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Notch1-Jagged1 Signaling Pathway in Oral Squamous Cell Carcinoma: Relation to Tumor Recurrence and Patient Survival

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Abstract

BACKGROUND: Dysregulated Jagged1/Notch1 signaling has been implicated in a variety of carcinomas, but little is known about the expression and possible role of Jagged1 and Notch1 in patients with oral squamous cell carcinoma (OSCC).

AIM: We set out to examine the clinical significance of Notch1 and Jagged1 expression in OSCC.

METHODS: Specimens were obtained from 44 patients who underwent surgical resection of primary OSCC. Immunostaining was done for Notch1 and Jagged1. The utilized markers' expressions were analyzed in respect to 3 years overall survival (OS) and disease-free survival (DFS).

RESULTS: Poor prognosis was significantly associated with high Notch1 expression, high Jagged1 expression, advanced TNM clinical stage (III and IV), presence of distant metastasis, presence of nodal involvement, large-sized tumors (≥4 cm), presence of lymphovascular invasion, higher grade carcinomas, high Notch1 and Jagged1 coexpression, and carcinomas aroused from tongue and palate. Notch1, Jagged1, histologic grade, and tumor site were the independent predictors of DFS, while Jagged1 expression, histologic grade, and tumor site were the independent predictors of 3 years OS.

CONCLUSION: Our findings imply that either high levels of Notch1 or Jagged1 expression, or combined combination of both are related with poor prognostic outcomes.

Introduction

Oral squamous cell carcinoma (OSCC) represents the sixth most common cancer, accounting for 2%–4% of all malignancies worldwide. The overall survival (OS) rate remains generally poor. Most mortality in OSCC patients is due to local recurrent disease and regional spread following surgical treatment failure at the primary site [1], [2]. The Notch signaling cascade plays a crucial role in multiple cellular processes as pro-oncogenic function, progression of cancer in various human tissues [3]. Notch signaling inhibition has been demonstrated to attenuate cancer cell proliferation/cell cycle progression, to decrease cancer cell viability, and to increase cell apoptosis in various types of cancer [4]. On the contrary, it is suggested that Notch signaling has anticancer role in neuroendocrine tumor, urothelial, and hepatocellular carcinoma [4], [5].

Notch1 is a single-pass transmembrane receptor protein, heterodimeric protein with large calcium-dependent extracellular portion. It regulates key cellular processes, including cell fate determination, maintenance of stem cells, cell survival, proliferation, apoptosis, epithelial-mesenchymal transition (EMT), and angiogenesis [6]. There are four Notch receptors (Notch1–4) and five Notch ligands (Delta-like (DLL) 1, 3, 4 and Jagged (JAG) 1–2). Notch receptor undergoes initial cleavage and post-translational glycosylation in Golgi complexes, and then, it is trafficked to the plasma membrane. Notch receptor interacts with Notch ligand in an adjacent signaling cell, it undergoes proteolytic cleavage, which leads to the release of Notch intracellular domain (NICD). The NICD enters the nucleus and binds to the DNA-binding protein, which recruits mastermind-like protein (MAML) to activate the transcription of Notch target genes such as the Hes and Hey families [7].

Notch signaling is deregulated in a number of cancers with unique roles in occurrence, progression, and recurrence of cancer suggesting the potential values of Notch receptor-based therapeutic approaches [8], [9]. Furthermore, Notch1 is one of the stem cell markers that play an important role in cell migration, invasion, metastasis, and resistance of treatment [10], [11]. Jagged1 is mainly expressed in the suprabasal layer

cells and some basal cells of the epidermis. Jagged1 is an important member of the family of Notch ligands, so it has been shown to play a promoting role in cancer progression, although its exact roles in cancer have not yet been systematically elucidated [8].

Upregulation of Notch1 and Notch2 molecules has been observed in cancers of the pancreas, colon, lung, skin, cervix, and brain. Furthermore, upregulation of Jagged1 and Jagged2 molecules has been described in pancreatic, prostate, cervix, and brain cancers. However, there is still no consensus and many results have shown wide variation in the influence of these markers on the prognosis of patients with oral cancer. Although Notch1 plays a crucial role in development and progression of OSCC, it also has a tumor suppressive role as suggested from other studies. It has a tumor suppressive role as suggested from other studies [12]. The role of Notch signaling in OSCC is poorly characterized and little is known about the expression and possible role of Jagged1 and Notch1 in patients with OSCC [13]. We, therefore, set out to examine the clinical significance of Notch1 and Jagged1 expression in OSCC.

Materials and Methods

Patients selection

The current retrospective study was carried out on 44 specimens surgically resected from patients (without new adjuvant therapy) diagnosed with primary OSCC in the period from August 2017 to August 2020 (3 years interval). Patients' clinicopathological data and the formalin paraffinized tissue blocks were retrieved from archives of pathology laboratory of oncology center in that time. Patients' follow-up and survival data were extracted from their medical reports. Follow-up of patients get started after the completion of treatment (every 3 months). Clinical examination and ultrasonography for whole the body were done when relapse was suspected. Three years OS was calculated from the dates of diagnosis to death or to the dates of the last follow-up. Disease-free survival (DFS) was calculated from the dates of diagnosis to death, relapse, or the last follow-up. The present study was approved by IRB and the national research ethics committee in accordance with the 1964 Helsinki Declaration and its later amendments (November 2021, ethical committee faculty of Dentistry, Mansoura University IRB (A10021121)). Informed consent was obtained from all participants involved in our study.

Immunohistochemical staining

Two 4 μm thick sections were cut from each tissue block and placed on positive charged slides. Incubation of sections was done with the primary antibodies at 4 C overnight (Notch1, sc-6014; Jagged1, sc6011; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), followed by reaction with the streptavidinbiotin complex using an SAB PO kit (Nichirei, Tokyo, Japan). The antigen was retrieved with 0.01 M citrate buffer (pH 6.0) by heating the sample in a microwave oven at a controlled final temperature of 100 ◦C for 15 min. The dilutions of primary antibodies were 1:100 for Notch1 and Jagged1. For negative controls, antibody diluent was used.

