P27/SKP-2 Histochemical Profile is Relevant to Malignant Salivary Gland Tumors (MST) Histogenesis and Tumor Grade

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Abstract Malignant salivary gland tumors (MST) represent over more than 24 distinct morphological subtypes. Most high grade tumors arise from the excretory duct portion of the salivary gland apparatus; the remainder from the intercalated duct portion. Altered p27/skp-2 expression has been associated with tumor aggressiveness and histologic differentiation. In our study, we analyzed p27/skp-2 expression proteins on series of malignant salivary gland tumors in order to assess their value as a histogenetic marker, which is relevant to tumor grade. 61 MST cases were segregated by proposed histogenesis and immunohistochemistry was performed using antibodies directed against p27 and skp-2. MST of proposed intercalated duct origin ($n = 27$) showed strong p27 expression ($n = 25/27$; 93%) in the vast majority of cases and all cases weakly expressed skp-2. MST of proposed excretory duct origin ($n = 32$) showed strong p27 expression ($n = 18/32$; 56%) and moderately strong/strong skp-2 expression ($n = 18/32$; 56%), respectively, in over half the cases. MST of intercalated duct origin showed evident p27/skp-2 inverse correlation. Differences

in p27/skp-2 expression among the MST subtypes correlated with histogenesis and tumor grade, which reinforces the notion that tumor behavior is relevant to the portion of the salivary gland unit from which they arise. MST of proposed intercalated duct origin strongly expressed p27, and not skp-2, unlike MST of proposed excretory duct origin. The immunohistochemical profile of high grade mucoepidermoid carcinoma was distinct from its low/intermediate grade counterparts, suggesting a separate identity. These results may influence future decision making when formulating workable MST categorization schemes. Further studies on a larger series of MST are warranted in order to support the value of our findings.

Keywords Salivary glands · Salivary gland tumors · Malignant salivary gland tumors · Histogenesis · p27 · skp-2

Introduction

Malignant salivary gland tumors (MST) are an uncommon group of neoplasms [1]. The current WHO classification system includes 24 distinct tumor subtypes, some with less than 10 reported cases in the English language literature [2]. Each MST subtype represents a distinct morphologic subtype, which hampers our efforts to simplify this system. This vast number, together with their relative rarity, complicates our efforts to gather useful information, as we rely on single or small series of cases to form judgement [3]. In modern pathology, MSTs are one of the most challenging diagnostically, as most are comprised of a heterogenous cellular make-up, and immunohistochemistry has not proven reliable in difficult cases with overlapping cytologic features [4]. MST classification system is based on several

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factors: (1) histopathologic features; (2) biologic behavior; and (3) histogenesis [5]. Although MST histogenesis remains a subject of debate [6], the “bicellular theory of origin” is the most widely-accepted and states that MST arises from the neoplastic transformation of basal cells located in either the intercalated duct or excretory (striated) duct [7, 8]. Basal cells, which normally act as reserve cells responsible for replenishing the differentiated cells of the mature salivary gland unit, may experience a mutational event, resulting in salivary gland neoplasia [7, 9]. Most studies on MST histogenesis claim that mucoepidermoid carcinoma (MEC), primary squamous cell carcinoma (PSCCA) and salivary duct carcinoma arise from the excretory duct, whereas polymorphous low grade adenocarcinoma (PLGA), basal cell adenocarcinoma (BCA), adenoid cystic carcinoma (AdCC) and acinic cell carcinoma (ACC) are of intercalated duct origin [9, 10]. Adenocarcinoma, not otherwise specified (NOS) (AdC NOS) is assumed to arise from either of these reserve cells [8, 11] and carcinoma ex pleomorphic adenoma (Ca ex PA) is of uncertain histogenesis [12]. The discovery of useful molecular markers involved in crucial steps of carcinogenesis provides information on MST behavior, yet applying their use to the understanding of histogenesis is less common. Human cancer, manifested as uncontrolled cellular proliferation, is commonly caused by changes in the expression or activity of proteins involved in regulating the normal cell cycle [13]. p27, a member of the cip/kip family of cyclin-dependent kinase inhibitors, is expressed at high levels in G⁰ cells and promotes cell arrest and apoptosis. The protein that specifically binds and targets p27 for degradation is S-phase kinase associated protein-2 (skp-2), and may be the main rate-limiting regulator for p27 degradation [13]. Previous studies on a variety of human cancers showed that an altered p27/skp-2 ratio was associated with poorer differentiation, aggressive biological behavior and worse clinical outcome [14–16]. In the present study, we retrospectively analyzed p27/skp-2 expression on a series of MSTs and sought to evaluate its usefulness as an MST histogenetic marker with relevance to tumor grade.

