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SHORT COMMUNICATION

p27 and salivary cancer

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Abstract This study examined p27 expression in a cohort of salivary malignancies ($n = 74$) for a prolonged period (20 years). Reduction of p27 expression was found to be a most powerful predictor for poor survival and more so when the tumor concurrently expressed high levels of p53, TUNEL and heparanase markers, dramatically dropping the patient survival probability to 0! While no patient whose tumor-staining profile included: p27 > 50%, p53 = 0, TUNEL = 0 and heparanase = 0, died of the disease during the 20-year follow up, the median of survival of the group with p27 ≤ 50%, p53 > 0, TUNEL > 0 and heparanase > 0 was only 39 months. The survival probabilities of these two groups at 5 years were 100 and 50%, respectively, and at 20 years they were 100 and 0%, respectively ($P = 0.05$). Significant p27 reduction also resulted in significantly larger tumor size (T value), higher spread of neck metastasis and extra capsular spread and in more advanced disease (higher stage). Significant correlation rates were found between age and poor survival, age and reduced p27 expression, and reduced p27 expression and other general co-existing malignancies, indicating p27 reduction as part of a general phenomenon—age related mutagenesis. Significantly more extensive therapy applied to patients with salivary reduced-p27 tumors could not prevent the rise in mortality rate, questioning the justification for extensive therapy which is naturally accompanied by higher morbidity. Additional therapeutic tools for fighting salivary cancer, possibly based on the new understanding of the p27, p53, TUNEL and heparanase carcinogenic network, are necessary.

Keywords p27 · Prognosis · Malignancies · Cancer · Salivary glands

Introduction

Salivary gland malignancies vary widely in their histological appearance, molecular characteristics, clinical appearance, pathogenesis [1], 5-year survival probability [2, 3] and biological behavior. Accordingly, patient survival rate may be related to a better understanding of their biological nature. In this respect, deregulation of p27 expression has been found to have a particularly important role in cancer lesions [4, 5], though it has never been studied in salivary gland cancer [6–8]. The p27 in these lesions is of the wild-type species, and its deregulation has been attributed to an aberrant accelerated ubiquitin-mediated degradation of the protein. Thus, increased degradation of p27 can play a pathogenetic role in the uncontrolled cell proliferation and malignant transformation.

The purpose of the current study was to elucidate the role of p27-mutated gene in salivary cancer by examining the immunohistological staining level of p27 (its expression rate) in a relatively large cohort of a variety of salivary tumors ($n = 74$). The examined p27 expression was compared with that of three other markers, p53, TUNEL (TDT-mediated dUTP-biotin nick end labeling) and heparanase, which we have recently shown to play a crucial role in the pathogenesis of salivary cancer [9, 10], and concurrently

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with various other clinical, carcinogenic and pathological characteristics. Most importantly, p27 was compared with the staging of the disease and with patient survival rates.

Materials and methods

A total of 74 patients with various salivary tumors (Table 1) and a mean age of 59 ± 16 years (range 15–90 years) and a gender distribution of 46 (62%) males and 28 (38%) females diagnosed with various salivary malignant tumors were enrolled in the current study. This study was approved by Rambam Medical Center's Human Studies Ethics Committee, in accordance with the Helsinki Declaration. Data concerning clinical, therapeutic and tumor characteristics were collected. In all cases the therapy administered included a local resection of the tumor in safe margins, sometimes accompanied by one or more of the following four therapeutic modalities: surgical neck dissection, local radiotherapy, external beam radiotherapy and/or chemotherapy. The extensiveness of the therapy administered was thus evaluated according to the number of therapeutic modalities administered (in total 1–5 possibilities), then these data were correlated with the staining levels obtained in the pathological study for p27, being compared with the staining levels of p53, TUNEL and heparanase. The expression rates of these markers were also correlated with the survival rates. The histopathological and statistical analyses were performed as previously reported [9–11].

P27 staining

Five-micron sections were deparaffinized with xylene and rehydrated in a series of ethanols. Endogenous peroxidase was blocked by 3% hydrogen peroxide in methanol for 20 min. For epitope retrieval, slides were heated in a microwave oven at 92°C for 20 min in a Tris-EDTA-buffer-pH 8.0. After cooling, slides were washed in distilled water and then in phosphate-buffered saline (pH 7.4). Slides were incubated overnight at 4°C with the primary antibodies anti p27, clone 57 (transduction laboratories, KY, USA) diluted 1:500. Staining was completed with a Histostain-plus kit (Zymed Laboratories, CA, USA). Color reaction product was developed with aminoethylcarbazole as the chromogen. All sections were counterstained with hematoxylin, dehydrated and covered. Incubations with phosphate-buffered saline containing 1% bovine serum albumin were used as negative controls (instead of the primary antibodies). Palatine tonsil tissue positive control was run in parallel where perigerminal center lymphocytes served as positive control for p27 [12–19].