Immunostaining evaluation

Immunohistochemistry staining was assessed in five power fields at ×200. Immunoreactivity was evaluated semi-quantitatively based on staining proportion and intensity. The stained tissues were scored by a pathologist who was blinded to patients' clinical data. Semi-quantitative scoring was done as from 0 to 3 (0, no staining; 1, 1%–25%; 2, 26%–50%; and 3, >50% of cells staining). In statistical analyses, scores of 0, 1, and 2 were defined as a low level of expression, whereas a score of 3 was defined as a high level of expression [14].

Statistical analysis

Statistical analysis was performed using Chi-square test. Two-side P-values were detailed for all investigations. Patients` survival was calculated and displayed with the Kaplan–Meier curves and analyzed with the log-rank test. The utilized markers' expressions were analyzed in respect to 3 years OS and DFS. Univariate and multivariate survival analyses were performed with Cox regression model to detect the independent prognostic factor. $p < 0.05$ was considered statistically significant. Statistical analysis of data was done using Excel program and Statistical Package for the Social Sciences (SPSS) version 22 program.

Results

Clinicopathological characteristics of the studied oral squamous cell carcinoma cases

As displayed in Table 1, a total of 44 OSCC patients involved in the current work, 29 males (65.9%) and 15 females (34.01%) with male-to-female ratio 1.93:1. The greater number of patients was older than 60 years old (31 cases, 70.5%). The sites from where the tumor aroused were as follows; posterolateral and ventral surfaces of the tongue (19 cases, 43.18%), palatal mucosa (11 cases, 25%), gingiva and alveolar mucosa (8 cases, 18.8%), and lip, labial, and buccal mucosa (6 cases, 13.6%). More

Table 1: Notch1 expression in relation to different clinicopathological variables

CP variables	Categories	Total (%)	Notch1 (%)		Asymptotic significance (two sided) Pearson Chi-square
			Low expression (25)	High expression (19)	
Patient age (years)	<60	13 (29.5)	6 (24.0)	7 (36.8)	0.355
	≥60	31 (70.5)	19 (76.0)	12 (63.2)	
Gender	Male	29 (65.9)	17 (68.0)	12 (63.2)	0.737
	Female	15 (34.1)	8 (32.0)	7 (36.8)	
Tumor site in oral cavity	Tongue	19 (43.2)	15 (60.0)	4 (21.1)	0.067
	Palate	11 (25.0)	4 (16.0)	7 (36.8)	
	Gingiva and alveolar mucosa	8 (18.2)	4 (16.0)	4 (21.1)	
	Lip + labial and buccal mucosa	6 (13.6)	2 (8.0)	4 (21.1)	
Tumor size (cm)	<4	22 (50.0)	16 (64.0)	6 (31.6)	0.033
	≥4	22 (50.0)	9 (36.0)	13 (68.4)	
Nodal involvement	Absent	18 (40.9)	16 (64.0)	2 (10.5)	0.000
	Present	26 (59.1)	9 (36.0)	17 (89.5)	
Distant metastasis	Absent	22 (50.0)	16 (64.0)	6 (31.6)	0.033
	Present	22 (50.0)	9 (36.0)	13 (68.4)	
TNM clinical stage	I and II	18 (40.9)	16 (64.0)	2 (10.5)	0.000
	III and IV	26 (59.1)	9 (36.0)	17 (89.5)	
Histologic grade	Well differentiated	19 (43.2)	16 (64.0)	3 (15.8)	0.006
	Moderately differentiated	14 (31.8)	5 (20.0)	9 (47.4)	
	Poorly differentiated	11 (25.0)	4 (16.0)	7 (36.8)	
LVI	Present	22 (50.0)	10 (40.0)	12 (63.2)	0.128
	Absent	22 (50.0)	15 (60.0)	7 (36.8)	
Incidence of death	Died	12 (27.3)	3 (12.0)	9 (47.4)	0.009
	Alive	32 (72.7)	22 (88.0)	10 (52.6)	
Incidence of recurrence	Absent	24 (54.5)	18 (72.0)	6 (31.6)	0.008
	Present	20 (45.5)	7 (28.0)	13 (68.4)	

LVI: Lymph vascular invasion, TNM: Tumor node metastasis, CP: Clinicopathological.

than one half of patients presented with disease in Stages III and IV (26 patients, 59.1%). One half of the studied OSCC cases had tumors that were ≥4 cm in diameter and demonstrated distant metastatic deposits in multiple organs as spleen, liver, lung, and bone marrow. Twenty-six cases (59.1%) had positive nodal involvement. Recurrence was documented in 20 cases (45.5%) and 12 patients (27.3%) died during follow-up due to complications relevant to cancer. Histologically, the present study comprised 19 well-differentiated carcinomas, 14 moderately differentiated carcinoma, and 11 poorly differentiated carcinomas. One half of cases had lymph vascular invasion (LVI). Notch1 and Jagged1 were positively expressed in all the included cases of OSCC. Low Notch1 and Jagged1 expressions demonstrated in 25 cases (56.8%) and 23 cases (52.3%), while high Notch1 and Jagged1 expressions observed in 19 cases (43.2%) and 21 cases (47.7%), respectively (Tables 1 and 2 columns head).

Notch1 and Jagged1 expression in relation to different clinicopathological parameters

Notch1 revealed either nuclear, nuclear with cytoplasmic, or nuclear with membranous staining patterns. Jagged1 revealed cytomembranous staining pattern. Expression of Notch1 and Jagged1 in dysplastic surface epithelium is confined mainly to the level of basal and parabasal cell layers with focal expression in spinous cell layer (Figure 1a and 2a). Cancerous tissue expressed Notch1 and Jagged1 in higher level than the nearby dysplastic surface epithelium (Figures 1b-d and 2b-d).

