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Finding an optimum immuno-histochemical feature set to distinguish benign phyllodes from fibroadenoma

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ABSTRACT

Purpose: Benign phyllodes and fibroadenoma are two well-known breast tumors with remarkable diagnostic ambiguity. The present study is aimed at determining an optimum set of immuno-histochemical features to distinguish them by analyzing important observations on expressions of important genes in fibro-glandular tissue.
Methods: Immuno-histochemically, the expressions of p63 and α-SMA in myoepithelial cells and collagen I, III and CD105 in stroma of tumors and their normal counterpart were studied. Semi-quantified features were analyzed primarily by ANOVA and ranked through *F*-scores for understanding relative importance of group of features in discriminating three classes followed by reduction in *F*-score arranged feature space dimension and application of inter-class Bhattacharyya distances to distinguish tumors with an optimum set of features.
Results: Among thirteen studied features except one all differed significantly in three study classes. *F*-Ranking of features revealed highest discriminative potential of collagen III (initial region). *F*-Score arranged feature space dimension and application of Bhattacharyya distance gave rise to a feature set of lower dimension which can discriminate benign phyllodes and fibroadenoma effectively.
Conclusions: The work definitely separated normal breast, fibroadenoma and benign phyllodes, through an optimal set of immuno-histochemical features which are not only useful to address diagnostic ambiguity of the tumors but also to spell about malignant potentiality.

1. Introduction

The benign phyllodes (PT) and fibroadenoma (FA), the fibro-epithelial breast tumors with differential malignant potentiality (i.e. ≤1% of FA and ≥43% of benign PT transform into malignancy) require specific discriminatory feature(s) especially to address their diagnostic ambiguities, encountered in clinical practices. PT accounts for 0.3–1.5% of breast tumors in the middle-aged women and approximately 2.5% of all fibro-epithelial breast tumors. PT arises from the intra-lobular stroma with an overgrowth of the connective tissue (Noguchi et al., 1995; Tavassoli, 1999). Whereas, FA commonly occurs as benign tumor in women of 20–35 years age group and comprises 75% of all benign breast tumors (Greenberg et al., 1998). It usually derives from glandular epithelium and is composed of dense epithelial and fibroblastic tissues.

The PT can be benign, borderline and malignant. The benign condition possesses overlapping histopathological features with benign FA. In general PT shows higher stromal cellularity, however, in some conditions FA also manifests similar stromal features or focal PT structures (fibroadenoma phyllodes) (Abdelkrima et al., 2010; Tavassoli, 1999). Generally, FA is of polyclonal origin but sometime, due to micro-environmental influence, it acquires monoclonal characteristic of PT (Noguchi et al., 1995), and this in addition to excessive stromal proliferation, sometimes create diagnostic bafflement. In the context of such diagnostic ambiguity, understanding differential malignant potentiality of these tumors may be a crucial. As it is obvious that differential malignant potentiality may reflect their differences, especially in the involvement

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of the vital genes and molecular pathways related to the structural and functional integrity of fibro-glandular structure of breast (Sawhney and Garrahan, 1992). Hence, this study evaluates the expressions of some prime molecules in the glandular (viz. myoepithelial cells) and stromal components of these tumors especially considering their important association with the tumor progression and malignant potentiality (Parmar and Cunha, 2004).

The myoepithelial cells (MECs) and epithelial cells maintain normal interactions with stroma and become impaired differentially during the evolution of different tumors (Parmar and Cunha, 2004; Sawhney and Garrahan, 1992). MECs provide important regulatory signals in the cross talk between glandular epithelium and stroma (Hsiao et al., 2011) and it has a significant role in the suppression of tumor (Deugnie et al., 2002; Gudjonsson et al., 2002). In understanding the functional integrity of MECs, evaluation of p63 and α-SMA expressions may be important (Adriance et al., 2005; Westfall and Pietenpol, 2004). p63 is a transcription factor and a homolog of tumor suppressor p53, and expresses in the nucleus (Ortega et al., 2001). This gene product is essential for breast development and in regulating epithelial proliferation and differentiation (Charles et al., 2002; Yang et al., 1999). But its absence promotes defective epidermal differentiation, as well as agenesis of mammary glands. Abnormal p63 expression may result structural defects in breast and the components become vulnerable to external or internal assaults and carcinogenesis is favored (Hsiao et al., 2011). While, α-SMA is a highly conserved gene (Mills et al., 1999) with vital roles in cell motility and contractility with implications on cellular and nuclear mechano-transduction including nuclear transport (Ueyama et al., 1990). The altered expressions of p63 and α-SMA are also considered to be associated with the altered cross talk between glandular epithelium and stroma (Charles et al., 2002; Mills et al., 1999; Ortega et al., 2001; Storch et al., 2007; Ueyama et al., 1990; Westfall and Pietenpol, 2004; Yang et al., 1999).

