Tissue microarray is a reliable method for immunohistochemical analysis of pleomorphic adenoma

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Objective. To determine the most adequate number and size of tissue microarray (TMA) cores for pleomorphic adenoma immunohistochemical studies.
Study design. Eighty-two pleomorphic adenoma cases were distributed in 3 TMA blocks assembled in triplicate containing 1.0-, 2.0-, and 3.0-mm cores. Immunohistochemical analysis against cytokeratin 7, Ki67, p63, and CD34 were performed and subsequently evaluated with PixelCount, nuclear, and microvessel software applications.
Results. The 1.0-mm TMA presented lower results than 2.0- and 3.0-mm TMAs versus conventional whole section slides. Possibly because of an increased amount of stromal tissue, 3.0-mm cores presented a higher microvessel density. Comparing the results obtained with one, two, and three 2.0-mm cores, there was no difference between triplicate or duplicate TMAs and a single-core TMA.
Conclusions. Considering the possible loss of cylinders during immunohistochemical reactions, 2.0-mm TMAs in duplicate are a more reliable approach for pleomorphic adenoma immunohistochemical study. (Oral Surg Oral Med Oral Pathol Oral Radiol 2014;117:81-88)

Although tissue microarray (TMA) principles were originally described by Wan et al. in 1987,[1] it was only after the description of Kononen et al. in 1998[2] that the exponential increase in the number of studies using this laboratory approach was noted. The main advantage of the TMA method is its ability to aggregate tissue samples of hundreds of different cases on a single paraffin block, leading to preservation of the original tissues, laboratory economy, and reduction of execution time.[3-6] On the other hand, limitations that are frequently pointed out—including the variable quality of the tissues used in the TMA, the technical skills required for arranging and cutting TMA blocks, and, most relevant, the capacity of TMA to adequately represent heterogeneous neoplasias—have demanded the development of validation studies.[6-8]

Therefore, with the purpose of analyzing the usefulness and reliability of TMA in the immunohistochemical study of pleomorphic adenoma (PA) of salivary glands, the authors investigated the expression of cytokeratin 7 (CK7), p63, Ki67, and CD34 proteins in 82 cases arranged in TMA blocks containing 1.0-, 2.0-, or 3.0-mm cores assembled in triplicate and compared these to findings from conventional whole section slides.

MATERIAL AND METHODS

Tissue samples

A total of 82 cases of PA over a 10-year period (January 2001 to December 2011) were retrieved from the archives of the Oral Diagnosis Department of the Piracicaba Dental School, São Paulo, Brazil, and from a surgical pathology laboratory in Cascavel, Paraná, Brazil. The original hematoxylin-eosin stained slides were reviewed by 3 oral pathologists, and the diagnoses of all cases were confirmed following the World Health Organization's 2005 Histological Typing of Salivary Gland Tumors guidelines.[9]

TMA construction

Tumor areas were selected and marked on hematoxylin-eosin-stained sections using an objective marker (Nikon

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Statement of Clinical Relevance

Molecular features of salivary gland tumors remain to be further investigated, and the use of reliable and high-throughput laboratory methods such as tissue microarray may improve the discovery of new markers related to the clinical behavior of these neoplasias.

Corp, Tokyo, Japan). The slide was then overlaid on the original paraffin block to determine the corresponding area to be used. TMAs were constructed using a manual tissue arrayer (Sakura Co, Japan) and, depending on the neoplastic tissue available to be arrayed in each case, 3 representative cylindrical cores of 1.0-, 2.0-, or 3.0-mm diameter were taken from each tissue block and then arranged sequentially into a recipient ready-to-use paraffin block (Sakura Co, Japan). The 82 cases of PA were distributed as follows: 15 cases as whole section conventional slides; 39 on one TMA block containing 1.0-mm cores; 19 on one TMA block containing 2.0-mm cores; and 9 on one TMA block containing 3.0-mm cores. Two cores of normal parotid gland and one of oral squamous cell carcinoma were inserted in the left upper corner of each recipient block for orientation. A map specifying the exact position of each case was prepared to facilitate the interpretation of the immunohistochemical results.

