 (87.1)	0	14 (53.8)	12 (46.2)*
	Positive	2 (50.0)	2 (50.0)	2 (50.0)	2 (50.0)	0	4 (100)	0	4 (12.9)	0	4 (100)
ErbB4	Negative	10 (71.5)	4 (28.5)	7 (53.8)	6 (46.2)*	13 (92.9)	1 (7.1)*	14 (100)	0*	14 (45.2)	0
	Positive	10 (58.8)	7 (41.2)	3 (20.0)	12 (80.0)	6 (42.9)	8 (57.1)	12 (75.0)	4 (25.0)	0	17 (54.8)

0: negative immunohistochemical reaction; 1: weak positive reaction; 2: moderate positive reaction; 3: strong positive reaction.
Percentages considering cases with complete information.
*$P < 0.05$.

tions involving homo- and hetero-dimers. Ligand binding stimulates receptor dimerization and tyrosine phosphorilation at several sites that serve to dock effector proteins and couple to physiologic responses (Penuel *et al*, 2001; O-charoenrat *et al*, 2002; Holbro *et al*, 2003; Stein and Staros, 2006). In the studied samples, ErbB2 was coexpressed with all ErbB family members. It has no specific ligand, and it is strongly activated by interactions with other ErbB family receptors (Penuel *et al*, 2001; O-charoenrat *et al*, 2002; Stein and Staros, 2006). For example, epidermal growth factor, which binds only ErbB1, activates ErbB2 more strongly than ErbB3 or ErbB4. Similarly, neuregulin β, which binds ErbB3 and ErbB4, activates ErbB2 more strongly than ErbB1 (Penuel *et al*, 2001; O-charoenrat *et al*, 2002; Holbro *et al*, 2003; Stein and Staros, 2006). Recent

Table 3 Correlation of molecular markers evaluated in HNSCC and the matched lung metastasis

Molecular markers	*HNSCC*	*Lung metastasis*		P-*value*
		Negative	*Positive*	
FASN	Negative	1 (4.8)	11 (52.4)	0.0063*
	Positive	5 (23.8)	4 (19.0)	
ErbB1	Negative	2 (11.8)	4 (23.5)	0.6875
	Positive	2 (11.8)	9 (52.9)	
ErbB2	Negative	9 (50.0)	4 (22.2)	0.3750
	Positive	1 (5.6)	4 (22.2)	
ErbB3	Negative	15 (75.0)	4 (20.0)	0.1250
	Positive	0	1 (5.0)	
ErbB4	Negative	4 (21.1)	4 (21.1)	0.9999
	Positive	3 (15.6)	8 (42.2)	

Percentages considering cases with complete information.
*$P < 0.05$.

studies concluded that patients with breast, ovary, lung, and bladder tumors show overexpression in ErbB1 and ErbB2 and more aggressive disease, associated with parameters predicting poor clinical outcomes and malignant transformation (Holbro *et al*, 2003; Ferretti *et al*, 2007; Zhang *et al*, 2007; Kassouf *et al*, 2008). Interestingly, in the studied samples, smoking habit and alcohol consumption, the most potent risk factors for HNSCC, were strongly associated with ErbB1 and ErbB2 ($P < 0.05$).

Some studies have discussed the possible role of the ErbB family members in human cancers (O-charoenrat *et al*, 2002; Holbro *et al*, 2003; Silva *et al*, 2004, 2008, 2009; Ferretti *et al*, 2007; Zhang *et al*, 2007; Kassouf *et al*, 2008). Overexpression of ErbB1 is common in SCC and was found to significantly correlate with tumor size and clinical stage (Holbro *et al*, 2003; Zhang *et al*, 2007; Kassouf *et al*, 2008). High levels of ErbB2 were shown to increase metastatic potential by promoting the multiple adhesion and invasion steps of the metastatic cascade (Holbro *et al*, 2003; Ferretti *et al*, 2007; Zhang *et al*, 2007; Kassouf *et al*, 2008). ErbB3 has not been shown to transform cells to a malignant phenotype on its own, but if overexpressed in the presence of ErbB2 it may induce transformation (Holbro *et al*, 2003; Zhang *et al*, 2007). The clinical significance of ErbB4 in cancer is not clear yet. It has been suggested that coexpression patterns of the four receptors may have more clinical and prognostic significance than the detection of these receptors alone (Holbro *et al*, 2003; Stein and Staros, 2006; Zhang *et al*, 2007).

The production of FASN was increased in HNSCC in comparison with the morphologically normal epithelium, and it was more intense in well-differentiated (grade I) tumors. Significantly, higher levels of FASN have been found in several tumors relative to their normal counterparts and more evident in the early differentiated stages of tumor development (Silva *et al*, 2004, 2008, 2009; Zhang *et al*, 2005). We have recently identified a significant correlation between ErbB2 at the cell surface and FASN positivity in well-differentiated (grade I) HNSCC samples (Silva *et al*, 2008, 2009). But, in our previous work, most patients did not have distant metastasis or nodal diseases (Silva *et al*, 2004, 2008) that are very strong predictors of survival (Kowalski *et al*, 2000). This study also confirmed the enhanced levels of FASN in ErbB2 positive in aggressive head and neck carcinomas. Recent studies revealed a bi-directional connection between FASN and the receptor ErbB2 (Kumar-Sinha *et al*, 2003; Menendez *et al*, 2004, 2005). It was experimentally demonstrated that the overexpression of human ErbB2 in mouse fibroblasts stimulates FASN protein through a MAPK and PI3K-AKT pathway (Menendez *et al*, 2005). FASN is the major enzyme required for anabolic conversion of dietary carbohydrates to fatty acids, which act as a metabolic fuel and are essential substrates for the synthesis of biological membranes (Zhang *et al*, 2005). The up-regulation of this protein may be a potential therapeutic target for the developing of anticancer drugs (Gabrielson *et al*, 2001; Pizer *et al*, 2001; Baron *et al*, 2004). Importantly, an inverse correlation of the FASN positivity was observed by comparing primary HNSCC and matched lung metastases ($P = 0.006$). FASN was more expressed in the lung metastases from primary HNSCC with reduced expression of this enzyme. Up-regulation of FASN expression is an early event in cancer development (Silva *et al*, 2004, 2008, 2009; Zhang *et al*, 2005) and often correlated with a poor prognosis (Alo *et al*, 1999; Baron *et al*, 2004; Silva *et al*, 2009). The mechanisms underlying cancer-associated FASN overexpression are not completely understood; however, it has been shown that steroid hormones can stimulate the expression of lipogenic genes via the Sterol Receptor Element Binding Protein (SREBP) pathway (Swinnen *et al*, 1997). Induction of SREBP pathway by androgens or epidermal growth factor increases FASN transcription through the activation of PI3K-dependent pathway in LNCaP prostate cancer cell line (Swinnen *et al*, 1997; Baron *et al*, 2004). In conclusion, the results here presented suggest that high FASN immunohistochemical expression is a feature of HNSCC lung metastasis and ErbB1-ErbB2, ErbB1-ErbB4, ErbB2-ErbB3, ErbB2-ErbB4, and ErbB3-ErbB4 expression levels are correlated in the respective primary tumors, being ErbB2 the preferred coexpression partner of all the other ErbB receptors.

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