Results

P27 and tumor characteristics

The frequency of SCCpatients with p27 ≤ 50% (9/10) was significantly higher than that of patients with p27 > 50 (1/10) ($P = 0.015$). In no other salivary gland malignancies was the frequency of the p27 ≤ 50% tumors significantly higher than that of the p27 > 50% tumors. Tumor size (T) was significantly related to the p27 staining level ($P = 0.001$). Similarly, tumors with reduced levels of p27 staining (p27 ≤ 50%) had higher N values ($P = 0.012$), extra capsular spread ($P = 0.05$) and stages ($P = 0.002$).

P27 and clinical characteristics

Patient age was a highly significant factor in the level of p27 staining; the mean age of patients with p27 ≤ 50% ($n = 39$) was 63 ± 13 years, while that of p27 > 50% patients was

Table 1 The salivary malignancies ($n = 74$) and their related p27 staining levels

P27 level/final diagnosis	≤50 patients (%)	>50 patients (%)	*P*
Squamous cell carcinoma	9 (23)	1 (3)	0.015
Mucoepidemoid carcinoma	8 (20)	9 (26)	0.78
Adeno carcinoma	6 (14)	8 (23)	0.55
Adenoid cystic carcinoma	1 (3)	6 (17)	0.047
Acinic cell carcinoma	5 (13)	5 (14)	0.99
Low grade polymorphous adeno carcinoma	1 (3)	4 (11)	0.18
Neuroendocrine carcinoma	1 (3)		
Salivary duct carcinoma		1 (3)	
carcinoma ex mixed adenoma	2 (5)		0.49
Anaplastic/undifferentiated carcinoma	4 (10)	1 (3)	0.36
Malignant oncocytoma	1 (3)		
Basal cell carcinoma	1 (3)		
Total	39 (100)	35 (100)	

56 ± 17 years ($P = 0.05$). Extensiveness of therapy was significantly related to p27 staining level: while 21/32 of the p27 ≤ 50%-stained tumors were treated only by local surgical removal, no fewer than 33/37 (89%) of p27 ≤ 50%-stained tumors were treated with multiple modalities ($P = 0.0001$). Gender, smoking habit or ethnic origin was not significantly related to the level of p27 staining.

P27, p53, TUNEL and heparanase expression rates

In 39/74 (53%) of the cases p27 expression rate (staining level) was ≤ 50%, while in the remainder of cases (47%) it was > 50%. The mean p27 staining level of the 31 p53 positively-stained tumors was ≤50% (42 ± 22%) while that of the 38 p53 negatively stained tumors was >50% (55 ± 19%). The correlation between positive p53 and reduced p27 staining (≤50%) was significant ($P = 0.018$), as was the correlation between reduced p27 staining level and positive expression of TUNEL ($P = 0.027$). Most reduced-p27-stained tumors (≤50%) were positively stained for TUNEL (56%), while most enhanced-p27-stained tumors (>50%) were negative for TUNEL. Interestingly, no significant correlation was found between the staining levels of p27 and heparanase.

Survival probabilities

Age and survival

The "Kaplan-Meier" analysis performed revealed a significant relationship between older age and poorer survival probability. Using the mean age of the entire analyzed group ($n = 74$), (62 years old) as the cut-off level showed that survival probability 5 years following diagnosis and treatment of the salivary malignancy was 80% for younger patients and 40% for older patients; at 20 years survival probability was 47 and 14%, respectively ($P = 0.0001$). The median of survival for these young versus old patients was 166 and 41 months, respectively ($P = 0.0001$).

P27 and survival

Survival probability rates dropped also with reduction of p27 expression level. The median of survival for patients with enhanced p27-stained tumors (>50%) was 162 months, and dropped significantly to 63 months ($P = 0.006$) for reduced p27-stained tumors (≤50%) . Thus, survival probability at 5 years following diagnosis and treatment of the salivary malignancy was 81% for enhanced p27-stained tumors (>50%) and 47% for reduced p27-stained tumors (≤50%); at 20 years, these survival probabilities dropped to 45 and 24%, respectively (Fig. 1, $P = 0.006$).