Immunohistochemistry

Briefly, the reactions were conducted in 3-μm sections of formalin-fixed, paraffin-embedded tissues that were dewaxed with xylene and then hydrated in an ethanol series. Antigen retrieval was done by immersing the sections in citrate buffer solution (pH 6.0) for 3 minutes of pressure cooking, and the endogenous peroxidase activity was blocked using 10% hydrogen peroxide in 5 baths, each of 5 minutes. After washing in PBS buffer (pH 7.4), slides were incubated overnight with primary antibodies anti-cytokeratin 7 (clone OV-TL 12/30, DakoCytomation, USA, diluted 1:200), anti-p63 (clone 4A4, DakoCytomation, diluted 1:300), anti-Ki67 (clone MIB1, DakoCytomation, diluted 1:100), and anti-CD34 (clone QBEnd10, DakoCytomation, diluted 1:50). All slides were subsequently exposed to avidin-biotin complex and horseradish peroxidase reagents (LSAB Kit, DakoCytomation) and diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich, St Louis, MO, USA). Finally, the slides were counterstained with Carazzi hematoxylin. Positive control sections were used for each antibody, whereas the negative control was obtained by omitting the specific primary antibody.

Digital analysis

The immunohistochemical slides were subsequently scanned into high-resolution images using the Aperio Scanscope CS Slide Scanner (Aperio Technologies Inc, Vista, CA, USA). All digital images obtained in .svs format were visualized with ImageScope software (Aperio Technologies Inc). Ki67 and p63 nuclear markers were analyzed using the Nuclear V9 algorithm (Aperio Technologies Inc) with the following input parameters: averaging radius, 1; curvature threshold, 2.5; lower threshold, 0; upper threshold, 230; minimum nuclear size, 20; maximum nuclear size, 1,000,000; minimum roundness, 0.1; minimum compactness, 0; minimum elongation, 0.1; clear area objective, 240; and intensity threshold, ranging from 0 to 235. The algorithm PixelCount V9 (Aperio Technologies Inc) was used for analyzing the cytoplasmic expression of CK7 with the following input parameters: overlap size, 0; hue value, 1; hue width, 0.5; color saturation threshold, 0.04; and intensity threshold, ranging from 0 to 220. Microvessel Analysis V1 (Aperio Technologies Inc) was used for the analysis of CD34 reactivity with the following input parameters: dark staining threshold, 160; light staining threshold, 210; region joining parameter, 6; vessel completion parameter, 7; minimum vessel area threshold, 15; maximum vessel area threshold, 20,000; maximum vessel wall thickness, 4; and clear area intensity, 240. The software TMA Lab (Aperio Technologies Inc) was also used as an auxiliary tool during the analyses of TMA slides. Cases arranged in TMAs where at least one core was lost or which did not present adequate available neoplastic tissue for analysis were excluded.

With the purpose of determining the amount of luminal cells (using CK7 staining) and myoepithelial cells (using p63 staining) in different-sized TMA cores in relation to the total neoplastic tissue punched, the results obtained represented the number of positive luminal and myoepithelial cells in relation to total tissue, including stromal components. Ki67 analysis also included neoplastic and stromal cells, whereas CD34 evaluation comprised the analysis of stromal vessels in relation to the total tissue area to obtain the vascular density of each case.

Statistical analysis

To compare the immunoexpression of Ki67, p63, CK7, and CD34 in TMAs containing 1.0-, 2.0-, and 3.0-mm cores, and in conventional whole section slides, the Wilcoxon-Mann-Whitney rank sum test was applied, followed by the Dunn post hoc test when a value of $P \leq .05$ was found. The GraphPad software (version 5.0; GraphPad Software Inc, La Jolla, CA, USA) was employed for analysis of the data. The current study was carried out in accordance with the ethical guidelines of our institution (Process number CEP/FOP 002/2013).