Notch1

Table 1 revealed that high Notch1 expression was significantly expressed in carcinomas of different histologic grades (Figure 1b-d), large-sized carcinomas,

Table 2: Jagged1 expression in relation to different clinicopathological variables

CP variables	Categories	Jagged1 (%)			Asymptotic significance (two sided) Pearson Chi-square
		Low expression (23)	High expression (21)	Total	
Patient's age (years)	<60	4 (17.4)	9 (42.9)	13 (29.5)	0.064
	≥60	19 (82.6)	12 (57.1)	31 (70.5)	
Gender	Male	18 (78.3)	11 (52.4)	29 (65.9)	0.070
	Female	5 (21.7)	10 (47.6)	15 (34.1)	
Tumor site in oral cavity	Tongue	13 (56.5)	6 (28.6)	19 (43.2)	0.189
	Palate	3 (13.0)	8 (38.1)	11 (25.0)	
	Gingiva and alveolar mucosa	4 (17.4)	4 (19.0)	8 (18.2)	
	Lip + labial and buccal	3 (13.0)	3 (14.3)	6 (13.6)	
Tumor size (cm)	<4	16 (69.6)	6 (28.6)	22 (50.0)	0.007
	≥4	7 (30.4)	15 (71.4)	22 (50.0)	
Nodal involvement	Absent	16 (69.6)	2 (9.5)	18 (40.9)	0.000
	Present	7 (30.4)	19 (90.5)	26 (59.1)	
Distant metastasis	Absent	16 (69.6)	6 (28.6)	22 (50.0)	0.007
	Present	7 (30.4)	15 (71.4)	22 (50.0)	
TNM stage	I and II	17 (73.9)	1 (4.8)	18 (40.9)	0.000
	III and IV	6 (26.1)	20 (95.2)	26 (59.1)	
Histologic grade	Well differentiated	15 (65.2)	4 (19.0)	19 (43.2)	0.004
	Mod. differentiated	3 (13.0)	11 (52.4)	14 (31.8)	
	Poorly differentiated	5 (21.7)	6 (28.6)	11 (25.0)	
LVI	Present	7 (30.4)	15 (71.4)	22 (50.0)	0.007
	Absent	16 (69.6)	6 (28.6)	22 (50.0)	
Incidence of death	Died	1 (4.3)	11 (52.4)	12 (27.3)	0.000
	Alive	22 (95.7)	10 (47.6)	32 (72.7)	
Incidence of recurrence	Absent	19 (82.6)	5 (23.8)	24 (54.5)	0.000
	Present	4 (17.4)	16 (76.2)	20 (45.5)	

LVI: Lymph vascular invasion, TNM: Tumor node metastasis, CP: Clinicopathological.

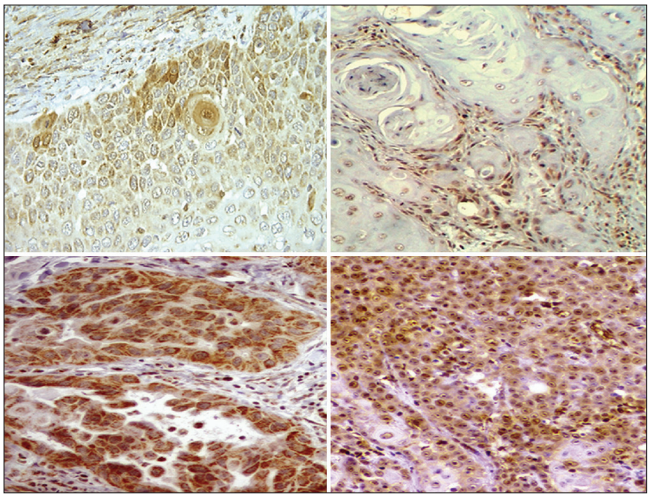

Figure 1: Notch1 expression patterns in OSC (a) dysplastic surface epithelium involving mainly basal and parabasal and focal spinous cell layers, (b) well-differentiated OSCC reveals nuclear Notch1 expression, (c) moderately differentiated OSCC shows cytomembranous expression, and (d) mixed nuclear and cytoplasmic expression in poorly differentiated OSCC (ABC- DAB, ×400). OSCC: Oral squamous cell carcinoma

carcinomas sending nodal or distant metastases, TNM clinical Stages III and IV, presence of recurrence, and death during follow-up ($p \leq 0.05$). On the other hand, Notch1 had no statistically significant associations regarding gender, patients' age, site of tumor, and LVI ($p > 0.05$).

Jagged1

Table 2 reveals that high Jagged1 expression was significantly expressed in carcinomas of different histologic grades (Figure 2b-d), tumors that demonstrated presence of LVI, large-sized carcinomas, carcinomas sending nodal or distant metastases, TNM clinical Stages III and IV, and presence of recurrence and death during follow-up ($p \leq 0.05$). On the other hand, Jagged1 had no statistically significant

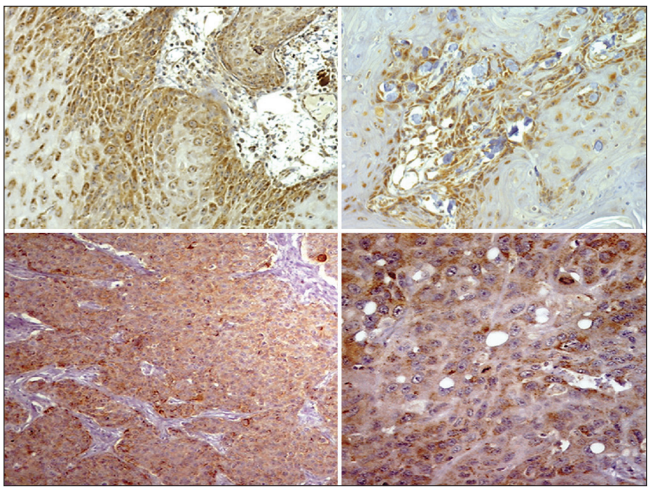

Figure 2: Jagged1 expression patterns in OSC (a) dysplastic surface epithelium; mainly basal and parabasal with focal spinous cell layers, (b) well-differentiated OSCC, (c) moderately differentiated OSCC, and (d) poorly differentiated OSCC (B-DABX200) (ABD-DAB, ×400). OSCC: Oral squamous cell carcinoma

