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Transcripts of cytokeratins as predictors of breast cancer

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ABSTRACT

Cytokeratins (CK) belong to a large family of intermediate filaments that are primarily expressed in epithelial cells and whose members are expressed in various combinations in normal and malignant entities. In breast cancer (BC), keratin typing is a major tool in tumor histodiagnosis, providing molecular parameters to assess its differentiation status. In our study, we evaluated *CK5*, *CK6*, *CK8*, *CK14* and *CK18* mRNA levels by qPCR, and *CK18*, *CK5* and *CK14* transcripts were effective to differentiate breast tumors from benign diseases. Relative quantification of *CK18* mRNA levels was 2.08-fold higher in benign breast disease than in BC. The same expression pattern was observed for *CK5* and *CK14*, which were 2.4 and 4.8-fold higher in nonmalignant tissues, and *CK18* correlated with *CK5*, *CK6*, *CK8* and *CK14* mRNA expression. Cytokeratins 8/18 and 5/14 presented the same pattern according to molecular subtypes. However, *CK18* itself correlated to clinical outcome in tumor phenotyping. We suggest that the mechanisms underlying the increased expression of cytokeratins in breast tumors may be similar, and *CK18*, *CK5* and *CK14* transcripts may be important in the tumorigenic process, distinguishing normal from tumor cells.

1. Introduction

Several efforts have been devoted to identify the molecular abnormalities contributing to breast cancer (BC) development and progress (Nzeangung et al., 2018). This malignant tumor is no longer seen as a single entity and patients with the same clinicopathological parameters can have markedly different outcomes (Li et al., 2017). Gene expression arrays have been able to genetically profile breast cancer into four distinct molecular subtypes: basal-like, luminal A and B, and erbB2/human epidermal growth factor receptor-2 (HER-2) oncogene status. Importantly, this molecular taxonomy has critical clinical value because some of these phenotypes show unfavorable prognosis and/or resistance to treatment. However, several factors are intertwined during malignant transformation and tumor development (DeSantis et al., 2016; Apuri, 2017). For this reason, the search for molecular changes that may affect the biology of cancer development and progression may be important for understanding this disease and improving clinical management of BC patients (Wu and Ma, 2017).

Distinct keratins emerge as highly dynamic scaffolds involved in different settings and contribute to cell size determination, translation control, proliferation, cell type-specific organelle transport and malignant transformation (Liu et al., 2018). In the mammary gland, cytokeratins (CK) expression is tightly regulated and correlates with the origin of the cells in the ducts (Wang and Yang, 2017). In the bilaminar breast epithelium, CK8 and CK18 characterize the differentiation compartment, whereas CK5 and CK14 are expressed in the proliferation compartment (Buhler and Schaller, 2005; Otsuka et al., 2018). Expression of CK7, CK17 and CK19 is variable but generally low (Chu et al., 2005; Awadelkarim et al., 2011; Adamo et al., 2017; Rakha et al., 2017). All of the CKs share the same domain structure and form heteropolymers (Linder et al., 2004), which have been recognized for > 20 years as epithelial markers in diagnostic histopathology, where basal-like cells express CK5, 6 and 14 and/or 17 (basal/myoepithelial phenotype) and luminal-like cells express CK8,18 and 19 (Zeichner et al., 2016; Bystricky et al., 2017; Patel and DeMichele, 2017). A key observation of many studies is that keratin expression changes rapidly

Abbreviations: CK, cytokeratins; BC, breast cancer; HER-2, human epidermal growth factor receptor-2; RT-PCR, reverse transcription followed by polymerase chain reaction; BBD, benign breast diseases; TNM, *Classification* of Malignant Tumors; ER, estrogen receptor; SBR, Scarff-Bloom-Richardson; MMLV-RT, Murine Moloney Leukemia Virus Reverse Transcriptase; B2M, β2-microglubuin; PR, progesterone receptor; TNBC, triple-negative breast cancer; UFU, Federal University of Uberlandia

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Table 1
Oligonucleotide sequences used for the multiple mRNA markers studied.

Primer	GenBank acess	Sequence (forward/reverse)	Nucleotide position	Annealing temperature (°C)	Amplified product
KRT5	NM_000424	5′-AGATGTTCTTTGATGCGGAGC-3′ 5′-CTGCGGTTGGCAATCTCCT-3′	1074–1094 1203–1221	60	148 bp
KRT6	NM_005554	5′-AGGGTGAGGAGTGCAGGCT-3′ 5′-CCAAGACCACTGCCATAGGAG-3′	1618–1636 1754–1774	54	157 bp
KRT8	NM_002273	5′-AGCTGGAGTCTCGCCTGGA-3′ 5′-CAGCTCCCGGATCTCCTCT-3′	729–747 790–808	60	80 bp
KRT14	NM_000526	5′-ATTGAGGACCTGAGGAACAAGATT-3′ 5′-CGCAGGTTCAACTCTGTCTCAT-3′	566–589 672–693	60	128 bp
KRT18	NM_000224.2	5′-GCTCTGGGTTGACCGTGG-3′ 5′-GTGGTGCTCTCCTCAATCTGC-3′	804–821 934–954	58	151 bp
B2M	NM_004048	5′-CCTGCCGTGTGAACCATGT-3′ 5′-GCGGCATCTTCAAACCTCC-3′	356–374 449–431	54–60	94 bp

during differentiation, tissue injury and metastasis (Buhler and Schaller, 2005; Chu et al., 2005; Magin et al., 2007; Rakha et al., 2017; Wang and Yang, 2017; Liu et al., 2018; Otsuka et al., 2018).