RESULTS

Considering the clinical features of the cases included in the current study, a slight female preponderance was observed (40 female patients, 35 male, and 7 not specified; male-to-female ratio, 0.8:1). The mean age was 44.4 years, ranging from 10 to 85 years. The

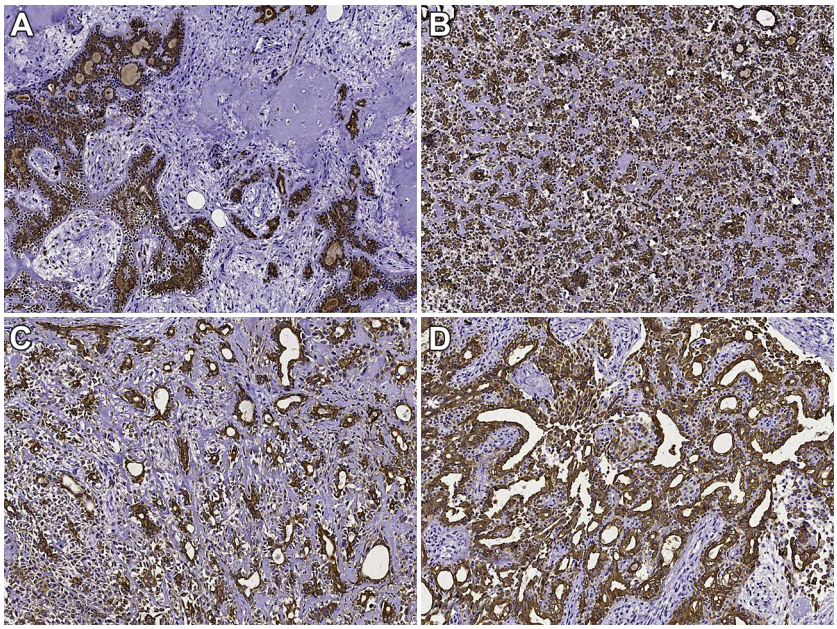

Fig. 1. Immunohistochemical expression of cytokeratin 7 in pleomorphic adenoma using tissue microarray cores of **(A)** 1.0-mm diameter (streptavidin-biotin, original magnification ×100), **(B)** 2.0-mm diameter (streptavidin-biotin, original magnification ×50), and **(C)** 3.0-mm diameter (streptavidin-biotin, original magnification ×25) and **(D)** conventional whole section slides (streptavidin-biotin, original magnification ×50).

parotid gland was by far the most commonly affected site (34 cases or 41.5%), followed by the palate (14 cases or 17.1%), upper lip (13 cases or 15.9%), and submandibular gland (10 cases or 12.2%), whereas other intraoral minor salivary glands were affected in 3 cases (3.7%), and in 8 cases (9.6%) the site was not specified.

All cases of PA included in the present study presented ductal structures, beside areas of high and poor cellularity, and most of the cases also showed chondroid material and hyalinized stroma. Core loss after immunohistochemical reactions was 3%, 5%, and 7% in 1.0-, 2.0-, and 3.0-mm cores, respectively.

The expression pattern of all markers was consistent with those previously described in the literature and, aiming to demonstrate the homogeneity in the expression of all markers in different cases of PA, statistical analysis was carried out using the immunohistochemical results obtained with conventional whole section slides, where no significant difference was seen ($P > .05$).

CK7 immunostaining was observed in the luminal cells of ductal structures in all cases evaluated (Figure 1). The results revealed a mean positivity of 0.47, 0.32, 0.50, and 0.37 for conventional whole section slides and 1.0-mm, 2.0-mm, and 3.0-mm TMA cores, respectively. Statistical analysis showed that the CK7 staining index was significantly lower in the 1.0-mm TMA compared with conventional whole section slides ($P < .05$), whereas no significant difference was seen between 2.0- and 3.0-mm TMAs in comparison with conventional whole section slides, even though a slightly lower result was obtained in 3.0-mm cores (Figure 2).