Table 3: Univariate analysis of disease free survival in different clinicopathological variables

CP variable	Mean (DFS)			Log rank (Mantel-Cox) (significance)
	Estimate	95% CI		
		Lower	Upper	
Notch1				
Low	23.160	18.611	27.709	0.000
High	9.316	7.310	11.321	
Jagged1				
Low	24.000	19.579	28.421	0.000
High	9.714	6.935	12.493	
Notch1 + Jagged1 coexpression				
High + high	8.647	6.702	10.592	0.000
High + low	15.000	9.120	20.880	
Low + high	14.250	1.922	26.578	
Low + low	24.857	20.193	29.521	
Age category (years)				
<60	15.000	8.614	21.386	0.564
≥60	18.097	14.075	22.118	
Gender				
Male	17.379	13.167	21.592	0.969
Female	16.800	10.898	22.702	
Tumor site in oral cavity				
Gingiva and alveolar mucosa	28.895	25.208	32.581	0.000
Palate	13.091	10.202	15.979	
Tongue	12.375	8.617	16.133	
Lip + labial and buccal	19.000	8.409	29.591	
Histologic grade				
Well differentiated	24.947	19.800	30.095	0.000
Moderately differentiated	13.286	9.164	17.407	
Poorly differentiated	8.727	5.230	12.224	
LVI				
Presence	12.000	8.763	15.237	0.002
Absence	22.364	17.192	27.536	
Tumor size (cm)				
<4	24.000	19.657	28.343	0.000
≥4	10.364	7.019	13.708	
Nodal involvement				
Absent	26.500	21.984	31.016	0.000
Present	10.731	7.865	13.596	
Distant metastasis				
Absent	23.864	19.394	28.333	0.000
Present	10.500	7.223	13.777	
TNM stage				
I and II	26.833	22.622	31.044	0.000
III and IV	10.500	7.615	13.385	
Recurrence				
Absent	24.625	20.468	28.782	0.000
Present	8.250	6.661	9.839	

CI: Confidence interval, DFS: Disease-free survival, LVI: Lymph vascular invasion, TNM: Tumor node metastasis, CP: Clinicopathological.

associations regarding patients' gender, age and site of tumor ($p > 0.05$).

Analysis of disease-free survival and 3 years overall survival of the studied oral squamous cell carcinoma cases

Disease-free survival (Tables 3, 4 and Figure 3)

Table 3 and Figure 3 demonstrate the univariate analysis of DFS in different clinicopathological variables using log-rank test and Kaplan–Meier survival curves.

Table 4: Cox regression model for prediction of the independent predictors of disease-free survival

CP variables	Exp (B)	95.0% CI for Exp (B)		Significance
		Lower	Upper	
Tumor recurrence	0.192	0.075	0.491	0.001
Histologic grade	0.326	0.129	0.826	0.018
Jagged1	0.344	0.123	0.966	0.043
Notch1	0.351	0.134	0.921	0.033
Nodal involvement	0.987	0.072	13.498	0.992
Distant metastasis	0.869	0.294	2.567	0.800
TNM stage	0.253	0.022	2.847	0.266
LVI	0.840	0.343	2.060	0.703
Tumor size	0.570	0.211	1.540	0.267
Tumor site				0.012
Tumor site (1)	0.951	0.365	2.475	0.918
Tumor site (2)	1.548	0.503	4.766	0.446
Tumor site (3)	4.477	1.414	14.169	0.011

CI: Confidence interval, LVI: Lymph vascular invasion, TNM: Tumor node metastasis, CP: Clinicopathological.

