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Improving discrimination in the grading of rat mammary tumors using two-dimensional mapping of histopathological observations

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ABSTRACT

This work aims at characterizing rat mammary tumors induced by 7,12-dimethylbenz(a)anthracene (DMBA) and the respective malignancy potential, commonly graded with histopathology features grouped by intensity levels. Tumors were described over fourteen multiple ranged microscopic parameters and a comprehensive characterization of the histological patterns and their relation with tumor grade was carried out by principal component analysis (PCA). The number of histological patterns present on a tumor tends to correlate with malignant features. High grade tumors are characterized by the presence of several structural patterns, with cribriform prevalence and necrosis. The cribriform pattern correlates with grading, i.e., tumors having a higher predominance of the cribriform pattern are likely to be more malignant. The findings may represent a benchmark for similar characterization studies in other models.

1. Introduction

Rat models have been accepted in oncobiology research for a long time due to characteristics comparable with human breast cancer, such as the degree of morphologic aggressiveness and tumor patterns (Currier et al., 2005; Russo and Russo, 1996, 2000; Withrow and Vail, 2007; Cardiff, 2001; Tavassoli and Devilee, 2003; Costa et al., 2002; Medina, 1976; Thompson and Singh, 2000; Cardiff and Munn, 1995; Cardiff et al., 2000; Russo et al., 1996; Rakha et al., 2010; Martins et al., 2008; Dias et al., 2001). The transposition between rat and human breast cancer is well documented. In these animals, the mammary gland shows age susceptibility for developing hormone dependent adenocarcinomas, which are very similar to those most frequently found in women (Costa et al., 2002; Russo and Russo, 2000). In humans and animals, over 90% of breast cancers are attributed to non-heriditary aetiology such as environmental carcinogen exposure and diet (Currier et al., 2005; Russo and Russo, 1996). In this context, rat models chemically induced by 7,12-dimethylbenz(a)anthracene (DMBA) are particularly useful for the study of breast cancer (Currier et al., 2005; Russo and Russo, 1996; Thompson and Singh, 2000; Russo et al., 1996). The exposure of terminal end-buds (TEBs) to DMBA causes unrepaired DNA damage, resulting on mutation, translocation, and inactivation of regulatory genes with long-lasting alterations in cell growth and in anti-apoptotic pathways leading to tumorigenesis (Currier et al., 2005; Russo and Russo, 1996; Thompson and Singh, 2000; Russo et al., 1996). Mammary tumors chemically induced by DMBA are heterogeneous and need to be described according to histological pattern or type, degree of differentiation reflected on histological grade and other morphological characteristics. The histological type is classified according to the presence of the most prevalent pattern of growth either on single or mixed pattern tumors (Costa et al., 2002). Histological grade is considered a valuable prognostic factor in breast cancer. The basis of this indicator relies on the degree of differentiation of the tumor tissue useful to predict the tumor malignancy (Cardiff et al., 2000; Rakha et al., 2010). The World Health Organization (WHO) recommends the Nottingham Grading System (NGS) as the grading system for human breast cancer (Tavassoli and Devilee, 2003). NGS is based on the evaluation of three morphological features: (1) degree of tubular or gland formation, (2) nuclear pleomorphism and (3) mitotic index. Nevertheless, there is an increasing need for more accurate prognostic and predictive markers (Webster et al., 2011; Meyer et al., 2005). In order to improve DMBA rat model for breast cancer investigation, some

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groups adapted grading parameters and correlated morphologic features also used in human breast pathology. The most relevant features are the structural pattern, percentage of cribriform pattern, gland secretion, inflammatory cell infiltration, hemorrhage, necrosis, stromal reaction and microcalcification, all possessing ranges of grading dependent on tumoral prevalence (Russo and Russo, 1996).

In human medicine, Breast Cancer Panels typically include ER/PR, a proliferation marker (Ki67, PCNA, or QDNA), Her-2, an angiogenesis marker (CD31, vegf, or factor VIII) and p53. The Annapolis Pathology Panel found that few model systems have been studied with immunohistochemistry (IHC), and even fewer have been characterized using the expected standard biomarker panel. As a result, direct comparison between human and mouse mammary tumors is still not possible (Cardiff, 2001). In this work, the prognostic features based on hematoxylin and eosin (HE) histopathology, a readily accessible method of tumoral evaluation, are maximized. The need to improve traditional histopathology has been complemented using multivariate analysis techniques (Cova et al., 2013; Madsen et al., 2010; Cheng et al., 2005; Méndez et al., 2009; Selaru et al., 2004). Clinical tools often produce large amounts of data, being multivariate in nature. Statistical, multivariate data analysis cannot be dissociated from most of the problems found in cancer studies (Cova et al., 2013). They form a commonly used set of methods for obtaining reliable results. Dimensionality reduction techniques based on variance, as principal component analysis (PCA), have been used for variable selection and data overview, which will reveal outliers, groups and trends.