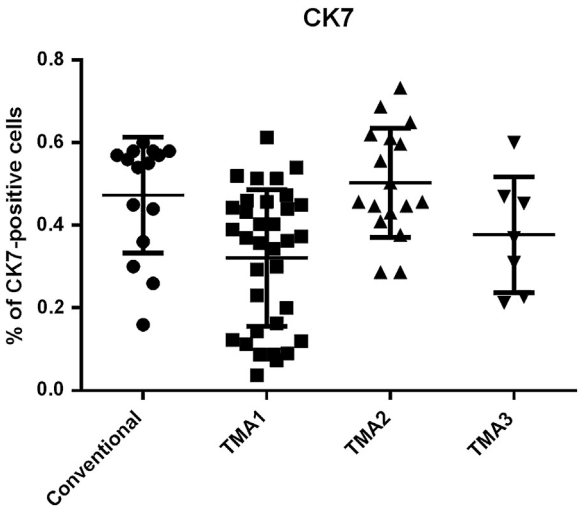

Fig. 2. Immunohistochemical expression of cytokeratin 7 in pleomorphic adenoma was significantly lower in 1.0-mm tissue microarray cores than in 2.0- and 3.0-mm cores, when compared with traditional whole section slides ($P < .05$).

Similar results were obtained using the myoepithelial marker p63 (Figures 3 and 4) and the proliferative marker Ki67 (Figures 5 and 6) (mean positivity of 25.9%, 5.7%, 23.3%, and 16% for conventional whole section slides and 1.0-mm, 2.0-mm, and 3.0-mm TMA

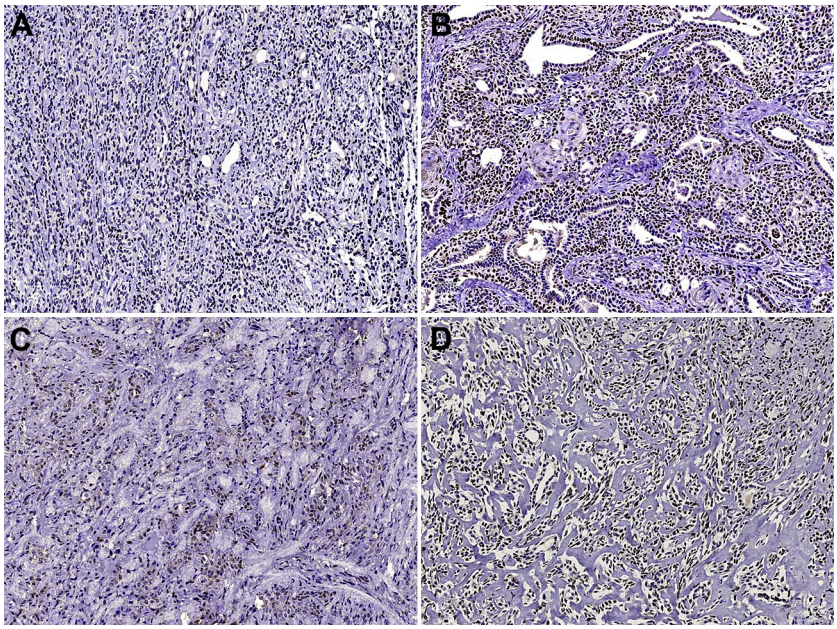

Fig. 3. Nuclear immunohistochemical expression of p63 in myoepithelial cells of pleomorphic adenoma using tissue microarray cores of **(A)** 1.0-mm diameter, **(B)** 2.0-mm diameter, and **(C)** 3.0-mm diameter and **(D)** conventional whole section slides (streptavidin-biotin, original magnification ×100).