Materials and Methods

The files of the pathology laboratory at Rambam Medical Center in Haifa, Israel served as a source of material for this study. This study was approved by Rambam Medical Center’s Human Study Ethics Committee in accordance with the Helsinki Declaration. Paraffin blocks were retrieved from the surgical pathology archives at Rambam Medical Center. All tissue specimens had been fixed in neutral-buffered formalin and routinely processed. All pathology slides were retrospectively reviewed and diagnosis confirmed by

well-accepted diagnostic criteria [5]. Metastasis or extensions into the salivary glands were excluded.

For the statistical comparison of categories, we employed the Chi-square test to evaluate the association of qualitative variables, considering $P < 0.05$ as significant.

Immunohistochemistry

4-um-thick sections were deparafinized and rehydrated in a graded series of alcohols. For antigen retrieval, the sections were heated in a pressure cooker at 110 degrees for 6 min in EDTA buffer (pH 8.0). The streptavidin–biotin peroxidase complex (SABC) method was used by means of an automatic stainer (Ventana, Nexes). The tissue sections were incubated with anti-p27 clone 57 (Zymed laboratories, USA) at a dilution of 1:500 and with anti-skp-2, clone 2c8d9 (Zymed laboratories, USA) at a dilution of 1:100. Reaction products were visualized by chromogenic reaction with diaminobenzidine tetra-chloride (DAB+) and hematoxylin staining.

Immunohistochemical Evaluation

Tumor expression of p27 and skp-2 was assessed according to the methods described by Hara et al. [17] with minor modifications. Each case was rated according to a score that added a scale of intensity to the number of tumor cells that stained for p27/skp-2. The intensity of staining was on the following scale: 0, no staining; 1+, mild staining; 2+, moderate staining; 3+, intense staining. The number of neoplastic cells stained was evaluated as follows: 0, <10% of cells stained in the microscopic fields; 1+, <25% of cells stained positive; 2+ between 25 and 50% stained positive; 3+, between 50 and 75% stained positive; 4+, >75% stained positive. The minimum score when summed (percentage of cells + intensity) was therefore 0 and the maximum 7. The combined staining scores (percentage of cells + intensity cells) were as follows: ≤2 was negative or weak staining; score 2–3 was moderate staining; score 4–7 strong staining. Nuclei were considered p27 positive if any nuclear staining was present. Both nuclei and a granular cytoplasmic pattern were considered positive skp-2 expression. Like previously reported, this cytoplasmic staining was not believed to be background staining due to the absence of skp-2 in stromal cells and nuclei of these sections [18].