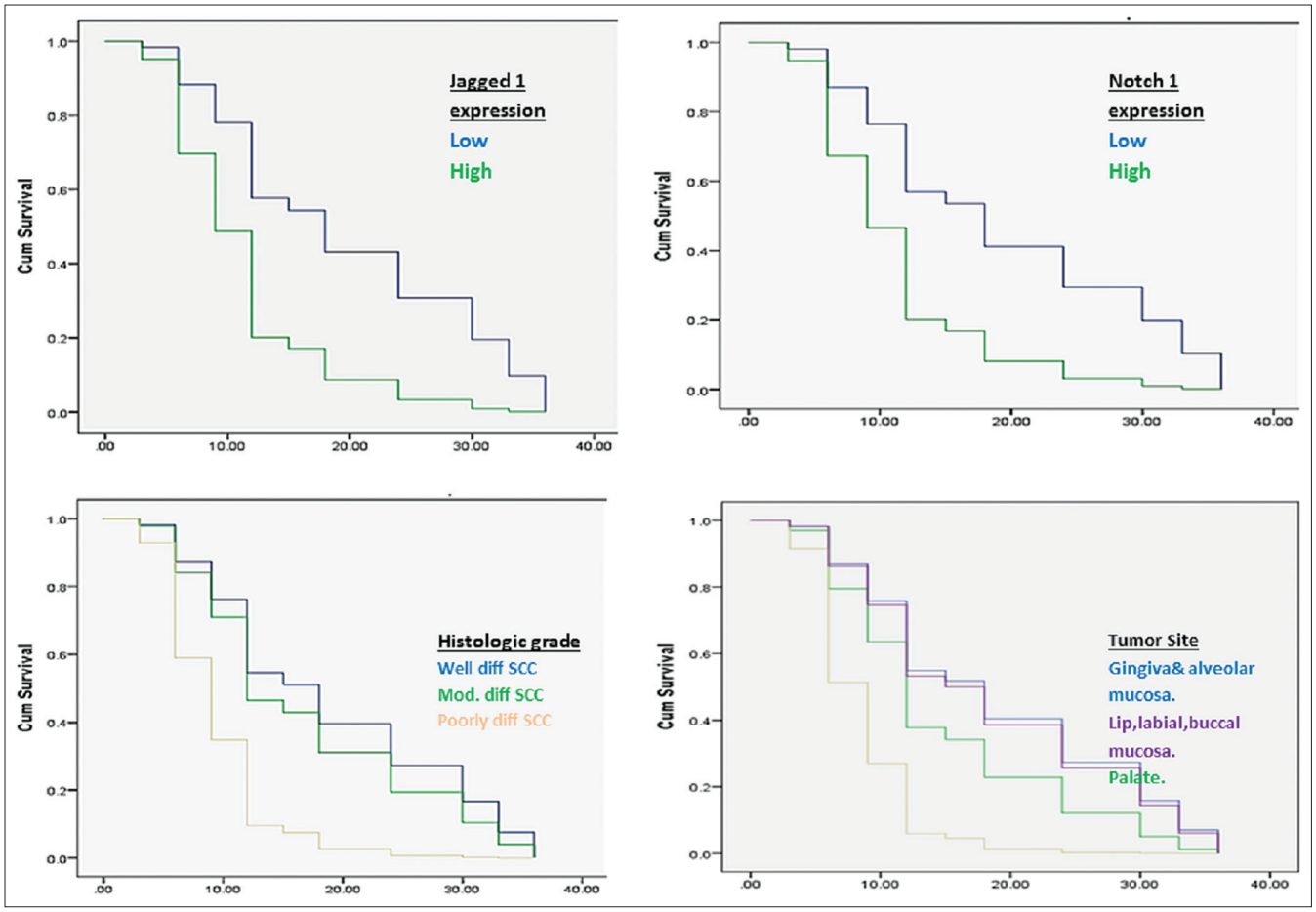

Figure 3: Kaplan–Meier survival curves demonstrate the independent predictors of DFS. DFS: Disease-free survival

Multivariate analysis using COX regression method was performed to identify the independent predictors of DFS. Notch1, Jagged1, histologic grade, and tumor site were the independent predictors of DFS (Table 4).

Reduced DFS was significantly encountered in cases revealed high Notch1 expression (mean DFS = 9.316 months vs. 23.160 months in low Notch1 expression, p = 0.000), high Jagged1 expression (mean DFS = 9.714 months vs. 24 months in low Jagged1 expression, p = 0.000), advanced TNM clinical Stages III and IV (mean DFS = 10.5 months vs. 26.833 months in TNM clinical Stages I and II, p = 0.000), presence of distant metastasis (mean DFS = 10.5 months vs. 23.864 months in the absence of distant metastasis, p = 0.000), presence of nodal involvement (mean DFS = 10.731 months vs. 26.5 months in the absence of nodal involvement, p = 0.000), large-sized tumors ≥4 cm (mean DFS = 10.364 months vs. 24 months in small-sized tumors, p = 0.000), presence of LVI (mean DFS = 12 months vs. 22.346 months in the absence of LVI, p =.000), and moderately and poorly differentiated SCCs (mean DFS = 13.286 and 8.727 months, respectively, vs. 24.947 months in well-differentiated carcinomas, p = 0.000). Carcinomas that had high Notch1 and Jagged1 coexpression recognized reduced DFS (8.647 months). On the other hand, carcinomas that had low Notch1 and Jagged1 coexpression revealed longer DFS (24.857 months). DFS in carcinomas that had high Notch1 (only) or high Jagged1 (only) is relatively equivalent (15 months and 14.25 months, respectively). DFS of patients who had carcinomas aroused from the tongue and palate was reduced (12.375 and 13.091 months, respectively) compared to that aroused from gingiva, alveolar mucosa, and lip, labial, and buccal mucosa (28.895 and 19 months).

Three years overall survival (Tables 5, 6 and Figure 4)

Table 5 and Figure 4 demonstrate the univariate analysis of 3 years OS in different clinicopathologic variables using log-rank test and Kaplan–Meier survival curves. Multivariate analysis using COX regression method was achieved to identify the independent predictors of 3 years OS. Jagged1 expression, histologic grade, and tumor site were the independent predictors of 3 years OS (Table 6).

Reduced 3 years OS was significantly recorded in cases that demonstrated high Notch1 expression (mean 3 years OS = 13.263 months vs. 26.160 months in low Notch1 expression, p = 0.000), high Jagged1 expression (mean 3 years OS = 14.286 months vs. 26.348 months, p = 00.000), advanced TNM clinical Stages III and IV (mean 3 years OS = 15.577 months vs. 27.833 months in TNM clinical Stages I and II, p = 0.000), presence of distant metastasis (mean 3 years OS = 15.955 months