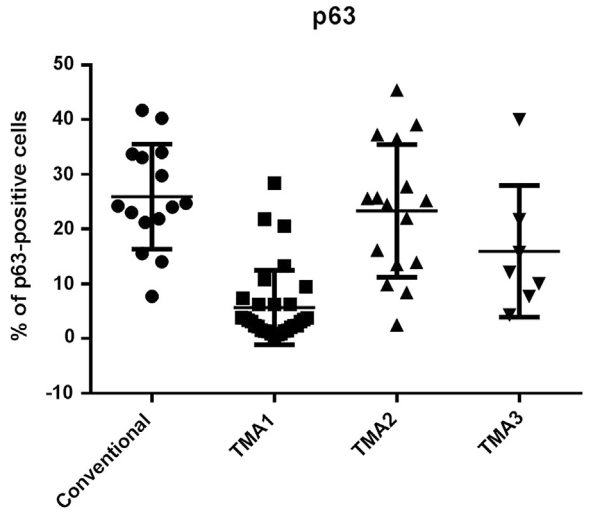

Fig. 4. Significantly lower immunohistochemical expression of p63 was seen in 1.0-mm tissue microarray cores than in 2.0- and 3.0-mm cores, when compared with conventional whole section slides ($P < .05$).

cores, respectively, analyzed for p63, and a mean positivity of 1.18%, 0.45%, 1.46%, and 0.58% for conventional whole section slides and 1.0-mm, 2.0-mm, and 3.0-mm TMA cores, respectively, analyzed for Ki67), where a significantly inferior staining index was seen with both biomarkers in 1.0-mm TMA compared with conventional whole section slides ($P < .05$), and no significant difference was seen when using 2.0- and 3.0-mm core TMAs compared with conventional whole section slides ($P > .05$). Again, an inferior result was obtained with 3.0-mm cores than with 2.0-mm cores.

The vascular antigen CD34 revealed positivity in all cases analyzed (Figure 7) and, in contrast to the results observed with the previous markers, the staining pattern for this stromal marker revealed no significant difference in the vascular density of all TMAs compared with conventional whole section slides (mean values of 4.55, 3.12, 3.97, and 4.93 for conventional whole section slides and 1.0-mm, 2.0-mm, and 3.0-mm TMA cores, respectively) ($P > .05$), even though an increase could be seen as the diameter of the cores became larger, with 3.0-mm cores presenting the most similar results to conventional whole section slides (Figure 8).

Finally, considering that 1.0-mm cores afforded significantly inferior staining indexes for most of the biomarkers analyzed, and considering that both 2.0- and 3.0-mm cores had similar results to conventional whole section slides, the use of 2.0-mm TMAs would be preferred because of the lower damage caused to the original blocks. Therefore, we subsequently determined how many 2.0-mm cores would be appropriate to adequately represent conventional whole section slides of PA. For that, the values relating to the third core were excluded; considering only 2 cores, there was no significant difference between the immunoexpression of all markers and those seen in conventional whole section slides ($P > .05$). Similarly, by excluding the