Results

Demographic Features (Table 1)

Between 1980 and 2009, there were a total of 61 cases (9 morphologic subtypes) of primary malignant major

Table 1 Epidemiology of 61 cases of primary MST

	Age, years	Gender, <i>n</i> (%)	Location, <i>n</i> (%)			
	Mean (range)	Male/female	Parotid	Submandibular	Palate	Other
High grade MST (<i>n</i> = 21)						
MEC (high grade) (<i>n</i> = 3)	60 (51–71)	2 (66):1 (33)	1 (33)	1 (33)	1 (33)	0
S. duct ca (<i>n</i> = 3)	65 (62–82)	3 (100):0	1 (33)	0	0	2 (66)
AdC nos (<i>n</i> = 3)	70 (70–71)	2 (66):1 (33)	1 (33)	1 (33)	1 (33)	0
Ca ex PA (<i>n</i> = 1)	40	1 (100):0	1	0	0	0
AdCC (solid type) (<i>n</i> = 2)	61 (59–63)	0:2 (100)	1 (50)	0	1 (50)	0
PSCCA (<i>n</i> = 9)	80 (77–84)	4 (44):5 (56)	6 (66)	2 (22)	1 (11)	0
Total	63 (40–80)	12 (57):9 (43)	11 (52)	4 (19)	4 (19)	2 (9)
Intermediate grade (<i>n</i> = 11)						
MEC (<i>n</i> = 8)	47 (21–61)	2 (25):6 (75)	4 (50)	0	3 (37)	1 (12)
AdC nos (<i>n</i> = 3)	78 (77–80)	2 (66):1 (33)	0	1 (33)	0	2 (66)
Total	62 (47–78)	4 (36):7 (64)	4 (36)	1 (9)	3 (27)	3 (27)
Low grade MST (<i>n</i> = 29)						
MEC (<i>n</i> = 3)	40 (40–41)	3 (100): (0)	3 (100)	0	0	0
PLGA (<i>n</i> = 7)	55 (22–73)	0:7 (100)	0	0	6 (86)	1 (14)
ACC						
Papillary cystic type (<i>n</i> = 3)	30 (31–36)	2 (66):1 (33)	2 (66)	0	0	1 (33)
Solid/microcystic (<i>n</i> = 6)	55 (15–80)	5 (83):1 (13)	2 (33)	0	1 (17)	3 (50)
BCA (<i>n</i> = 3)	53 (38–74)	1 (33):2 (66)	2 (66)	0	1 (33)	0
AdCC (cribiform/tubular type) (<i>n</i> = 5)	44 (25–65)	1 (20):4 (60)	1 (20)	3 (60)	1 (20)	0
Ca ex PA (<i>n</i> = 2)	66 (57–76)	1 (50):1 (50)	1 (50)	0	1 (50)	0
Total	49 (15–80)	13 (45):16 (55)	11 (38)	3 (11)	10 (30)	5 (17)

MEC mucoepidermoid carcinoma, S. duct ca salivary duct carcinoma, Adc nos adenocarcinoma nos, Ca ex PA carcinoma ex pleomorphic adenoma, AdCC adenoid cystic carcinoma, PSCCA primary squamous cell carcinoma, PLGA polymorphous low grade adenocarcinoma, ACC acinic cell carcinoma, BCA basal cell adenocarcinoma

(*n* = 34; 56%) and minor (*n* = 27; 44%) salivary gland tumors. The known specific location in the major glands included the parotid gland (*n* = 26; 76%) and submandibular gland (*n* = 8; 24%). The known specific location in the minor glands included the palate (*n* = 17; 63%), buccal mucosa (*n* = 4; 15%), labial mucosa (*n* = 3; 11%), retromolar (*n* = 2; 7%), and base of tongue (*n* = 1; 5%). Of the 9 morphologic MST subtypes, the commonest was MEC (*n* = 14; 22%) followed by ACC (*n* = 9; 14%) and PSCCA (*n* = 9; 14%), AdCC (*n* = 8; 12%), PLGA (*n* = 7; 11%), AdC nos (*n* = 5; 8%), salivary duct carcinoma (*n* = 3; 4%), BCA (*n* = 3; 4%), and Ca ex PA (*n* = 3; 4%). There were 32 females (52%) and 29 males (47%). Age ranged from 15–84 years. The mean age for female and male patients was 53 and 57.5 years, respectively. Where applicable, tumor subtypes were segregated according to histologic grade (AdC nos and MEC). Due to interobserver variability in the histologic grading of MEC, and the histologic and biologic similarities of the intermediate and low grades, our study used a two tier grading system in categorizing MEC: low/intermediate grade and

high grade [1, 4]. Six cases of pleomorphic adenoma (PA), 2 cases of retention cyst and 7 cases of sialadenitis were also evaluated for p27 and skp-2 expression.