In assessing stromal status, evaluation of collagen I (Col I) and collagen III (Col III) in the stroma may be important as they influence the biophysical properties including stiffness of extra-cellular matrix (ECM), normal duct formation and signaling to the adjacent epithelium (Bissell and Radisky, 2001; Geyp et al., 1999; Keely et al., 1995). The increase in collagen density may promote tumorigenesis, local invasion, and metastasis, causally linking increased stromal collagen with tumor formation and progression (Bissell and Radisky, 2001). Further in the identification of tumoric micro-vessels, study on CD105 (i.e. endoglin), a proliferation associated and hypoxia-inducible protein expression is also important (Duff et al., 2003). There is report indicating correlation between increase in micro-vessels density and transformation of benign tumor into malignant one (Gary et al., 2003). Further, immuno-histochemical observations on definite molecular expressions are semi-quantified using appropriate visual analog scales and automated analytical tools (Das et al., 2010; Maity et al., 2010). However, such data need to be assessed with suitable statistical measures to identify the features with sufficient significance in separation of disease classes. In this approach *F*-ranking and use of interclass statistical distance measure such as Bhattacharyya distance function are effective (Goutte and Gaussier, 2005; Pai et al., 2010). Efficacy of Bhattacharyya distance function lies on its scale independence and embodiment of variance–covariance matrix (McLachlan, 1999). Such kind of distances is used in cluster analysis and classification methods for pathological decision making (Comaniciu et al., 1999; Bonetti and Pagano, 2005).

In the light of the aforementioned facts, present study uses *F*-scores and Bhattacharyya distance function to find an optimal feature set of reduced dimension in statistical evaluation of the immuno-histochemical observations on benign PT and FA for drawing effective diagnostic distinction.

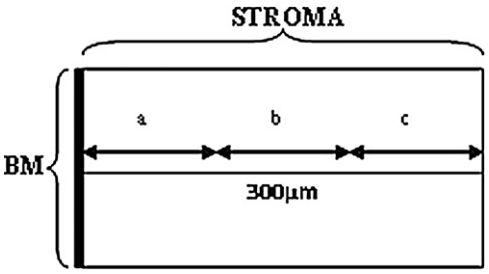

Fig. 1. Schematic diagram showing the methodology used to generate color intensity value on [using 10 point (i.e. 0–10) intensity scoring scale] Col-I and III expressions in three equidistant zones (i.e. $a = b = c = 100\,\mu m$) along the 300 μm outer distance from ducto-glandular BM (basement membrane).

2. Materials and methods

In this study biopsy samples from FA [($n = 33$), age-group 20–45years] benign PT [($n = 10$), age-group 45–55] and normal [($n = 6$), collected from the unaffected region of FA breast during surgical intervention] were collected under the informed written consent of patients and ethical clearance from Medical College, Kolkata, West Bengal, India

2.1. Routine histopathological and immuno-histochemical (IHC) studies

The 10% phosphate buffered formalin fixed biopsies, embedded in paraffin and 4 μm thick sections were put on poly-L-lysine (P8920, Sigma–Aldrich, St. Louis, USA) coated glass slides. Initially H and E sections were studied histo-pathologically. In IHC studies for p63, α-SMA, Col I, Col III and CD105 standard protocols were adopted (BioGenex, San Ramon, CA, USA). Tissue sections were baked (60 °C) and de-paraffinized then hydrated for antigen retrieval in 10 mM citrate buffer (pH 6.0) using EZ-Retriever System V.2 (BioGenex, San Ramon, CA, USA) and immunostained with kit (i.e., Super Sensitive Polymer-HRP IHC Detection System, Cat. No. QD400-60K BioGenex). Further sections were incubated overnight at room temperature with primary antibodies (P63, clone 4A4, Cat. No. AM418-5M, α-SMA clone 1A4, Cat. No. AM128-5M, Col III, clone HWD1.1, Cat. No. AM167-5M, CD105, clone 4G11, Cat. No. AM441-5M, BioGenex, USA and Col-I, polyclonal, Cat. No. ab34710, Abcam, Cambridge, UK). Primary binding of the antibody was visualized by HRP conjugated secondary antibody, using the chromogen 3,3′-diaminobenzidine (DAB) and counterstained with hematoxylin. Then sections were dehydrated and mounted in DPX (Maity et al., 2010).

2.2. Microscopic imaging

The microscopic images were captured digitally (1388 × 1040 pixels) by Zeiss Observer.Z1 Microscope (Carl Zeiss, Germany) interfaced with CCD camera (AxioCam MRC, Zeiss) under 10× (NA 0.25) and 40× oil (NA 1.3) objectives with their respective resolutions being 0.63 μm and 0.16 μm.

2.3. Semi-quantization of IHC observations

Under the guidance of onco-pathologist, p63 positive nuclei of MECs (40× images) were hand segmented and nuclear area was measured (Axiovision software, version 4.7.2, Carl Zeiss, Germany). The p63 and α-SMA expression intensities were analyzed by 0–10 point intensity scoring scale. MECs and epithelial cells were also counted. In assessing Col I and III expressions along the 300 μm stromal zone in three equidistant regions i.e. *a*, *b*, and *c* (Fig. 1) the Axiovision software and 10 point intensity scoring scale were used.