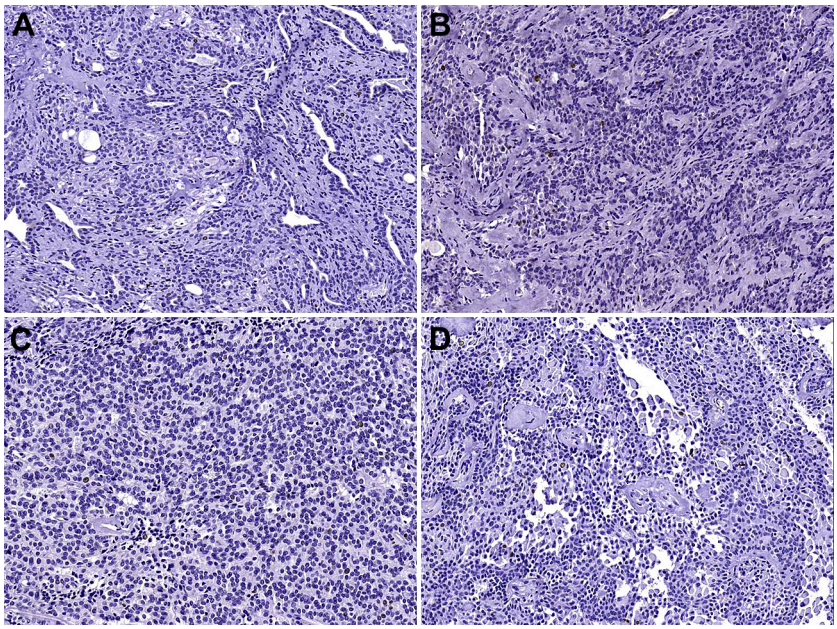

Fig. 5. Ki67 nuclear immunoexpression observed in pleomorphic adenoma using tissue microarray cores of **(A)** 1.0-mm diameter, **(B)** 2.0-mm diameter, and **(C)** 3.0-mm diameter and **(D)** conventional whole section slides (streptavidin-biotin, original magnification ×100).

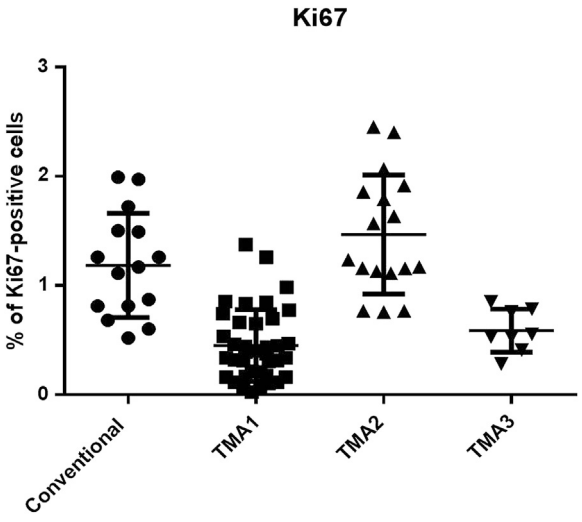

Fig. 6. Significantly lower immunohistochemical expression of Ki67 was obtained in 1.0-mm tissue microarray cores than in 2.0- and 3.0-mm cores, when compared with traditional whole section slides ($P < .05$).

values relating to the second and the third cores and analyzing only the first core, no significant difference could be seen between 2.0-mm TMA and conventional whole section slides ($P > .05$) (Table I).

DISCUSSION

The ability to simultaneously use of a large number of cases, the standardization of secondary reactions (immunohistochemistry, in situ hybridization, etc), a significantly reduced reagent volume for a given investigation and experimental handling time, improving the laboratory workflow, represent advantages of the use of TMAs for molecular studies of paraffin-embedded tissues. However, despite these benefits, TMA has some limitations, including that the evaluation of a given marker is reduced from the conventional whole section slide analysis to a few tissue cores of only 0.6 to 2.0 mm in diameter. This has led to concerns because of the morphologic and molecular heterogeneity of human cancers, necessitating the development of validation studies for each tumor type.[6,10,11]

As a consequence, validation studies have been published showing the significant equivalence of TMA cores with original whole section slides. Boone et al. (2008)[12] validated the TMA technique for studying esophageal squamous cell carcinoma by demonstrating that 0.6-mm cores arranged in triplicates affords excellent concordance to conventional whole section histologic slides when analyzing the expression of CK5/6, CK14, E-cadherin, Ki67, and p53, whereas only 2 cores proved to be necessary to obtain results concordant with those observed in whole section slides of cervical adenocarcinoma.[13] TMAs have also been validated in lung,[14] breast,[15,16] bladder,[17] vulva,[18] and endometrial,[19] cancers, demonstrating the utility of cores as small as 0.6 and 1.0 mm, in contrast to the significant superiority of 2.0-mm cores observed in the current study, which