P27/SKP-2 Expression According to Proposed MST Histogenesis (Table 2)

Statistical differences in p27 (*P* = 0.005) and skp-2 (*P* = <0.0001) expression between the two histogenetic groups (excretory duct vs intercalated duct origin) was highly significant (Chi-square test). Specifically, MSTs of proposed excretory duct origin (*n* = 32) showed strong p27 expression (*n* = 18/32; 56%) and moderately strong/strong skp-2 expression (*n* = 18/32; 56%) in over half the cases. By comparison, MSTs of proposed intercalated duct origin (*n* = 27) showed strong p27 expression (*n* = 25/27; 93%) in the vast majority of cases, but weak expression of skp-2 in *all cases*. Similarly, all cases of PA (*n* = 6) strongly expressed p27 and all but one weakly expressed skp-2.

Table 2 Percentage and intensity of tumor cells expressing Skp2 and p27 according to proposed MST histogenesis

	No. of patients (%)		
	Score >2 Weak	Score 2–3 Moderate	Score 4–7 Strong
<i>Intercalated duct histogenesis (n = 27)</i>			
P-27			
AdCC (n = 8)	0	0	8 (100)
PLGA (n = 7)	0	0	7 (100)
BCA (n = 3)	0	0	3 (100)
ACC (n = 9)	0	2 (22)	7 (77)
Skp-2			
AdCC (n = 8)	8 (100)	0	0
PLGA (n = 7)	7 (100)	0	0
BCA (n = 3)	3 (100)	0	0
ACC (n = 9)	9 (100)	0	0
<i>Excretory duct histogenesis (n = 32)</i>			
P27			
MEC (n = 14)			
Low/int grade	0	0	11 (100)
High grade	0	2 (66)	1 (33)
S. duct ca (n = 3)	0	2 (66)s	1 (33)
AdC nos (n = 6)			
Intermediate grade	0	0	3 (100)
High grade	0	2 (66)	1 (33)
PSCCA (n = 9)	7 (77)	1 (12)	1 (12)
Skp-2			
MEC (n = 14)			
Low/int grade	11 (100)	0	0
High grade	1 (33)	2 (66)	0
S. duct ca (n = 3)	0	2 (66)	1 (33)
AdC nos (n = 6)			
Intermediate grade	1 (33)	2 (66)	0
High grade	1 (33)	2 (66)	0
PSCCA (n = 9)	0	1 (11)	8 (89)
<i>Unknown histogenesis (n = 3)</i>			
P27			
exPA (n = 3)	0	0	3 (100)
Skp-2			
exPA (n = 3)	1 (33)	1 (33)	1 (33)

MEC mucoepidermoid carcinoma, AdCC adenoid cystic carcinoma, PLGA polymorphous low grade adenocarcinoma, S. duct ca salivary duct carcinoma, BCA basal cell adenocarcinoma, AdC nos adenocarcinoma nos, ACC acinic cell carcinoma, ex PA carcinoma ex pleomorphic adenoma, PSCCA primary squamous cell carcinoma

Revised Histogenesis Scheme

When low/intermediate grade MEC was allocated to the category of MST of proposed intercalated duct origin, the statistical differences in p27/skp-2 expression between the two histogenetic groups was highly significant, ($P = 0.0001$) ($P = <0.0001$), respectively (Chi-square test). In this scenario, MST of proposed intercalated duct origin ($n = 38$) showed strong p27 expression ($n = 36/38$; 95%) while none of the cases expressed skp-2. MST of proposed excretory duct origin ($n = 21$) showed strong p27

expression ($n = 7/21$; 33%) and moderate/strong skp-2 expression ($n = 18/21$; 86%).