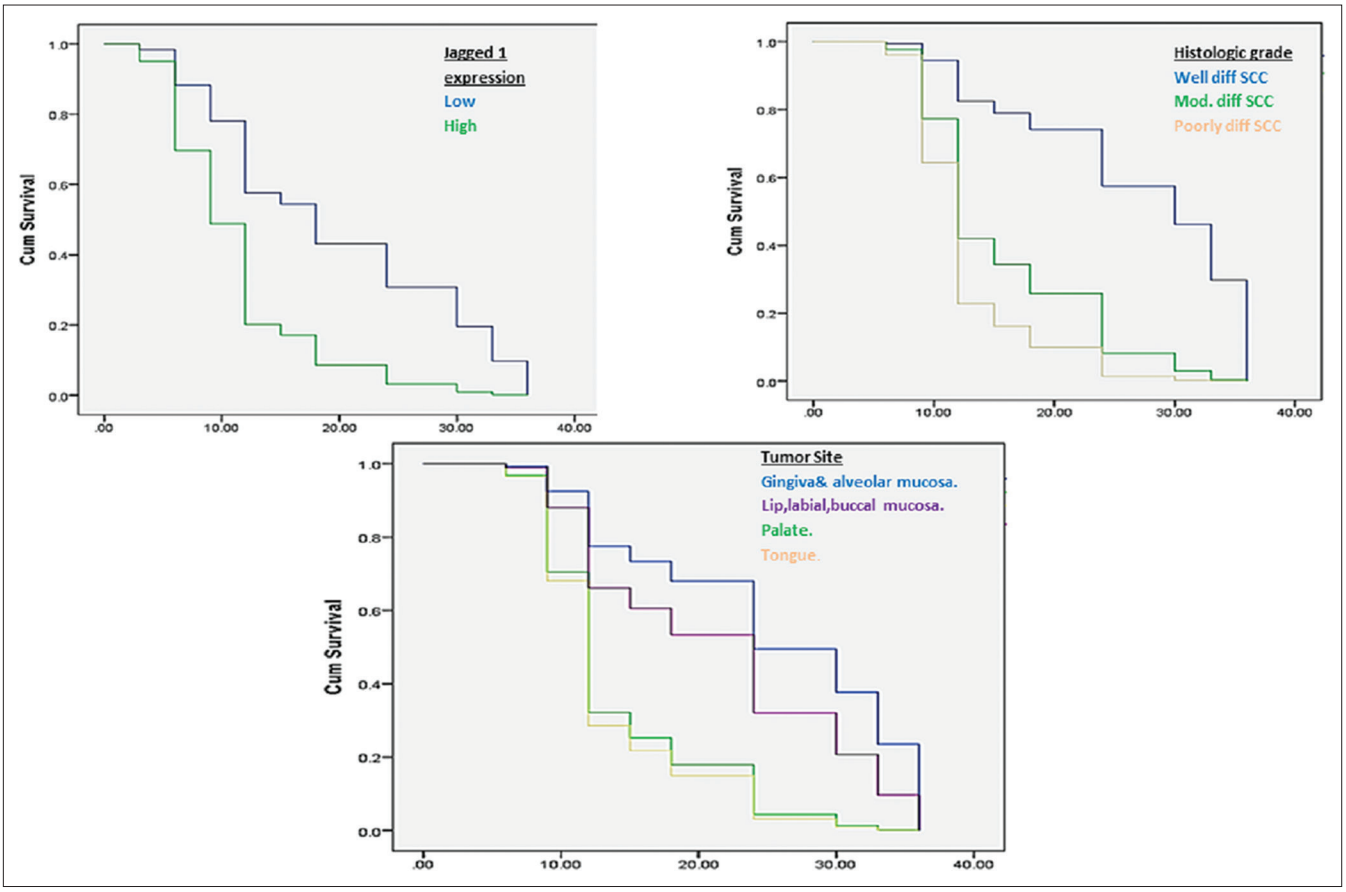

Figure 4: Kaplan–Meier survival curves demonstrate the independent predictors of 3 years OS. OS: Overall survival

vs. 25.227 months in absence of distant metastasis, p = 0.000), presence of nodal involvement (mean 3 years OS = 15.692 months vs. 27.667 months in the absence of nodal involvement, p = 0.000), large-sized tumors ≥4 cm (mean 3 years OS = 14.864 months vs. 26.318 months in small-sized tumors, p = 0.000), presence of LVI (mean 3 years OS = 16.909 months vs. 24.273 months in the absence of LVI, p = 0.014), and moderately and poorly differentiated SCCs (mean 3 years OS = 14.786 and 11.455 months, respectively, vs. 30.158 months in well-differentiated carcinomas, p = 0.000). Carcinomas that had high Notch1 and Jagged1 coexpression recognized reduced 3 years OS (13.059 months). On the other hand, carcinomas that had low Notch1 and Jagged1 coexpression revealed longer 3 years OS (27.429). Three years OS in carcinomas that had high Notch1 (only) or high Jagged1 (only) were 15 and 19.5 months, respectively. Three years OS of patients who had carcinomas aroused from the tongue and palate was reduced (8.625 and 11.727, respectively) compared to that aroused from gingiva and alveolar mucosa, and lip, labial, and buccal mucosa (23.684 and 18 months).

Discussion

In the present study, all of the studied OSCC cases expressed Notch1 and Jagged1. Low Notch1 and Jagged1 expressions demonstrated in 25 cases (56.8%) and 23 cases (52.3%), while high Notch1 and Jagged1 expressions were observed in 19 cases (43.2%) and 21 cases (47.7%), respectively. Notch1 expression was noticed as nuclear staining, nuclear with cytoplasmic staining, and nuclear with membranous staining. Jagged1 revealed cytomembranous staining. In cancerous cells, Notch1 and Jagged1 showed high expression in comparison with their expression in the dysplastic overlying surface epithelium where the expression was lesser and confined to basal, parabasal, and spinous cell layers only. On the same line to our findings, several studies have shown high percentages of Notch proteins expression in cancerous cells of oral cancer [15], [16], [17], [18].

Expression of Notch signaling molecules was evident in neoplastic cells with apparent differences in expression levels between tumor tissue and adjacent non-neoplastic tissues. Similar pattern of Jagged1 expression distribution was observed. In concordance with us, Zhang *et al.* observed strong and diffuse Notch1 expression in stratum spinosum and stratum basale, with only faint and focal staining in stratum granulosum and stratum corneum in the adjacent non-neoplastic tongue tissue. These results direct us to suggest the oncogenic role of Notch pathway in OSCC initiation and progression. Moreover, Yao's *et al.* have reported that downregulation of Notch1 can inhibit the growth of human tongue squamous cell carcinoma cell line (Tca8113), accompanied by cell cycle arrest and apoptosis [19], [20], [21].

