Annals of Diagnostic Pathology 39 (2019) 30–35

Contents lists available at ScienceDirect

Annals of Diagnostic Pathology

journal homepage: www.elsevier.com/locate/anndiagpath

Original Contribution

Mutation profiling in the *PIK3CA*, *TP53*, and *CDKN2A* genes in circulating free DNA and impalpable breast lesions

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ARTICLE INFO

Keywords:
Impalpable breast lesions
cfDNA
PIK3CA
TP53
CDKN2A

ABSTRACT

Breast impalpable lesions have become a clinical dilemma because they are small, presenting a heterogeneous cellular phenotype. The aim of this study was to evaluate the mutational profile of the *PIK3CA*, *TP53*, and *CDKN2A* genes, comparing the mammary tissue with the respective circulating free DNA (cfDNA). The *PIK3CA*, *TP53*, and *CDKN2A* genes were sequenced (PCR-Sanger) in 58 women with impalpable lesions (49 malignant and 9 benign) with the respective cfDNA. The chi-square or Fisher's exact test was used to evaluate statistical significance between the clinical variables and mutational profile. A total of 51 out of 58 samples generated successful mutation profiles in both breast lesion and cfDNA. Of the 37 mutations detected, 10 (27%) and 16 (43%) mutations were detected in benign and malignant breast lesions, respectively, while 2 (5%) and 9 (24%) were found in cfDNA of women with benign and malignant lesions, respectively. The lymph node involvement with mutations in the *PIK3CA* in malignant lesions ($P = 0.001$), and the relationship between mutations in *PIK3CA*, comparing ductal tumors with benign lesions ($P = 0.05$), were statistically significant. This study detected different mutations in *PIK3CA*, *TP53*, and *CDKN2A* genes, which represent, in part, the heterogeneity of impalpable lesions. The results confirm that more studies should be conducted on the functional role of cfDNA in the impalpable lesions.

1. Introduction

The amplification and dissemination of the use of imaging tests for breast cancer screening has increased the number of cases diagnosed with latent and non-palpable (occult) breast lesions [1]. On the other hand, when these lesions are diagnosed, they raise doubts as to the management to be adopted, since they may not be malignant [2-5]. For example, many of these lesions are repeatedly biopsied to define their evolution. In the same way, although these lesions are small, this factor does not limit the potential of metastases (axillar or long distance), leading to the presence of circulating genetic material [6-8]. In this scenario, the liquid biopsy has emerged as a hope for description of the lesion.

The first description of circulating free DNA (cfDNA) was made > 60 years ago [9]. Since then, with the technology advancement, the origin of circulating nucleic acids has been unraveled. Initially, levels of this material were described in the bloodstream as fluctuating in adverse conditions such as pregnancy [10], intense physical activity [11], immunological diseases [12], and tumors in general [13,14].

The cfDNA presence in the bloodstream of healthy individuals originates predominantly from nucleated apoptotic cells. In malignant tumor cases, the cfDNA mixture is more heterogeneous, and may be composed of DNA from the apoptotic and necrotic cells released from the tumor microenvironment [15,16]. The amount of the circulating free tumor DNA (ctDNA) present in the cfDNA mixture may vary. The tumor microenvironment is composed of multiple phenotypes and cell clones; thus, the alterations found in the tumor cannot be easily

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https://doi.org/10.1016/j.anndiagpath.2018.12.008

detected in the cfDNA. During tumor evolution, tumor cells may spread from the primary site and when present in the bloodstream will depend on cell degradation and physiological events, such as lymphatic circulation [16] and errors in the immune response [17].

The specific somatic mutation identification for certain tumor types has generated clues to alterations that can be investigated in the cfDNA, responsible for the metastatic site development [18] and resistance to treatment [19]. For breast cancer, the cfDNA role is controversial, but with the introduction of next-generation sequencing (NGS), a mutated gene list has been revealed, including *ESR1* [20-22], *PIK3CA* [18,21], *TP53* [18], *PTEN*, *MYC*, and *CDKN2A* [22]. Many of these genes have been investigated in the cfDNA of breast cancer patients and have shown relevance in the response monitoring treatment [23] and in the risk to metastasis progression [24].