Benign and Reactive Lesions

All cases of sialadenitis ($n = 7$) strongly expressed p27 and skp-2 (score 6–7), although p27 expression was located in both the acini and salivary ducts. skp-2 expression was located exclusively in the salivary ducts. All cases of PA ($n = 6$) strongly expressed p27, and one weakly expressed skp-2.

Fig. 1 P27 and skp-2 immunohistochemical staining of MST of proposed intercalated duct origin. Note the strong p27 and weak skp-2 expression in: **a, b** MEC (low/intermediate grade), **c, d** PLGA, **e, f** AdCC

Discussion

The aim of our study was to correlate MST expression of cell cycle proteins p27/skp-2 with MST histogenesis, which is relevant to the classification system and in predicting tumor behavior [5].

Strong p27 expression (score 4–7) was noted in all cases of PLGA, BCA, ACC, AdCC, low/intermediate MEC, intermediate grade AdC nos and Ca ex PA. Our results differ from previous studies that noted strong p27 expression in only a minority of AdCC cases, [19, 20] yet are in agreement with Affolter et al. [21] who found strong expression in all cases of PLGA. Skp-2 expression was associated with MEC (high grade), salivary duct carcinoma, PSCCA and AdC nos (all

histological grades). These results are in agreement with others [22] who reported positive skp-2 expression for MEC. Skp-2 expression in the three cases of Ca ex PA was variable and seemed dependent on its grade of malignancy. According to Tortoledo et al. the degree of malignancy is defined by the measurable extent of extracapsular invasion and histologic type [12]. All 3 of our cases of Ca ex PA dedifferentiated into a malignant portion that resembles AdC nos. Our single case that strongly expressed skp-2 was the “invasive” subtype.

Proposed Histogenesis (Figs. 1, 2 3)

The MST subtypes were segregated by their proposed histogenesis: intercalated duct reserve cells and excretory

Fig. 2 P27 and skp-2 immunohistochemical staining of MST of proposed excretory duct origin: **a, b** high grade MEC; moderately/strong p27 and skp-2 expression, **c, d** salivary duct carcinoma;

moderately/strong p27 and skp-2 expression, **e, f** PSCCA; weak p27 expression and strong skp-2 expression

duct reserve cells. The histochemical profile and pattern of expression was different between the groups. A consistent p27/skp-2 inverse relation was noted in almost all cases of tumors from proposed intercalated duct origin: p27 overexpression and skp-2 underexpression. This staining pattern was more similar with our cases of PA. In contrast, MST of proposed excretory duct origin, although somewhat less predictable, had a tendency for skp-2 overexpression with variable p27 expression. MEC was the primary exception to the rule. Although some contend that the excretory duct reserve cells give rise to MEC [7], Darick et al. by means of ultrastructural studies, argued this concept and claimed that MEC develops from the

intercalated duct reserve cells [23]. In that study, Dardick et al. found a close histogenetic relationship between MEC and PA, a benign tumor of intercalated duct histogenesis [24]. In our study, similar to previous reports, significant differences in p27/skp-2 expression were associated with the level of cellular differentiation in MEC [25, 26]. All our cases of low/intermediate grade MEC had a pattern of p27/skp-2 expression more similar to MST from proposed intercalated duct origin. As a test, we allocated low/intermediate grade MEC to the group of MST of intercalated duct origin and discovered that the differences in skp-2 expression between the 2 histogenetic groups were highly significant: intercalated duct histogenesis (0%) and

