

FAS ligand expression in inflammatory infiltrate lymphoid cells as a prognostic marker in oral squamous cell carcinoma

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Genet. Mol. Res. 14 (3): 11145-11153 (2015)

Received December 8, 2014

Accepted June 8, 2015

Published September 22, 2015

DOI <http://dx.doi.org/10.4238/2015.September.22.8>

ABSTRACT. Currently, the most important prognostic factor in oral squamous cell carcinoma (OSCC) is the presence of regional lymph node metastases, which correlates with a 50% reduction in life expectancy. We have previously observed that expression of hypoxia genes in the tumor inflammatory infiltrate is statistically related to prognosis in OSCC. FAS and FASL expression levels in OSCC have

previously been related to patient survival. The present study analyzed the relationship between FASL expression in the inflammatory infiltrate lymphoid cells and clinical variables, tumor histology, and prognosis of OSCC. Strong FASL expression was significantly associated with lymph node metastases ($P = 0.035$) and disease-specific death ($P = 0.014$), but multivariate analysis did not confirm FASL expression as an independent death risk factor ($OR = 2.78$, $95\%CI = 0.81-9.55$). Disease-free and disease-specific survival were significantly correlated with FASL expression ($P = 0.016$ and $P = 0.005$, respectively). Multivariate analysis revealed that strong FASL expression is an independent marker for earlier disease relapse and disease-specific death, with approximately 2.5-fold increased risk compared with weak expression ($HR = 2.24$, $95\%CI = 1.08-4.65$ and $HR = 2.49$, $95\%CI = 1.04-5.99$, respectively). Our results suggest a potential role for this expression profile as a tumor prognostic marker in OSCC patients.

Key words: FASL expression; Lymphoid cells; Oral cancer; Inflammatory infiltrate; Prognostic marker

INTRODUCTION

Head and neck cancer (HNC), the sixth most frequent type of cancer, is associated with high morbidity and mortality rates. The latest world census estimates that 600,000 new cases and 300,000 deaths can be attributed to this disease every year. Among these, oral squamous cell carcinoma (OSCC) contributes 389,000 new cases. Particular risk factors for HNC are alcohol and tobacco consumption, and it affects mainly individuals between 50 and 70 years old (Saman, 2012).

The five-year survival rate for HNC is 50%; treatment is based on radiotherapy in combination with surgery or cytostatic drugs. However, treatment resistance and relapse are common (Hsu et al., 2014). The epidemiology of the disease is very complex owing to its multigenic and multifactorial nature; it is associated with personal genetic susceptibility factors, life style, and the large number of environmental factors to which individuals are exposed (Han et al., 2010).

Currently, the most important prognostic factor for OSCC is the presence of regional lymph node metastases, which correlates with a 50% reduction in life expectancy (Myers and Fagan, 1998; Zhen et al., 2004). In addition, inflammatory infiltrate cells have been associated with many aspects of tumor behavior, causing microenvironment remodeling, angiogenesis, and tumor progression (Mantovani et al., 2008). This was observed in our previous study, in which hypoxia gene expression in the tumor inflammatory infiltrate was statistically related to prognosis in OSCC (Mendes et al., 2014).

Tumor immune evasion has been shown to promote solid tumor progression in many cases. Tumors use multiple mechanisms for evasion, including defective antigen presentation, interference with tumor/T-cell interaction, and production of immune suppression factors. Another possible evasion mechanism is FAS/FASL-mediated T-cell apoptosis (Töpfer et al., 2011).

Several factors are responsible for the modulation of tumoral growth and patient prognosis. Over the years, factors that alter proliferation and apoptosis have received a great deal of attention. It is believed that disequilibrium between proliferation and apoptosis may be the key factor in tumor development and prognosis (Shibakita et al., 2000).