The *PIK3CA* gene is an oncogene responsible for the coding of the p110 kDA subunit, a phosphatidylinositol 3-kinase (PI3K) component, which is responsible for activated protein tyrosine kinases. *PIK3CA* has been described as mutated in breast cancer, with rates ranging from 20 to 40% and 28–43% in tumors and cfDNA, respectively [21,24]. Mutations in *PIK3CA* have been described especially in tumors with estrogen receptor-positive (ER+) [21,24].

The *TP53* gene is a tumor suppressor, acting directly on genome integrity, controlling the cell cycle [25]. It is considered the most mutated gene in sporadic mammary tumors, ranging from 23%–70%, depending on the mammary tumor subtype [26,27]. The mutational rates in cfDNA are similar to those found in the tumor [18,28].

Another gene, *CDKN2A* is responsible for the encoding of p14 (ARF) and p16 (INK4) proteins that will act directly on two pathways responsible for cell cycle control (*TP53* and *RB1*) [29]. It is described as mutated in > 2% of the breast tumors, and is associated with metastatic tumors [30,31] and with the triple negative subtype [31].

In this study, we evaluated the mutational profile of the *PIK3CA*, *TP53*, and *CDKN2A* genes, comparing the mammary tissue (benign and malignant) with the respective cfDNA, to test if they would match. All the cases included in this study were impalpable breast lesions, classified as Breast Imaging Reporting and Data System (Birads) 3 and 4.

2. Subjects and methods

2.1. Study population

Patients who underwent breast cancer screening in the years 2015 to 2016 at the Americas Barra Medical City Clinics in the city of Rio de Janeiro, Brazil, with radiologic diagnosis Birads 3 and 4 were invited to participate in the study. All lesions were selected without any selection bias, being a blind study. The diagnosis of the impalpable lesion was radiological by mammography, and when undetermined the Birads classification, complemented by ultrasonography and/or magnetic resonance.

The study was conducted according to the guidelines of the Declaration of Helsinki and all patients provided written informed consent. This study was approved by the ethics committee of Rio de Janeiro State University Hospital, number 43560115.5.0000.5259. Data on age, tumor classification, grade, size, nodal involvement, and immunohistochemical profile were obtained from histopathological and medical reports. Histologic classification was graded according to current World Health Organization criteria [32], and nuclear grade was defined as grades I to III according to Elston and Ellis [33]. All diagnostic reviews were made by three pathologist physicians (SDOR, CMDN, ASB).

2.2. Methods

2.2.1. Breast lesion and plasma DNA extractions

Ten mL of blood was collected in EDTA before surgery, and centrifuged at room temperature for 10 min at 2000*g*. Supernatants were centrifuged at 16,000*g* for 10 min at 20 °C to remove debris. Plasma was harvested and stored at −80 °C. When DNA was to be analyzed, 2 mL was used to obtain cfDNA using the QIAamp® Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. The DNA from the tumor tissue was extracted from the formalin-fixed paraffin-embedded (FFPE) samples, using the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol.

For verification of the quality and integrity, the DNA from tissue and plasma samples were quantified with Qubit dsDNA HS Assay Kit (Invitrogen) according to the manufacturer's protocol.

2.2.2. DNA sequencing

The polymerase chain reaction (PCR) was performed in a 25 μL reaction mixture containing 1–10 ng of DNA (breast lesion DNA or cfDNA), STR 1× buffer (Invitrogen, Carlsbad, USA); 200 mM dNTPs (Invitrogen, Carlsbad, USA); 3 mM of $MgCl_2$ (50 mM) (Invitrogen, Carlsbad, USA); primer pairs for each region to be amplified (10 pmol/uL each); and 0.5 U Platinum™ *Taq* DNA Polymerase High Fidelity (Invitrogen, Carlsbad, USA) in a final volume of 25 μL. The primers used for *PIK3CA* (exon 9 and 20) and *TP53* (exon 5–8) amplification have been previously described [34,35]. For exon 4 of the *TP53* gene, the primers described by Fernandez et al. [36] were used, due to the limited cfDNA amplification. For amplification of exon 9 of the *TP53* gene the following sequences, forward 5′CCAAGGGTGCAGTTATGCCT3′ and reverse 5′AAAGTTTCCAGTCTCAATCA3′, were used. The primers used for *CDKN2A* amplification (exon 1, 2a, and 3) have been previously described [37,38]. Exon 2 was fragmented into two parts, “a” [37] and “b” [38], due to its long size.

