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A Comparison of Fas-Fas Ligand Mediated Apoptosis with Clinical and Pathological Parameters in Larynx Cancers; Twenty Years After Laryngectomy

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ABSTRACT

Objective: Larynx cancer constitutes 2% of all cancers in adults with 96% of larynx malignancies being squamous cell carcinoma (SHC). Apoptosis is a cell death mechanism that is quite different from necrosis. It is also known as programmed cell death, physiological cell death, or cell suicide. Physiological mediators that activate programmed cell death (apoptosis) are Fas and Fas Ligand (FasL).
Materials and Methods: In a thesis study conducted by our authors in 2004, we investigated the relationship of Fas-Fas ligand-mediated apoptosis with survival in laryngeal cancer, prognostic factors (age, localization, histological grade, tumor size, lymph, blood vessel invasion), stage, and inflammatory response of the tumor. In this study, we investigated the relationship between survival and death rates after 20 years and Fas-FasL.
Results: When FAS was evaluated 20 years later, a statistically significant difference was found between mortality rates depending on stage ($p=0.023$; $p<0.05$). While the survival rate is higher in stage 1 cases, the rate of death is higher in stage 3 cases. No statistically significant difference was found between mortality rates according to stages for FAS Ligand 20 years later ($p>0.05$).
Conclusion: In conclusion, we found that the Fas/Fas-L system was not associated with clinical parameters in laryngeal cancers in our short-term follow-up. However, when we repeated our follow-up 20 years later, we found that Fas system deficiency, although not in FAS Ligand, adversely affected survival in the long term in laryngeal cancer patients.

Keywords: Fas, Fas ligand, laryngectomy, larynx cancer, squamous cell tumor

INTRODUCTION

Larynx cancer constitutes 2% of all cancers in adults. This rate is 2.2% in men and 0.4% in women. 96% of larynx malignancies are squamous cell carcinoma (SHC), and 26% of this type of malignancy in the head and neck region is located in the larynx (1).

Apoptosis is a cell death mechanism that is quite different from necrosis, which is known as the classical form of cell death, in terms of many features. It has also been found to be implicated in many other pathological conditions including autoimmune disorders, AIDS, certain major neurodegenerative disorders including Alzheimer's disease, and even malignancies. Apoptosis is also known as programmed cell death, physiological cell death, or cell suicide (2).

Physiological mediators that activate programmed cell death (apoptosis) are Fas and Fas Ligand (FasL). Fas is found in lymphoid cells, hepatocytes, some tumor cells, lungs, and even the myocardium. Its ligand of interest is called Fas ligand (FasL). FasL, tumor necrosis. It is a member of the factor (TNF) family. FasLis found in cells of cytotoxic T lymphocytes and "natural killers" (3).

Fas-FasL interaction with the cell membrane surface has a very important role in killing tumor cells by cytotoxic T lymphocytes and natural killer (NK) cells. Expression of Fas Ligand in tumor

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cells helps tumor cells to evade the immune system by developing resistance to Fas-related apoptosis (4).

In a thesis study conducted by our authors in 2004, we investigated the relationship of Fas-Fas ligand-mediated apoptosis with survival in laryngeal cancer, prognostic factors (age, localization, histological grade, tumor size, lymph, blood vessel invasion), stage, and inflammatory response of the tumor. In this study, we investigated the relationship between survival and death rates after 20 years and Fas-FasL.

MATERIALS AND METHODS

In the first phase of our study, 20 years ago, between December 2000 and October 2003, the cases that involved operations for laryngeal carcinoma in the Haseki Training and Research Hospital Ear Nose and Throat Clinic were scanned in the Pathology laboratory. Of these cases, 60 (59 males, 1 female) were selected for inclusion in the study. The patients in these cases were between the ages of 35-82 and the mean age was 58.3±9.71 years. All specimens from the cases were reviewed and evaluated. All individuals in this study signed a written informed consent form for participation. This work was done under the principles of the Declaration of Helsinki. Because this was a retrospective study and there was no experimental intervention involved, ethical committee approval was not needed.