Breast tumorigenesis involves altered expression of proteins and transcripts, which can play an important role in cancer progression. The different biological behaviors and metastatic patterns observed among the distinct breast cancer phenotypes may suggest different mechanisms of invasion and metastasis for breast tumors.

It is becoming evident that a cell changes its expression patterns while it is progressing from a normal to an invasive panel and tumor growth is not just a result of uncontrolled proliferation but also of reduced apoptosis, which involves regulation of cytokeratins. Therefore, elucidating new molecular associations may provide clues to novel diagnostic, prognostic and therapeutic approaches to treat BC (Deng et al., 2017; Patel and DeMichele, 2017).

Due to the heterogeneous, multifactorial and multifocal nature of breast cancer, the search for potential biomarkers and their molecular associations involved in the occurrence and development of this disease is fundamental for more precise diagnosis and to clarify the neoplastic phenotype. Considering that most cancers also change keratins profile, we aimed to clarify the relevance of cytokeratins 5, 6, 8, 14 and 18 mRNA in human BC, assessed by quantitative RT-PCR, demonstrating a possible molecular pathway that is associated with BC tumor occurrence and characterization.

2. Materials and methods

2.1. Study design and sample collection

This project was carried out from 2010 to 2011 at the Nanobiotechnology Laboratory of the Federal University of Uberlandia (UFU) together with the Obstetric Service of University Hospital. The study protocol was approved under the number 176/2008 by the local Research Ethics Committee in accordance with the Helsinki Declaration of 1975, as revised in 2008, and informed consent was obtained from all participants.

The breast samples encompassed materials from 80 patients grouped in two classes: 40 BC and 40 benign breast diseases (BBD). Classification of patients was made according to clinical parameters. The average age of the patients investigated was 46.2 years (range 30–80 years) for the BC group and 46.8 years (range 18–58 years) for the BBD group. There were 15 (37.5%), 18 (45.0%), 3 (7.5%) and 4 (10.0%) breast tumors classified as Tumor Node Metastasis (TNM) stages T1, T2, T3 and T4, respectively. The histological gradings according to the Nottingham system were grade I (GI) in 4 (10%), GII in 22 (55%) and GIII in 14 (35%).

For hormone receptors, estrogen receptor (ER) was positive in 29 (72.5%), negative in 6 (15.0%) and not evaluated in 5 (12.5%); progesterone receptor (PgR) was positive in 23 (57.5%), negative in 12 (30.0%) and not analyzed in 5 (12.5%). HER2 status was considered as positive (score 3+) and negative (score 0–1+); scores of 2+ were excluded from the analyses. HER-2 status was positive in 9 cases (22.5%), negative in 25 (62.5%) and score 2+ or not analyzed in the other 6 (15.0%).

In BC cases, surgical procedures were radical mastectomy and quadrantectomy, depending on the size of the tumor, with axillary dissection and were collected from untreated patients. The mRNA analysis was carried out on fresh breast samples containing 50–80% malignant portion. BC patients were characterized by their TNM staging, Scarff-Bloom-Richardson (SBR) grading, lymph node, hormone status and Her2.

2.2. Quantitative RT-PCR

The mRNA was extracted in duplicate from fresh tumor and BBD breast tissues of each patient using the *Trizol* reagent (Invitrogen - Carlsbad, CA, USA) according to the manufacturer's recommendations. RNA extractions were carried out as described elsewhere (Neves et al., 2008).

Synthesis of cDNA was performed from 1 μg of total mRNA at a total volume of 20 μL containing 2 U of *Murine Moloney Leukemia Virus* Reverse Transcriptase (MMLV-RT), 1X MMLV-RT Buffer, 0.1 M DTT, 1 U of RNase inhibitor, 200 μM of each dNTP (deoxyribonucleotide) and 6 μM hexamer random primers (Invitrogen - Carlsbad, CA, USA). The reactions were incubated at 37 °C for 1 h and heated at 95 °C for 5 min. For normalization of amplification reactions, the constitutive β2-microglubuin (*B2M*) gene (Table 1) was chosen as an internal positive control gene, which was also used to validate reactions and to further characterize RNA quality of each sample.

qPCR for *CK18*, *CK5*, *CK6*, *CK8*, *CK14* and *B2M* genes was performed using the ABI PRISM 7300 Sequence Detection System (Applied Biosystems - Carlsbad, CA, USA). Sequences of primers used as well as annealing and detection temperatures are presented in Table 1. All primers were designed to not amplify genomic DNA (usually one is positioned on an exon-exon junction). The PCR was conducted in a total volume of 10 μL containing Power SYBR_ Green PCR Master Mix (Applied Biosystems - Carlsbad, CA, USA), 2 μL cDNA (1:4) and 5 μM of each primer. Dissociation and standard curves for all primers were constructed. PCR efficiency (E) was calculated according to the equation: $E = (10^{-1/slope} - 1) \times 100$ to validate the relative quantification based on comparative CT method. To compare mRNA levels of target genes between BBD patients and BC patients, one BBD tissue sample was chosen as the calibrator sample to which all other were compared.

2.3. Data analysis

The non-parametric Mann-Whitney test was used for mean comparisons of relative mRNA expression of the *CK18*, *CK5*, *CK6*, *CK8* and *CK14* transcripts between BC and BBD patients. Spearman's correlation analysis was performed with all clinical parameters: patient age at