Apoptosis is programmed cell death and it plays a critical role in the development and homeostasis of multicellular organisms (Shibakita et al., 2000). This complex process involves several genes, as well as mutations and polymorphisms that may lead to deficient death signaling and potentialization of tumor aggressiveness. Some tumor cells have acquired the ability to overcome apoptosis stimuli or to induce apoptosis of tumor-specific lymphocytes, favoring tumor progression (Zhang et al., 2005). Apoptosis resistance is common to most malignancies. Subversion of apoptotic pathways is a major mechanism in cancer development, being also related to tumor aggressiveness, degree of histological differentiation, and prognosis (Volm and Koomägi, 2000; Sun et al., 2004).

The FAS ligand (FASL/CD95L) and its receptor (FAS/CD95) are members of the tumor necrosis factor family and play a fundamental role in regulating the immune system. The *FASL* gene spans approximately 8 kb, has four exons, and encodes a type II transmembrane protein. FASL expression was observed initially in activated T-cells. However, a variety of cell types can express this protein, including tumor cells (De Maria and Testi, 1998). Shortly after ligand-mediated receptor activation, the apoptotic signal is relayed into the cell through the adapter molecule FAS-associated death domain (FADD), which is tethered owing to its death domain binding ability. FADD causes auto-cleavage of caspase-8, resulting in its active form, which in turn activates other caspases, initiating the cascade of events that culminates in activation of a specific DNase that cleaves nuclear DNA. This results in morphological and biochemical changes that are typical of apoptosis, culminating in cell death (Ashe and Berry, 2003; French and Tschopp, 2003). Altered FAS/FASL expression may cause tumor protecting immunomodulation, with a direct impact on patient prognosis (Ohno et al., 2000; Ehrenschwender and Wajant, 2009).

In previous studies, FAS and FASL expression in OSCC was related to patient survival (de Carvalho-Neto et al., 2013). Based on these results and on the importance of the microenvironment for tumor progression and prognosis, the present study aimed to analyze the relation between FASL expression in the inflammatory infiltrate lymphoid cells and clinical variables, tumor histology, and prognosis of OSCC.

MATERIAL AND METHODS

Ethics

This study was approved by the Research Ethics Committee of the Heliópolis Hospital on December 8, 2008 (CEP No. 637) and informed consent was obtained from all patients enrolled.

Samples

Samples were collected by the Head and Neck Genome Project (GENCAPO), a collaborative consortium created in 2002 with more than 50 researchers from institutions in Brazil. In this study, 64 tumoral tissue samples were obtained and used for immunohistochemical

analysis of FASL in lymphoid cells of inflammatory infiltrate from a total of 64 patients with OSCC, surgically treated at the Head and Neck Surgery Department of Heliópolis Hospital, São Paulo, Brazil, between January 2002 and December 2008. The clinical follow-up lasted for at least 48 months after surgery. Previous surgical or chemotherapeutic treatment, distant metastasis, non-removal of cervical lymph nodes, and positive surgical margins were exclusion criteria. Histopathological slides were reviewed by a senior pathologist to confirm the diagnosis and select appropriate areas for immunohistochemical analysis. Tumors were classified according to the TNM system (7th edition) (UICC, 2009).

The 64 patients were aged 35-81 years, with a mean age of 55.2 years (SD \pm 10.6 years); 54 (84.4%) were males and 10 (15.6%) were females. With regards to the anatomical location of the tumors, 26 (40.6%) were on the tongue, 12 (18.8%) were on the gums, 21 (32.8%) were on the floor of the mouth, and 5 (7.8%) were in the retromolar area. The clinical and pathological tumor characteristics are described in Table 1.

Immunohistochemistry

Anti-FASL monoclonal antibody (Santa Cruz Biotechnology[®], USA) was used in the immunohistochemistry reaction at a 1:400 dilution (Rimm et al., 2001; Hedvat et al., 2002; Hsu et al., 2002). Positive and negative controls were used. Sample scoring was performed by semi-quantitative microscopic analysis, considering the number of stained cells; signal intensity was evaluated for each sample and a mean score was calculated. Independent duplicates were analyzed by expert pathologists. Considering the percentage of immune-positive lymphoid cells, a score of 1 was given when \leq 10% of cells were positive; 2 when 10-50% of cells were positive; and 3 when $>$ 50% of cells were positive. Signal intensity was scored as negative (0), weak (1), moderate (2), and strong (3). The scores were multiplied together (Soini et al., 2000; Campos et al., 2009) and the resulting score was used to categorize FASL expression as strong (\geq 3) or ($<$ 3).