The most suitable tumor blocks were selected for immunohistochemical examination. Clinical data of the cases were obtained from archive records. Age at the time of diagnosis was divided into 2 groups – those under 60 years old and those above. In terms of the largest diameter of the primary tumor, it was evaluated in two groups as being greater than 4 cm and less than 4 cm. The tumor was divided into 3 groups - supraglottic, glottic, and transglottic according to the location in the larynx. Histological grade was classified as good, moderate, and poorly differentiated (grade 1,2,3) considering the degree of keratinization, pearl formation, and the presence of intercellular bridges between tumor cells. Inflammatory and desmoplastic responses around the tumor were subjectively defined as weak, moderate, and severe. Lymphovascular, perineural, and any cartilage invasion were evaluated. The primary tumor (T), the status of lymph nodes (N), presence or absence of distant metastases (M) were evaluated. It was grouped as early-stage (stage 1-2) and advanced stage (stage 3-4) according to AJCC (American Joint Committee on Cancer) criteria.

Immunohistochemical Examination

2-3 µm paraffin sections of the tumors were placed on slides pretreated with Histogrip (Zymed) and kept in an oven at 37°C overnight. Sections were clarified in xylene and rehydrated in alcohol. Sections were heated in a microwave oven for 3x5 minutes in 10 mM citrate buffer (pH 6) to reveal antigen. It was allowed to cool at room temperature for 20-30 minutes in the same buffer. To eliminate the endogenous peroxidase activity in the tissues, a 3% hydrogen peroxide solution was dripped onto the sections and left for 10 minutes and then into a protein block solution (TA-125-UB, Lab Vision Corp. Fremont, CA, USA, 10 min), primary antibody, secondary antibody (TA-125-BN, Lab Vision Corp. Fremont, CA, USA, 10 min) and treated with streptavidin-HRP (TA-125-HR, Lab Vision Corp. Fremont, CA, USA, 10 min). Aminoethyl carbazole (TA-125-HA, Lab Vision Corp. Fremont, CA, USA, 20 min) was used as the chromogen. Sections were rinsed with PBS after each treatment. Finally, it was counterstained with Mayer's hematoxylin and closed. negative controls. This was done by skipping the primary antibody step. Positive controls: Small intestinal mucosa and lymph node sections were used for Fas, prostate and testis sections were used for FasL.

Early assessment

The following antibodies were used in immunohistochemistry: CD95 (FAS) Ab-3 (Clone 95 C03, Mouse monoclonal antibodies Neomarkers, Fremont, CA, USA, 1/20 dilution 120 min), Moroccan Ligand Ab-1 (Clone FSL01, Mouse monoclonal antibodies, Neomarkers, Fremont, CA, USA, 1/15 dilution 180 min). Fas immunohistochemistry showed a positive reaction as intracytoplasmic granular staining. Staining was observed in tumor cells and stromal lymphoplasmacytic cells. Fas Ligand (FasL) immunohistochemistry revealed a positive reaction as intracytoplasmic granular staining. Staining was observed in tumor cells and stromal lymphoplasmacytic cells. A total of 1000 cells were counted in the area where staining was most prevalent in the tumor. Cells that showed positive reactions were identified and their percentages were calculated. Staining intensity was not taken into account. Evaluations were made under the Olympus B×50 light microscope at 400× (40× objective lens, 10× ocular lens, 0.151 mm^2) magnification. Statistical analysis of the data was performed using the chi-square test, Fisher's exact test, Kruskal Wallis analysis of variance, and Mann-Whitney-U test in the SSPS/PC program. p values below 0.05 were considered significant.

Evaluation after 20 years

We reached out to our patients or their relatives from the contact information in the archive to find out if they were still alive. If they were alive, we questioned whether there was any sign of tumor recurrence. Since we could not obtain clear information about the cause of death in the deceased patients we reached, we could not learn whether the deaths were due to tumor recurrence.