Studies on the Notch pathway genes in cell lines (human OSCC cell lines Ca99-2, HSC-2 and

Table 5: Univariate analysis of 3 years overall survival in different clinicopathologic variables using log-rank test

CP variables	Mean (3 years OS)			Log rank (Mantel-Cox) (significance)
	Estimate	95% CI		
		Lower bound	Upper bound	
Notch1				
Low	26.160	22.136	30.184	0.000
High	13.263	10.480	16.047	
Jagged1				
Low	26.348	22.215	30.480	0.000
High	14.286	11.017	17.555	
Notch1+Jagged1 coexpression				
High+high	13.059	9.994	16.123	0.001
High+low	15.000	9.120	20.880	
Low+high	27.429	23.206	31.651	
Low+low	19.500	8.631	30.369	
Age category (years)				
<60	17.308	11.934	22.682	0.373
≥60	21.968	18.089	25.847	
Gender				
Male	19.655	15.980	23.330	0.319
Female	22.400	16.223	28.577	
Tumor site in oral cavity				
Gingiva and alveolar mucosa	23.684	18.659	28.709	0.001
Palate	11.727	8.141	15.313	
Tongue	8.625	3.854	13.396	
Lip+labial and buccal	18.000	6.741	29.259	
Histologic grade				
Well differentiated	30.158	26.690	33.626	0.000
Moderately differentiated	14.786	11.220	18.351	
Poorly differentiated	11.455	8.729	14.180	
LVI				
Presence	16.909	13.011	20.808	0.014
Absence	24.273	19.632	28.914	
Tumor size (cm)				
<4	26.318	22.355	30.281	0.000
≥4	14.864	11.123	18.604	
Nodal involvement				
Absent	27.667	23.321	32.012	0.000
Present	15.692	12.267	19.118	
Distant metastasis				
Absent	25.227	21.031	29.424	0.004
Present	15.955	11.927	19.982	
TNM stage				
I and II	27.833	23.640	32.026	0.000
III and IV	15.577	12.125	19.029	
Recurrence				
Absent	24.625	20.468	28.782	0.016
Present	15.750	11.639	19.861	

OS: Overall survival, CI: Confidence interval, LVI: Lymph vascular invasion, TNM: Tumor node metastasis, CP: Clinicopathological.

HSC-4) and tumors found that Notch1, Notch2, and Jagged1 expression levels were upregulated in cancer cells compared to these genes in normal oral tissues, suggesting that Notch signaling is active in OSCC [22]. Moreover, Lowell *et al*. had shown that the clusters of basal keratinocytes enriched for stem cells express higher levels of Notch signal molecules than their neighbors [23]. Similarly, Notch and its ligands become upregulated in SCCs in other sites as cervix, supporting that Notch has a role in regulate proliferation of human keratinocytes, and aberrant Notch signaling results in the development of epidermis cancers such as SCC [24].

Table 6: Cox regression model for prediction of the independent predictors of 3 years overall survival

CP variables	Exp (B)	95.0% CI for Exp (B)		Significance
		Lower	Upper	
Tumor recurrence	0.903	0.428	1.906	0.789
LVI	0.876	0.371	2.068	0.762
Tumor size	0.544	0.230	1.285	0.165
Notch1 expression	0.523	0.198	1.383	0.191
Nodal involvement	0.824	0.046	14.661	0.895
Jagged1 expression	0.365	0.160	0.837	0.017
Histologic grade	0.130	0.047	0.363	0.000
Distant metastasis	1.213	0.404	3.640	0.730
TNM clinical stage	0.381	0.026	5.576	0.481
Tumor site				0.008
Tumor site (1)	0.618	0.240	1.593	0.319
Tumor site (2)	2.743	0.849	8.856	0.092
Tumor site (3)	3.026	0.891	10.281	0.076

CI: Confidence interval, LVI: Lymph vascular invasion, TNM: Tumor node metastasis, CP: Clinicopathological.

In contrast to our results, Mohamed *et al*. noticed that expression of Notch1 was in the cytoplasm of colorectal cancer tissue specimens and rarely expressed in adjacent normal tissues. These contradictory findings may be explained by the difference in the used antibody, or the methodology steps, however, suggest that the Notch signaling pathway is functionally activated in many cancers including OSCC and need to be confirmed in further studies [11].

Our findings along with others concluded that higher expression levels of Notch1 and Jagged1 are detected in basal and parabasal cells than other strata of oral mucosa. These features suggest that upregulation of Notch signal interferes with the differentiation of stem cells in the basal cell layer [12], [25]. Furthermore, lower expression level in oral epithelium adjacent to tumor tissue in contrast to cancerous tissue predicting the oncogenic role Notch signaling in tumorigenesis and development of OSCC.

The current research work revealed the lower the differentiation of tumor the higher the Notch1 expression; high Notch1 expression was observed in 64.3% of moderately and 63.6% of poorly differentiated OSCCs. Our results support the hypothesis that the more density of cancer stem cells in oral cancer is closely associated with higher grade and lesser degree of cell differentiation, as also seen and reported by the previous studies [25], [26], [27], [28].

Moreover, the present study revealed that high Notch1 expression was observed in large-sized carcinomas, carcinomas sending nodal and distant metastases, advanced TNM stage, presence of tumor recurrence during follow-up, and incidence of death ($p \leq 0.05$, Table 2). In agreement with our findings, many studies reported that Notch1 immunopositivity in OSCCs was significantly associated with advanced clinical staging, lymph node metastasis, histopathological tumor classification, tumor invasion, and locoregional recurrence [5], [15], [21], [25], [29], [30]. In contrast, Ravindran and Devaraj reported a statistically significant association between weak or negative Notch1 expression and advanced clinical stage and lymph node metastasis in OSCC, they suggested that the absence of Notch1 could be related to the processes of tumor invasion and metastasis [27]. Furthermore, accumulating evidence suggests that variation of Notch1 mutation signature may determine the role of Notch signaling in OSCC. Hence, Notch is thought to act as an oncogene in a subset of OSCC, but also has a tumor suppression role, and the role of Notch in OSCC seems to be highly context dependent [12].