Statistical analysis

Chi-square and Fisher exact tests were used for association analysis, and confirmation was obtained by the Lilliefors test (significance considered when $P < 0.05$). Multivariate-logistic regression was used to obtain odds ratios and 95% confidence intervals. Survival was calculated by the number of months between surgery and death for each patient, or the last appointment in cases where the patient was alive. To calculate disease-free survival, the time endpoint was the date of disease relapse. The Kaplan-Meier model was used for survival analysis, using the Wilcoxon P value and Cox proportional hazards to adjust P values and obtain hazard ratios. Statistical calculations were performed using Epi-Info[®] v3.4.3, 2007 and StatSoft Statistica[®] v7.0.61.0 softwares.

RESULTS

FASL expression was studied in lymphoid cells from 64 tumors. Of these, 22 showed strong (34.4%) and 42 showed weak (65.6%) signals. FASL expression was not significantly associated with tumor characteristics such as size ($P = 0.581$), differentiation grade ($P = 0.511$), inflammatory infiltration ($P = 0.229$), and perineural invasion ($P =$

0.156), but was significantly associated with lymph node metastases ($P = 0.035$; Table 1). Multivariate analysis showed that strong FASL expression in lymphoid cells of the inflammatory infiltrate is an independent marker for lymph node metastases (OR = 5.39, 95%CI = 1.30-22.34).

Table 1. Clinical and pathological tumor features and their association with FASL expression in inflammatory infiltrate lymphoid cells.

Clinical pathological features	FASL expression						P value
	Total		Weak		Strong		
	No.	(%)	No.	(%)	No.	(%)	
Tumor size (T) [‡]							
T1+T2	26	(40.6)	19	(45.2)	7	(31.8)	0.581
T3	13	(20.3)	8	(19.0)	5	(22.7)	
T4	25	(39.1)	15	(35.7)	10	(45.5)	
Lymph node metastases (N) [‡]							
Absent	29	(45.3)	23	(54.8)	6	(27.3)	0.035
Present	35	(54.7)	19	(45.2)	16	(72.7)	
Differentiation							
Good	30	(46.9)	21	(50.0)	9	(40.9)	0.511
Moderate	29	(45.3)	17	(40.5)	12	(54.5)	
Poor	5	(7.8)	4	(9.5)	1	(4.5)	
Inflammatory infiltration							
Mild	23	(35.9)	17	(40.5)	6	(27.3)	0.229
Moderate	28	(43.8)	19	(42.5)	9	(40.9)	
Severe	13	(20.3)	6	(14.3)	7	(31.8)	
Perineural invasion							
Absent	30	(46.9)	17	(40.5)	13	(59.1)	0.156
Present	34	(53.1)	25	(59.5)	9	(40.9)	
Disease relapse							
No	28	(43.8)	22	(52.4)	6	(27.3)	0.054
Yes	31	(48.4)	17	(40.5)	14	(63.6)	
Not available*	5	(7.8)	3	(7.1)	2	(9.1)	
Disease-specific death							
No	35	(54.7)	27	(64.3)	8	(36.4)	0.014
Yes	24	(37.5)	11	(26.2)	13	(59.1)	
Not available*	5	(7.8)	4	(9.5)	1	(4.5)	
Total	64	(100.0)	42	(65.6)	22	(34.4)	

[‡]TNM classification, 7th edn. *Not available (not considered in the statistical calculations).