RESULTS

Early Results

In the early period, no statistically significant relationship could be demonstrated between Fas and age, diameter, grade, localization, blood vessel invasion, perineural invasion, cartilage invasion, lymph node involvement, and survival. Fas Ligand (FasL) staining showed a positive reaction with immunohistochemistry as intracytoplasmic granular staining. Staining was observed in tumor cells and stromal lymphoplasmacytic cells. Staining was not observed in 3 (5%) cases (Stage I), 1-10% (Stage II) in 41 (68.3%) cases, 11-25% (Stage III) in 14 (23.3%) cases, 2 (3%) cases. 3) staining was

observed at a rate of 26-50% (Stage IV) in one case. There was no correlation between the other parameters compared with FasL in the cases. There was no correlation between Fas and FasL ratios in tumor cells.

Results after 20 years

We designed our study 20 years later to find out the survival status of patients by accessing their file information. We were able to reach 36 of 60 (9 Ex) cases.

Since we could not meet face-to-face with our patients due to covid restrictions, we reached out to our patients or their relatives via telephone. We asked our questions from a standard questionnaire we had prepared. According to the information given, we were able to obtain information on current survival and tumor recurrence status. While our questionnaire had more questions, due to the large number of deceased patients, the answers to most questions did not yield a statistically significant result. We had to be content with just assessing survival rates.

There was no history of tumor recurrence or metastasis in the healthy patients. We found and compared the fas and fas ligand staining rates of right and ex-patients from their files. 24 of 36 cases had died. Three of the 12 survivors had received radiotherapy within 5 years of surgery. The survivors had no morbidity other than permanent laryngeal tracheostomy. Nine of the 12 surviving patients were retired and not working elsewhere (Table 1).

Fas evaluations

Initially, in FAS cases, no statistically significant difference was found between stages and mortality rates (p>0.05).

When FAS was evaluated 20 years later, a statistically significant difference was found between mortality rates according to the stage (p=0.023; p<0.05). While the survival rate was higher in stage 1 cases, the rate of death is higher in stage 3 cases (Table 2).

In stage I cases, 22.2% of the cases died in the initial period, and only 3 cases could be questioned after 20 years, and since all of them lived, no significant difference was found between the mortality rates of the two periods (p>0.05).

In stage II cases, 11.9% of the cases died in the initial period, and this rate was 66.7% after 20 years; a significant difference was found between the mortality rates of the two periods (p<0.01).

In stage III cases, 11.1% of the cases died in the initial period, and this rate was 88.9% after 20 years; a significant difference was found between the mortality rates of the two periods (p<0.01) (Figure 1) (Table 2).

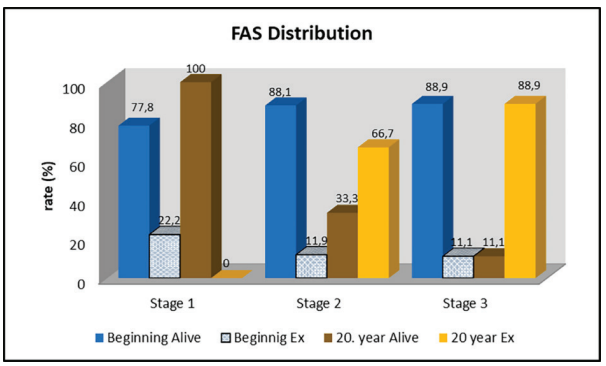

Figure 1: Fas Distribution

Table 1: Questionnaire for follow up of long-term survival

1) Is the patient alive?
2) If he is dead, what is the cause of death?
3) If alive, is there any recurrence?
4) If alive, is there any metastasis?
5) If alive, is there lymph node involvement?
6) Is there any morbidity related to the surgery?
7) Is there any morbidity related to the disease?
8) Has he received chemotherapy or radiotherapy in the long term after the operation?
9) If he has received chemotherapy or radiotherapy, is there any associated morbidity?
10) Has laryngeal cancer adversely affected your work life?
11) Has laryngeal cancer adversely affected family life?
12) If so, how many years have passed since the patient's death?