Fig. 3 P27 and skp-2 immunohistochemical staining in benign and reactive salivary gland lesions: **a, b** benign mixed tumor. Note the strong p27 expression, especially in the duct-like structures, and absence of skp-2 expression, **c, d** sialadenitis. Note strong p27 expression in both acini and salivary ducts and strong skp-2 expression exclusively in the salivary ducts

excretory duct histogenesis (86%). The biologic nature of intermediate grade MEC is also an issue of debate. Some consider intermediate grade MEC to behave similar to its low grade counterpart [27, 28] while others prefer to classify them as high grade tumors [29]. In our survival study, 2 (18%) patients with MEC succumbed to their disease, and both were high grade tumors. In summary, the results of our study favors the contention of Dardick et al. [23] and others [2] that although composed histologically of the same tumor cells types, high grade MEC may represent a separate entity.

Previous studies on a variety of human cancer types associated increased skp-2 expression with high grade tumors and aggressiveness [14–16]. The histogenesis of MST is probably relevant to the prediction of behavior [7]. Based on clinical experience and survival studies, MST are divided into high grade and low grade categories [30, 31] (Table 3). It is probably no coincidence that most MST classified as high grade are of proposed excretory duct origin. Our results seem to favor the notion that tumor behavior may be related to skp-2 expression, which is relevant to the portion of the salivary duct unit from where they arise. AdC nos is theorized to arise from either (intercalated or excretory) reserve cell type [8, 9], yet based on p27/skp-2 expression and biologic behavior, these tumors seem more closely related to MST subtypes of excretory duct origin, and its expression was independent of tumor grade.

Table 3 Grading of MST after Cheuk and Chan[30]

High grade MST	Low grade MST
Mucoepidermoid carcinoma	Mucoepidermoid carcinoma
Adenocarcinoma nos	Adenocarcinoma nos
Carcinoma ex pleomorphic	Carcinoma ex pleomorphic
Primary squamous cell carcinoma	Polymorphous low grade adenocarcinoma
Salivary duct carcinoma	Acinic cell carcinoma
	Basal cell adenocarcinoma

Interestingly, all cases of sialadenitis, a reactive not neoplastic condition, strongly expressed p27 (salivary ducts and acini) and skp-2 (salivary ducts only). This pattern of p27/skp-2 expression, not seen in either malignant or benign tumors, may represent a type of balance or equilibrium in the salivary gland cell cycle which cannot be maintained following a mutagenic event and subsequent tumor development.

PSCCA and MST

PSCCA, although considered a distinct pathologic entity of the salivary glands and of proposed excretory salivary duct origin, is a diagnosis of exclusion after ruling out invasion from a mucosal SCCA into the parotid gland or a metastasis from a distant site [7, 32, 33]. In our cases, following an extensive search of our clinical archives, there was no

evidence of either, so the final diagnosis was PSCCA. Yet, all cases manifested a pattern of p27/skp-2 expression significantly different than all other MST subtypes; specifically, a predictable inverse correlation showing weak p27 expression and strong skp-2 expression. In our previous study of MST and cox-2 expression, we came to a similar conclusion [34]. Based on previous studies on p27/skp-2 expression and oral (mucosal) squamous cell carcinoma, it may be concluded that the histogenesis of PSCCA is more closely related to mucosal SCCA rather than the salivary duct apparatus [25]. This information may provide us with an additional diagnostic tool for distinguishing between high grade MEC and PSCCA [35].

Conclusion

In conclusion, our study seems to show that the p27/skp-2 profile of expression for a variety of MST subtypes is related to its histogenesis, which in turn has an impact on tumor behavior. A clear inverse correlation was especially evident in tumors of proposed intercalated duct origin: strong p27 expression and negative skp-2 expression. Low/intermediate grade MEC had a profile of expression more similar to those of intercalated duct origin. Skp-2 overexpression was a feature noted only in MST of proposed excretory duct origin. Uniquely, p27/skp-2 expression of PSCCA was different than the other MST subtypes which may suggest an alternate histogenesis from that of the salivary duct apparatus. Further studies on a larger series of MST are warranted in order to assess the value of our findings.

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