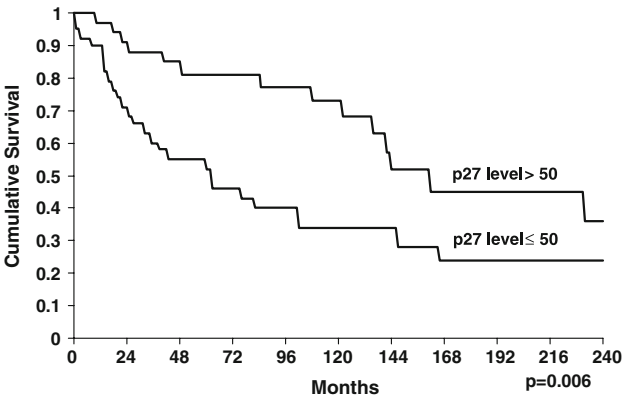

Fig. 1 Cumulative survival probability curves of the 74 salivary-gland-tumor-patients according to their age. Kaplan–Meier analysis showed significantly poorer survival probability for elderly patients (>62) as compared with younger patients (≤62) ($P = 0.0001$), (>0) of all three other markers ($P = 0.05$)

P53, TUNEL and heparanase and survival

Patients with reduced p27-stained tumors (≤50%), who were concomitantly positive for p53, TUNEL or heparanase fared even worse than those with reduced p27-stained tumors (≤50%) only.

Five-year survival probability for patients with tumors negatively-stained for p53 (=0) and enhanced p27 staining (>50%) was 86%, for those with p53 = 0 and reduced p27 staining (≤50%) it was 44%, and for those with tumors positively stained for p53 (>0) as well, it was 44%. At 20 years these values were 68, 22 and 0%, respectively ($P = 0.015$). The median survival probabilities of these three groups dropped in a similar manner to 232, 63 and 36 months, respectively.

Similarly, the median of survival for patients with (p27 > 50,TUNEL = 0) was 162, 76 months for patients with (p27 ≤ 50%, TUNEL = 0), and 36 months for patients with (p27 ≤ 50%, TUNEL > 0). Survival probabilities for these three groups at 5 years dropped significantly to 90, 56 and 40%, respectively, and at 20 years they dropped to 45, 32 and 18%, respectively ($P = 0.006$).

Not one patient in the subgroup (p27 > 50, heparanase = 0) died from the disease during the 20 years of follow-up, while the median of survival for patients with (p27 ≤ 50, heparanase = 0) was 82 months, and only 39 months for patients with (p27 ≤ 50%, heparanase > 0). Survival probability at 5 years remained 100% in the first group but dropped significantly to 51 and 37% in the second and third groups, respectively; at 20 years, the first group remained at 100% but the second and third groups dropped to 25 and 16%, respectively ($P = 0.038$).

Moreover, while no patient of the group whose tumor-staining profile was p27 > 50%, p53 = 0, TUNEL = 0 and heparanase = 0 died of the disease during the 20 years of

follow-up, the median of survival in the group with p27 ≤ 50%, p53 > 0, TUNEL > 0 and heparanase > 0 was 39 months only. The survival probabilities of these two groups at 5 years were 100 and 50%, respectively, while at 20 years they were 100 and 0%, respectively ($P = 0.05$).

Discussion

Salivary tumors with reduced levels of p27 staining (≤50%) demonstrated a significantly more aggressive biological behavior and their size (*T*), neck metastasis (N), extra capsular spread and stage values were significantly higher. Despite more extensive therapy administered to the patients with such tumors, their survival probability was significantly lower than for those with enhanced expression of p27 (>50%) tumors, indicating the tumor's biological nature as the most important factor relating to the patient's prognosis. Furthermore, survival was even poorer for the positively stained p53, TUNEL and heparanase tumors; the biological profile of these proteins' network has a crucial role in the pathogenesis of salivary cancer.

This conclusion is supported by previous reports that p53, TUNEL and p27 have key positions in cell cycle regulation leading to an arrest of cell proliferation enabling a repair process of DNA damage [20–24].

The relationship between p27 and apoptosis was also explored by Zheng et al. [25] who found that p27 overexpression caused cell arrest and induced apoptotic cell death reflected by pre-G1 peak in the histogram of FACS, which was also confirmed by TUNEL assay and electron microscopy. Accordingly, the researchers concluded that p27 may be a good candidate for cancer gene therapy.

In contrast, the fact that we found no significant correlation between the expression rates of p27 and heparanase, though both were associated with poor survival, may be explained by their being involved in unrelated lethal pathological pathways. The first, related to p27 (and p53 and TUNEL), is based on the reduced capacity for removal of defective cells via either correction of DNA aberrations or apoptosis, while the second, related to heparanase, is based on the latter's pro-metastatic and angiogenetic functions as previously reported [10].

In summary, the biological profile of the p27, p53, TUNEL and heparanase network was found to have a crucial role in the pathogenesis of salivary cancer, suggesting a vital need for further studying and understanding the biological background of salivary cancer in order to develop efficient biological therapies based on such an understanding.

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