Table 2: FAS Evaluations

	FAS Beginning (n=60)		FAS 20 years later (n=36)		[b]*p*
	Alive	**Ex**	**Alive**	**Ex**	
Stage 1	7 (77.8)	2 (22.2)	3 (100)	0 (0)	**1.000**
Stage 2	37 (88.1)	5 (11.9)	8 (33.3)	16 (66.7)	**0.001****
Stage 3	16 (88.9)	2 (11.1)	1 (11.1)	8 (88.9)	**0.001****
p	[a]**0.763**		[a]**0.023***		

[a]Fisher Freeman Halton Test, [b]Fisher Exact test, *p<0.05, **p<0.01

Fas Ligand evaluations

In the initial evaluations of FAS Ligand, no statistically significant difference was found between death rates according to the stage (p>0.05).

No statistically significant difference was found between mortality rates according to stages for FAS Ligand 20 years later (p>0.05) (Table 2).

In the stage I cases, 40% of the cases died in the initial period, and this rate was found to be 25% after 20 years. There was no significant difference between the mortality rates of the two periods (p>0.05).

In stage II cases, 10.9% of the cases died in the initial period, and this rate was 71.4% after 20 years. A significant difference was found between the mortality rates of the two periods (p<0.01).

In stage III cases, 12.5% of the cases died in the initial period, and this rate was 72.7% after 20 years. A significant difference was found between the mortality rates of the two periods (p<0.01).

In stage IV cases, 100% of the cases died in the initial period and no cases could be questioned after 20 years, so the difference between them could not be examined (Figure 2) (Table 3).

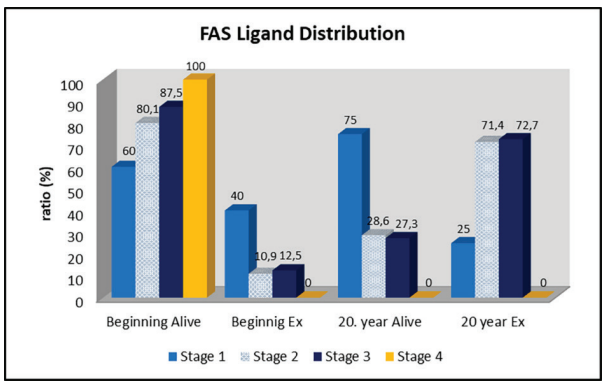

Figure 2: Fas Ligand Distribution

DISCUSSION

Tumors developed because the balance between spread and death was disrupted. Tumor antigens can cause an immune reaction that can destroy tumor cells. In both steps, defects in the Fas system contribute to tumorigenesis (5).

In our retrospective study, we investigated Fas and Fas-ligand expression (release) in laryngeal squamous cell cancer and analyzed its relationship with clinical-pathological parameters. We evaluated the association of Fas and Fas-ligand expression (release) with survival (6).

As an important result, we observed that Fas immunoreactivity decreased in advanced-stage tumors as the T stage of the tumor increased. This showed us that down-regulation of cell-surface Fas receptors is accompanied by an increase in tumor stage and T-stage. The clinical significance of Fas release in head and neck cancers is still not fully understood. Previous studies of Fas expression in these organ tumors included limited specimens and gave conflicting results. Although high levels of Fas expression were found in tongue cancer (6), significant suppression of Fas receptors was observed in other series of oral squamous cell cancers compared with normal epithelium (7). There is a proven association between the functional FAS and FASL and the risk of developing laryngeal and hypopharyngeal squamous cell carcinomas (8).

While Muraki et al found a relationship between Fas release and clinical staging in well and moderately differentiated oral carcinomas, they did not observe such a relationship in poorly differentiated oral carcinomas (9). Similar contradictory results were obtained from esophageal squamous cell carcinomas. (10, 11) It seems that downregulation of Fas is exhibited as a feature of some tumors. This hypothesis is in line with our study, which had broadly distributed results obtained as a result of Fas staining in laryngeal carcinomas. However, it is not an essential step in the malignant transformation of the squamous epithelium (12). Another important result we obtained was the positive correlation between Fas immunoreactivity and lymphoplasmacytic stroma reaction. This result showed us that down-regulation of Fas receptors on the cell surface may be accompanied by the absence of inflammatory cells infiltrating the tumor stroma. The same results were obtained from gene transfer experiments on murine tumor cells (13).

