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

Felipe Paiva Fonseca, DDS, MSc,[a] Bruno Augusto Benevenuto de Andrade, DDS, PhD,[a] Ana Lúcia Carrinho Ayrosa Rangel, DDS, PhD,[b] Ricardo Della Coletta, DDS, PhD,[a] Márcio Ajudarte Lopes, DDS, PhD,[a] Oslei Paes de Almeida, DDS, PhD,[a] and Pablo Agustin Vargas, DDS, PhD, FRCPath[a]
State University of Campinas, São Paulo, and State University of Western Paraná, Paraná, Brazil

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

This study was supported by grants from the São Paulo State Research Foundation (FAPESP-Brazil), process numbers 2009/53839-2, 2012/07519-9, and 2012/10781-7.
[a]Piracicaba Dental School, State University of Campinas, São Paulo, Brazil.
[b]Department of Pathology, State University of Western Paraná, Paraná, Brazil.
Received for publication Apr 2, 2013; returned for revision Aug 22, 2013; accepted for publication Aug 29, 2013.

2212-4403/$ - see front matter
http://dx.doi.org/10.1016/j.oooo.2013.08.029

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