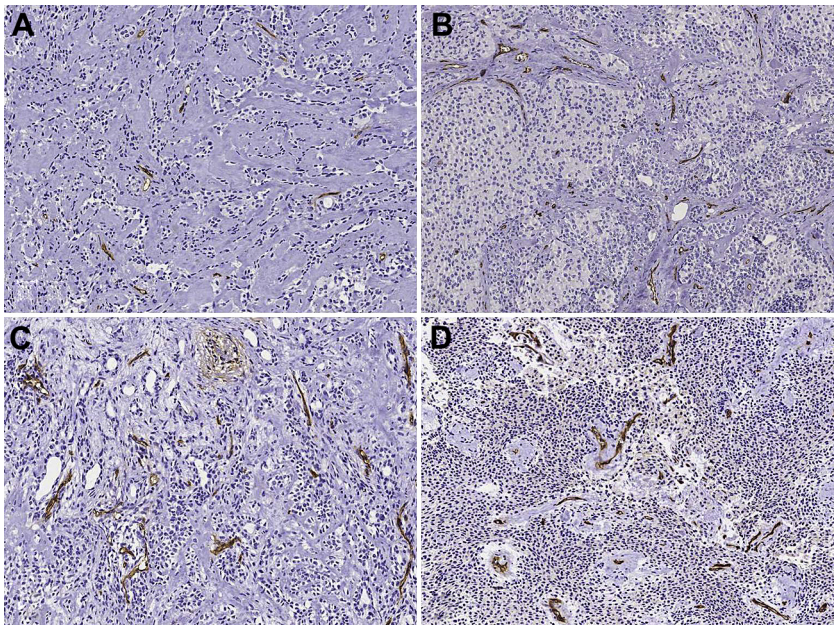

Fig. 7. CD34 immunoexpression in pleomorphic adenoma using tissue microarray cores of **(A)** 1.0-mm diameter (streptavidin-biotin, original magnification ×100), **(B)** 2.0-mm diameter (streptavidin-biotin, original magnification ×50), and **(C)** 3.0-mm diameter (streptavidin-biotin, original magnification ×25) and **(D)** conventional whole section slides (streptavidin-biotin, original magnification ×50).

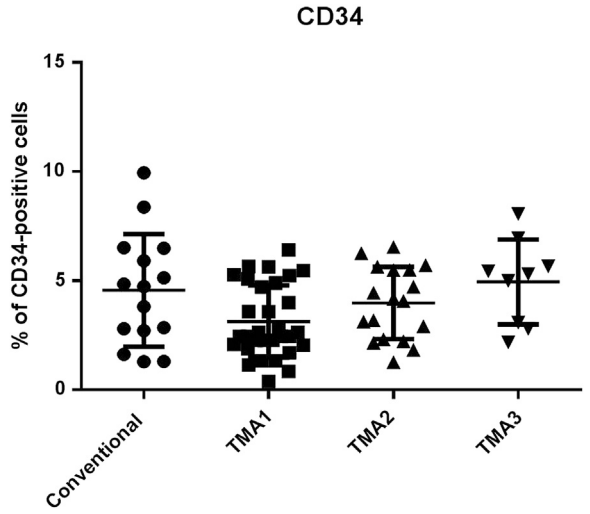

Fig. 8. Investigating the vascular marker CD34, no significant difference could be seen among tissue microarray cores of different sizes and conventional whole section slides ($P > .05$), although an increased expression of the protein could be noted in larger tissue microarray cores, revealing that more stromal component would be available in these tissue microarrays.