Table 3: FAS Ligand Evaluation

	FAS Ligand Beginning (n=60)		FAS Ligand 20 years later (n=36)		[b]p
	Alive	Ex	Alive	Ex	
Stage 1	3 (60.0)	2 (40.0)	3 (75)	1 (25)	**1.000**
Stage 2	41 (89.1)	5 (10.9)	6 (28.6)	15 (71.4)	**0.001****
Stage 3	14 (87.5)	2 (12.5)	3 (27.3)	8 (72.7)	**0.003****
Stage 4	2 (100)	0 (0)	0 (0)	0 (0)	-
p	**[a]0.366**		**[a]0.198**		

[a]Fisher Freeman Halton Test, [b]Fisher Exact test, *p<0.05, **p<0.01

Shibakita et al. reported that there is a correlation between strong Fas ligand release and a decrease in CD8 T-lymphocytes in esophageal squamous cell carcinomas (14). In the study of Kase S. et al., the frequency of Fas-ligand release was high in T3 squamous cell esophageal carcinoma, respectively, followed by T2 squamous cell esophageal carcinoma, and then dysplasia (14). Shibakita found no association between Fas-ligand release and survival (13). It has been reported that Fas-ligand release in esophageal squamous cell carcinomas may be associated with tumor progression and poor prognosis (15). Ewing sarcomas have more metastatic tumors than Fas-ligand primary tumors. It is secreted frequently and in larger volumes. Similarly, Fas-ligand is secreted less in non-aggressive non-Hodgkin lymphomas and more in aggressive non-Hodgkin lymphomas (16). Older patients, greater tumour size and lymph node positivity were found to be associated with high expression of FasL in tobacco-related intraoral squamous cell carcinoma (17).

It has been reported that papillary thyroid carcinomas with aggressive histology and extensive/locally invasive/recurrent poor prognostic features show stronger Fas-ligand immunoreactivity compared to thyroid carcinomas that are well-differentiated and do not have poor prognostic features (18).

Studies have been carried out using a Fas activation system in the treatment of several types of cancer including gastric, prostate, and glioblastoma. Positive results were obtained in these trials. These results recommend that FasL gene therapy may become a potent treatment for HNSCC. (19) In this study, a positive correlation was found between Fas and the inflammatory response (p=0.019). In cases with weak inflammatory response, staining with Fas is weak (1-10%) or not observed at all. As the inflammatory response increased, the rate of staining with Fas increased. In a recent study 20 years ago, no statistically significant relationship could be shown between Fas and survival in the cases.

Studies have shown that squamous cell carcinoma of the Head and Neck is sensitive to apoptotic inducers such as etoposide (VP-16), but resistance to agonistic antibodies such as CH11has been noticed. We mentioned above that this result was also found in cancerous cells in thyroid specimens. Such a result indicates that tumor cells are strongly Fas-resistant, protecting themselves from apoptosis via Fas-ligand on tumor cells or Fas-ligand in plasma/serum.

Head and neck squamous cell cancers contribute to escape from immune effector cells, such as Fas resistance, by secreting bcl-2 as an additional defense mechanism (3) Fas-related phosphatase-1, protein kinase C, and FLICE protein inhibitor FL1P, which inhibit apoptotic signals by acting on the COOH terminal of FAS, are examples of additional protection mechanisms. It is shown, that there are associations of FAS and FAS Ligand with elevated hematological toxicity, ototoxicity, and lessened survival of tobacco- and alcohol-related head and neck squamous cell patients homogeneously treated with chemoradiation (20).

CONCLUSION

In conclusion, we found that the Fas/Fas-L system was not associated with clinical parameters in laryngeal cancers in our short-term follow-up. However, when we repeated our follow-up 20 years later in the long term, we found that Fas system deficiency, although not in FAS Ligand, adversely affected survival in laryngeal cancer patients.

Ethics Committee Approval: This work was done under the principles of the Declaration of Helsinki. Because this was a retrospective study and there was no experimental intervention involved, ethical committee approval was not needed.

Informed Consent: Written informed consent was obtained.

Peer-Review: Externally peer-reviewed.

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