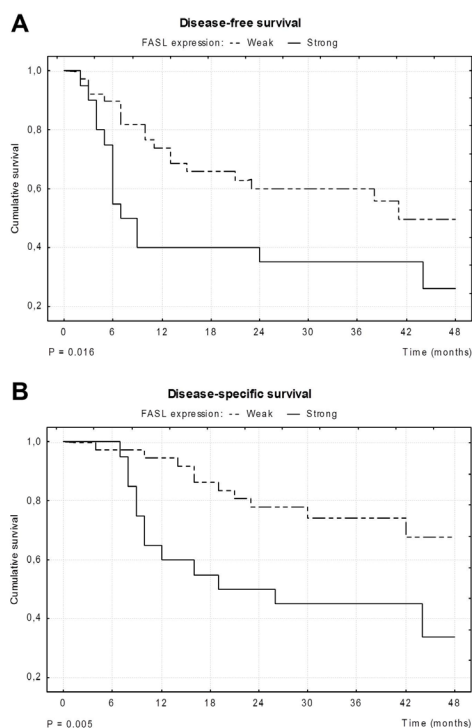
FASL expression did not show a significant association with disease relapse ($P = 0.054$), but was significantly associated with disease-specific death ($P = 0.014$; Table 1). However, multivariate analysis did not confirm FASL expression as an independent death risk factor (OR = 2.78, 95%CI = 0.81-9.55; Table 2).

Disease-free and disease-specific survival were significantly correlated with FASL expression ($P = 0.016$ and $P = 0.005$, respectively). According to a 24-month follow-up after surgery, approximately 60% of cases with strong expression presented disease relapse, compared with approximately 40% of recurrence in patients with weak expression of FASL (Figure 1A). Additionally, according to a 36-month follow-up after surgery, approximately 55% of cases with strong expression died of disease-specific causes, compared with approximately 25% of deaths in patients with weak expression of FASL (Figure 1B). Multivariate analysis revealed that strong FASL expression is an independent marker for earlier disease relapse and disease-specific death, with approximately 2.5-fold increased risk compared with weak expression (HR = 2.24, 95%CI = 1.08-4.65 and HR = 2.49, 95%CI = 1.04-5.99, respectively; Table 2).

Table 2. Multivariate analysis of the relationship between clinical, pathological tumor features and survival with FASL expression in inflammatory infiltrate lymphoid cells.

Features	Logistic regression				Cox proportional hazard			
	Lymph node metastases (N) ^y		Disease-specific death		Disease-free survival		Disease-specific survival	
	OR (95%CI) [§]	P value [§]	OR (95%CI) [§]	P value [§]	HR (95%CI) [§]	P value [§]	HR (95%CI) [§]	P value [§]
FASL expression								
Strong	1	1	1	1				
Weak	5.39 (1.30-22.34)	0.020	2.78 (0.81-9.55)	0.105	2.24 (1.08-4.65)	0.030	2.49 (1.04-5.99)	0.041
Tumor size (T) ^y								
T1+T2	1	1	1	1				
T3	0.61 (0.12-3.21)	0.562	1.77 (0.35-8.99)	0.489	1.91 (0.73-4.98)	0.187	1.86 (0.57-6.10)	0.307
T4	3.86 (1.04-14.35)	0.044	2.07 (0.55-7.85)	0.285	2.26 (0.92-5.53)	0.075	1.74 (0.63-4.77)	0.282
Lymph node metastases (N) ^y								
Absent	1	1	1	1				
Present	-	-	7.49 (1.16-48.48)	0.035	2.15 (0.77-5.99)	0.145	4.39 (1.22-15.83)	0.024
Differentiation								
Good	1	-	-	-	-	-	-	-
Moderate	1.39 (0.40-4.79)	0.602	-	-	-	-	-	-
Poor	3.75 (0.33-42.25)	0.284	-	-	-	-	-	-
Irradiated								
No	1	1	1	1				
Yes	-	-	0.30 (0.05-1.82)	0.190	0.25 (0.09-0.68)	0.006	0.42 (0.13-1.39)	0.154

OR = odds ratio; HR = hazard ratio; CI = confidence interval. ^yTNM classification, 7th edn. [§]Values adjusted by multivariate analysis.

**Figure 1.** Survival plots. **A.** Disease-free survival and **B.** disease-specific survival according to FASL expression in lymphoid cells.

DISCUSSION AND CONCLUSION

Tumor development presents a strong association with immune suppression and proliferation of cancer cells. The FAS/FASL pathway seems to play a dual role *in vivo* because it mediates proinflammatory effects as well as immune cell apoptosis (Hohlbaum et al., 2000).