Table I. Mean values for CK7, p63, Ki67, and CD34 when using tissue microarrays arranged in triplicate, in duplicate, or as a single core

Markers	*Slide design*	*3 cores**†	*2 cores**	*1 core*†
Cytokeratin 7	CWSS	0.47	0.47	0.47
	1.0-mm	0.32	0.32	0.31
	2.0-mm	0.50	0.51	0.51
	3.0-mm	0.37	0.37	0.33
p63 (%)	CWSS	25.9	25.9	25.9
	1.0-mm	5.7	5.2	5.1
	2.0-mm	23.3	23.7	23.9
	3.0-mm	16	16.4	17.7
Ki67 (%)	CWSS	1.18	1.18	1.18
	1.0-mm	0.45	0.44	0.38
	2.0-mm	1.46	1.38	1.27
	3.0-mm	0.58	0.59	0.59
CD34 (vessels/μm^2)	CWSS	4.55	4.55	4.55
	1.0-mm	3.12	3.30	3.03
	2.0-mm	3.97	3.96	3.82
	3.0-mm	4.93	4.90	5.24

CWSS, conventional whole section slides.
*No significant difference was seen in the mean value of the reactions when comparing all markers arranged as triplicate or duplicate ($P > .05$).
†No significant difference was seen in the mean value of the reactions when comparing all markers arranged as triplicate or as one single core ($P > .05$).

could be explained by the high heterogeneity of pleomorphic adenomas that would require further neoplastic tissue in the cylinders so that an adequate comparison with conventional whole tissue sections could be obtained. Moreover, although studies have been conducted using TMA in the molecular investigation of salivary gland tumors,[20-22] to date, no attempt has been made to validate this technique in this group of lesions, and its usefulness and reliability in the investigation of

morphologically heterogeneous tumors such as PA remain debatable.

In the present study, using 4 immunohistochemical markers with different staining patterns in PA, the authors observed that TMAs containing cores of 2.0- and 3.0-mm diameter offered results similar to those observed in conventional whole section slides, whereas 1.0-mm TMAs showed significantly lower results. Because the preservation of neoplastic tissue is an important concern, 2.0-mm TMAs would be more desirable than 3.0-mm cores. Since the analysis of all markers included both neoplastic and stromal components present in conventional whole section slides and TMA cores, the significantly inferior results obtained with 1.0-mm TMAs could be explained by a technical inaccuracy during tissue punching, in which undesirable stromal tissues would be more frequently inserted in the TMA block. Similarly, the inferior results (although not statistically significantly) of 3.0-mm TMAs compared with 2.0-mm TMAs could also represent a higher amount of stromal component punched together with neoplastic tissue, which was diminished when using 2.0-mm cores. The increased amount of stromal component in 3.0-mm TMAs is further supported by the increased vascular density seen with the CD34 reaction compared with that observed in 1.0- and 2.0-mm cores. Hence, the use of larger cores would be more appropriate circumstances where analysis of the stromal compartment would be the main object of study.

In determining how many cores would be adequate to represent the conventional whole section slides of PA, we showed that a single 2.0-mm core would offer results similar to those obtained with conventional whole section slides. However, because of the frequent loss of TMA cores after immunohistochemical procedures and the high morphologic heterogeneity of pleomorphic adenomas, it would be more advisable to use a second core for each case.

In this study, the authors evaluated the immunostaining of CK7 protein, known to be expressed in the luminal cells of ductal structures of normal and neoplastic tissues[23]; CD34, which has been shown to adequately illustrate the vascular component of PA[24]; the nuclear marker p63, which is related to myoepithelial differentiation[25]; and the proliferative marker Ki67, which indicates the proliferative potential of the tumor.[20]

In conclusion, despite the finding that one well-sampled 2.0-mm core could provide results similar to those of TMAs arranged in duplicate and triplicate, the authors suggest that, because of the possibility of tissue loss during additional laboratory procedures, 2.0-mm TMAs assembled in duplicate are a more desirable arrangement, representing a reliable, cost-effective, and high-throughput method for immunohistochemical study of PA.

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