High Jagged1 expression was also significantly expressed in different histologic grades of OSCCs, large-sized carcinomas, presence of LVI, presence of nodal and distant metastasis, TNM clinical Stages III and IV, and occurrence of recurrence and death during follow-up period ($p \leq 0.05$, Table 3). On the other hand, Jagged1 expression had no statistically significant associations regarding tumor location in oral cavity, gender, and patients' age (p

> 0.05). On the same line to our findings, other studies reported that Jagged1 was more highly expressed in SCC of tongue with positive metastatic deposits in cervical lymph nodes when compared with localized carcinoma [19], [31], [32]. These findings with ours support that dysregulation of Notch1-Jagged1 proteins pathway has a role in tongue carcinoma progression and metastasis, and suggest that they may be useful markers in distinguishing early and advanced tongue carcinoma.

Staging and grading of OSCC are established prerequisites for management, as they influence risk stratification and are the first step toward personalized treatment [29]. A growing evidence has indicated a relationship between Notch signaling and EMT and invasion, through E-cadherin repression by Slug [33]. This leads to loss of cell adhesion and increasing cell motility. Sahlgren *et al.* have shown that Notch signaling is involved in response to the hypoxic stimulus and directing EMT, increased motility, and invasiveness [31]. Furthermore, Jagged1 plays a role in invasion and metastasis of tongue carcinoma by EMT. Zeng *et al.* clarified another mechanism in which the direct interplay between tumor cells and endothelial cells by Notch1 and Jaggd1 pathway promotes angiogenesis through the MAPK and emphasized the importance of this process in tumor growth, invasion, and metastasis [13]. Our data support these previous findings and we conclude that Notch1 has a role in OSCC invasion. However, we need further investigation to clarify the underlying complicated mechanisms.

Cancer relapse remains one of the major problems in managing OSCC, early relapse has a worse prognosis than late relapses. Up to 50% of OSCCs recur following surgical resections with conventional “histologically negative” margins usually within 2 years of initial surgical intervention [34]. IIn this study, there was a significant correlation between high expression of Notch1 and Jagged1and tumor recurrence (p = 0.008 and < 0.0001, respectively).

Cancer relapse may explained by the population of cancer stem cells (CSCs), CSCs possess high capability to repair DNA damage together with overexpression of anti-apoptotic proteins such as Bcl-2 and Bcl-XL, rescuing the tumor cells from the activation of cell death. The repopulation of the tumor mass after genotoxic therapies is then promoted by the activation of signaling pathways associated with self-renewal and survival-related pathways of CSCs. Signals through the Notch1 pathway play a key role in the stem cell maintenance [35], [36]. Moreover, recent clinical and preclinical findings confirmed the role of CSC-specific pathways in cancer progression, relapse, and drug resistance, in different cancers, such as ovarian cancer, glioblastoma, colorectal cancer, and leukemia [37], [38].

Reduced DFS and OS for patients with OSCCs were significantly encountered in cases that revealed high Notch1 expression, high Jagged1 expression, carcinomas having high Notch1 and Jagged1 coexpression, advanced TNM clinical Stages III and IV, presence of distant metastasis, presence of nodal involvement, large-sized tumors (≥4 Cm), presence of LVI, moderately and poorly differentiated carcinomas, patients who had carcinomas aroused from the tongue and palate, and in particular patients with a coexpression of high level of Jagged1 and Notch1 had the worst survival compared with other patients. In agreement with our findings, relative to the oral cancer patient prognosis, Wang *et al.* found that positivity for Notch1 indicated lower OS and was an independent prognostic factor for patients with oral cancer [18]. These findings suggested that a Jagged1-Notch1 activation loop between tumor cells in OSCC may help to promote tumor formation and progression. Several studies on head-and-neck cancer, ovarian, breast cancer, gastric cancer, and prostate cancer reported similar findings [32], [39], [40], [41], [42], [43]. In contrary to our findings, De Freitas Filho *et al.* concluded that immunoexpression of Notch1 has no influence on the prognosis of patients with oral cancer [44], a result similar to those obtained by Joo *et al.* and Ravindran and Devaraj [16], [44].

Conclusion

Collectively, our data suggest that poor prognostic outcomes as reduced DFS and OS of OSCC patients, large-sized carcinomas, existence of nodal and distant metastatic deposits, poorly differentiated carcinomas, and advanced TNM clinical stage are associated with high levels Notch1 and Jagged1 expression either solely or combined. These results can support use of Jagged1 as a base anticancer therapy and anti-angiogenic therapeutic target.

Ethical Approval

As per international standard or university standard, patient’s written consent has been collected and preserved by the authors. Our study was approved by the faculty ethics committee.

Consent to Participate

Written informed consent was obtained from the patients on admission to hospital.

Consent to Publish

Informed consent for publication of images of this case was obtained.

Code Availability

Excel program and Statistical Package for the Social Sciences (SPSS) version 22 program.

Availability of Data and Material

All data are available.

Consent to publish.

Authors' Contributions

1. Conceptualization: NME,
2. Data curation: NME
3. Formal analysis: ATI, HAE, and NAS
4. Funding acquisition: NME
5. Investigation: NME, ZEH, ATI, and HAE
6. Methodology: NME
7. Project administration: NME
8. Resources: NME
9. Software: ZEH, ATI, HAE, and NAS
10. Supervision: NAS, ATI, and HAE
11. Validation: NAS, ATI, and HAE
12. Visualization: NAS, ATI, and HAE
13. Writing – original draft: NME and ZEH
14. Writing – review and editing: NAS, ATI, and HAE
15. Approval of final manuscript: All authors.

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