The functional status and role of an inflammatory infiltrate of lymphoid cells in human cancer have been debated since the early 1970s (Ioachim, 1976). The main components of the lymphoid cells are CD4+ T helper cells, CD8+ cytotoxic lymphocytes, and natural killer cells (Torisu et al., 2000). After Clark's classic article demonstrating the positive relationships between lymphoid cells and prognosis, this fact has been widely accepted and supported for several cancer types (Clark et al., 1989; Okada et al., 2000; Reichert et al., 2002; Kase et al., 2003; Guo et al., 2008; Bozdogan et al., 2010; Pryczynicz et al., 2010), including head and neck cancer (Fang et al., 2013; Mendes et al., 2014).

Although no study has related FASL expression in inflammatory infiltrate lymphoid cells with clinicopathological and prognostic tumor features, many reports are found in the literature showing that FASL expression in tumor cells is important for prognosis and lymphoid cell apoptosis. Signaling dysfunctions and spontaneous apoptosis in circulating T-cells and in lymphoid cells of the inflammatory infiltrate of patients with cancer strongly suggest that immunosuppressive effects of the tumor extend beyond its microenvironment (Dworacki et al., 2001; Reichert et al., 2002; Kurita et al., 2010).

Gastman et al. (1999) showed that FASL expression in head and neck squamous cell carcinoma promotes tumor inflammatory infiltrate cell apoptosis. In the same line, Reichert et al. (2002) analyzed the correlation between FASL expression in OSCC and apoptosis index in the inflammatory infiltrate, suggesting that the tumor can alter immune cell survival via the FAS/FASL pathway, since most circulating T-cells are FAS+ and are therefore susceptible to apoptosis through interaction of FASL with its receptor (Okada et al., 2000; Hoffmann et al., 2002; Pryczynicz et al., 2010). Moreover, in colorectal cancer other authors have shown that lymphoid cell apoptosis is higher when FASL expression is strong (Bennett et al., 1998; Okada et al., 2000; Pryczynicz et al., 2010).

Fang et al. (2013) showed that the inflammatory infiltrate cell apoptosis index is increased compared to tumor cell apoptosis in oral cancer with strong FASL expression, suggesting an important role for FASL in tumor progression. Moreover, Okada et al. (2000) suggested that tumor cells may be resistant to apoptosis via FAS/FASL, owing to failed apoptotic signal transduction. This explains the observation that apoptosis is higher in inflammatory cells than in tumor cells.

In this study, strong FASL expression in lymphoid cells of the tumor inflammatory infiltrate was associated with a 5-fold increase in the risk for lymph node metastases compared with weak expression. Our results are in agreement with Okada et al. (2000), who reported a strong correlation between increased lymph node metastases in colorectal cancer and apoptosis in inflammatory infiltrate lymphoid cells, which can be explained by a decreased immune surveillance due to tumor counterattack.

Several authors have associated strong FASL tumor expression with lymph node metastases; examples include colorectal cancer (Nozoe et al., 2003; Pryczynicz et al., 2010), cervical adenocarcinoma (Kase et al., 2003), and oral cancer (Fang et al., 2013).

Our results did not correlate strong FASL expression in lymphoid cells with disease

relapse or death in oral cancer patients ($P = 0.054$). However, strong expression was related to lower disease-free and disease-specific survival (Figure 1A and B), increasing relapse and death risk by >2 . FASL expression in cervical carcinoma tumor cells was strongly correlated with worse survival (Kase et al., 2003).

In conclusion, FASL expression in tumor inflammatory infiltrate lymphoid cells was associated with lymph node metastases and disease-free and disease-specific survival, suggesting a potential role for this test as a tumor prognostic marker in OSCC patients.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

We are grateful to the GENCAPO (Head and Neck Genome Project: <http://www.gencapo.famerp.br/>) team for the invaluable discussions that motivated the present study. Research supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and Fundação de Amparo à Pesquisa do Estado do Espírito Santo (FAPES), and fellowships from Conselho Nacional de Pesquisas (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

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