In this work, a step forward on data analysis of histopathological and morphological parameters is presented. The analysis is carried out by employing a procedure for dimension reduction over a data set composed of routine mammary tumors chemically induced on Sprague Dawley (SD) virgin female rats. All parameters are defined by scores related to their intensity and are then further detailed and rationalized by PCA, which facilitates two- or three-dimensional representation. This approach enables to assess the relation between the histological features of each tumor and its grade. Additionally, inspection of the most discriminant parameters provides a detailed understanding of the tumors profile. An improvement in malignancy prediction, traditionally established by tumor grading, can be achieved if a significant relation is identified between histological parameters and grade. As a result, a better understanding of tumor characteristics enables a more accurate comparison between human breast tumors and those chemically induced in rat models, frequently used in cancer research.

2. Methods

Forty-eight outbreed Sprague Dawley (SD) virgin female rats were housed randomly into groups of three elements in each cage at a temperature of $23 \pm 2\,^{\circ}C$ and humidity of 50–55%, controlled facility on a 12 h light, 12 h dark cycle and fed a standard laboratory chow. Food and water were maintained *ad libitum*. All animals were weighted weekly. At 50 days of age, the animals were orally administered with 65 mg/kg of DMBA diluted in virgin olive oil (a maximum of one millilitre per animal). At 20 weeks after carcinogenic induction, tumors started to be detectable by mammary palpation and a weekly registration of the number, size and shape was carried out. At 27 weeks, all animals were ethically euthanized under anaesthesia. A full necropsy was performed and all tumors were excised, measured on three dimensions for volume calculation and weighed. The mammary tissue macroscopically free of tumors was collected for histopathologic evaluation.

2.1. Histopathologic evaluation

The Annapolis consensus (Cardiff et al., 2000) clarifies the classification of mammary tumors in genetically engineered mice, especially in what concerns to histological types. Russo adapted the Annapolis consensus to rat mammary tumors, increasing the field of compared pathology (Russo and Russo, 2000). In practical terms, the tissue fragments were fixed in 10% of formalin. 5 μm sections obtained from paraffin blocks were stained by HE for histologic examination. The rat tumors were graded using NGS in grade 1 (3–5 points), grade 2 (6–7 points) and grade 3 (8–9 points) (Tavassoli and Devilee, 2003). The tubular and gland formation were scored according to its predominance in the tumor as 1, if present in more than 75%, 2 if present in a moderate degree (10–75%) and 3 for lower predominance or absence (less than 10%). Nuclear pleomorphism was classified as 1, for small and regular cells, 2 for moderate size and shape variation, and 3 for a marked shape variation and mitotic counts at the periphery of the tumor over 10 consecutive high-power fields (HPF), with a field of view of 0.625 mm on a Nikon Eclipse E-600 microscope. In these conditions, the total mitotic counts of 0–11 have score 1, for 12–22 and over 23 have scores 2 and 3, respectively (Thompson and Singh, 2000). Similarly to human breast cancer histological evaluation, additional parameters were also assessed (Russo and Russo, 1996; Thompson and Singh, 2000). The structural pattern was classified and graduated according to the type of lesion: non-neoplastic (0), benign neoplastic (1), *in situ* malignant neoplastic (2) and invasive malignant neoplastic (3). Each histological type was identified and registered according to the most prevalent patterns: papillary, cribriform, glandular, tubular, comedo and squamous. The total number of patterns present in each tumor was registered. The proportion of cribriform pattern was identified as low (0–30%, score 1), medium (40–60%, score 2), or high (70–100%, score 3). Microcalcifications and stromal invasion were classified as 1, if present, and 0 otherwise. Secretion, necrosis and hemorrhage were sorted in absent (score 0), focal (10%, score 1), moderate (20–70%, score 2) and extensive (if >80%, score 3). Stromal reaction was classified as absent (0), mild (1), moderate (2) and high (3) according to collagen tissue within and surrounding the tumor. The same score system was repeated for apoptosis and inflammatory cell infiltration.

2.2. Data analysis procedure

Principal component analysis (PCA) is a simple, non-parametric procedure of extracting relevant information from data sets (Jolliffe, 2002). PCA computes a compact and optimal description of the data set, providing a roadmap to lay out a complex data set to a lower dimension and reveal the hidden, simplified structure that often is underlying. The most influential variables in the system are highlighted, and the most relevant factors may be identified. This technique is based on the assumption that most of the information about the structure of the data is contained in the directions along which the variations are the largest (Massart, 1988; Brereton, 1990; Jolliffe, 2002). In this work, PCA summarizes the information residing in the data corresponding to the histological parameters into a form which may be more easily inspected and used. The original multi-dimensional space, defined by those parameters, is contracted into a few descriptive dimensions, which represent the main variation in the data. Each principal component (PC) can be displayed graphically and analysed separately, and its meaning may often be interpreted according to simple histological descriptors. Essentially, the procedure is carried out by a linear transformation of the m histological parameters $\mathbf{x}_i$ into a new set, the principal components $\mathbf{u}_i$

$$\mathrm{u_i} = w_{i1}\mathbf{x}_1 + w_{i1}\mathbf{x}_2 + \cdots + w_{im}\mathbf{x}_m \quad (1)$$