PCR assays were performed in the Veriti™ DX thermal cycler. The PCR program consisted of a pre-denaturation at 94 °C for the first 10 min, followed by 35 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 45 s. The final extension was performed at 72 °C for 10 min.

The PCR products were purified using the GFX™ DNA and Gel Band Purification kit (cat. no. 28903470, GE Healthcare Life Sciences, Chalfont, UK) and QIAquick PCR Purification Kit (cat. no. 28104, Qiagen, Hilden, Germany). Subsequent to purification, sequences were loaded onto an ABI 3730XL DNA Analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc.). Comparisons were made between the reference sequences of genes *PIK3CA*, *TP53*, and *CDKN2A* (accession nos. NM_006218.3, NC_000017–10, and NM_000077.4/NM_058197.4, respectively; GenBank) using the sample sequences obtained by sequencing. All samples were sequenced in duplicate. This comparison was performed using the Sequencher version 5.4.6 program (Gene Codes, Ann Arbor, MI, USA).

2.2.3. Statistical analysis

A contingency table was used to associate the mutational profile of each gene evaluated with age, histological grade, infiltration and lymph node involvement, Ki67 proliferation, and molecular classification subtype. Fisher's or Pearson's chi-square test was used to test the statistical significance of the association between such variables. The data were processed in the statistical program Predictive Analytics Software (PASW 18). A level of significance of 5% was considered in all the statistical tests used. Statistically significant data were considered as those whose P value was < 0.05.

3. Results

3.1. Social and clinical data

Following surgery, the histopathological diagnosis revealed 49/58 (84%) malignant lesions and 9/58 (16%) benign lesions. Malignant lesions were 55% IDC, 84% ER-positive, 67% PR-positive, 70% HER2-negative, and 65% Luminal A subtype (Table 1).

The patients with malignant breast lesions ranged in age from 33 to 90 years (mean 61 y, SD 11.42), while the patients with benign breast lesions ranged in age from 27 to 55 (mean 46 y, SD 7.76). The social demografic profile and clinical data of the cases are shown in Table 1.

Table 1
Social demografic and clinical data of the cases available.

	Patients	%
	No = 58	
Characteristic		
Age, years		
Mean	59	
SD	12.67	
≤50	17	29%
> 50	39	67%
Not declared	2	4%
Malignant lesions	*N* = 49	
IDC	32	55%
DCIS	3	5%
IDC-DCIS	5	9%
IDC + ILC	1	2%
ILC	2	3%
LCIS	2	3%
ILC/LCIS	1	2%
Micropapillary carcinoma	3	5%
Benign lesions	*N* = 9	
Fibroadenoma	1	11%
Intraductal Papillomas	2	22%
Ductal ectasia and apocrine metaplasia	4	45%
Hyperplasia of columnar cells with and without atypia	2	22%
Nuclear grade	*N* = 49	
I or II	39	80%
III	10	20%
TNM/stage	*N* = 49	
T1N0M0 (stage I)	38	78%
T1N1M0 (stage IIa)	6	12%
TisN0M0 (stage 0)	4	8%
TisN1M0 (stage IIa)	1	2
ER status (malignant lesions)	*N* = 49	
Positive	41	84%
Negative	2	4%
Unknown	6	12%
PR Status		
Positive	33	67%
Negative	10	21%
Unknown	6	12%
HER status		
Positive	7	14%
Negative	34	70%
Unknown	8	16%
Ki 67		
Low (< 20%)	21	43%
Intermediate/high (≥20%)	11	22%
Unknown	17	35%
BC subtype		
Luminal A	32	65%
Luminal B	9	19%
Triple negative	2	4%
Unknown	6	12%

ER = estrogen receptor; PR = progesterone receptor; HER = Human Epidermal Receptor; IDC = infiltrative ductal carcinoma; DCIS = ductal carcinoma in situ; ILC = infiltrative lobular carcinoma; LCIS = lobular carcinoma in situ.

3.2. Mutational profile of mammary DNA and cfDNA

Our study evaluated the mutational profile of the *PIK3CA*, *TP53*, and *CDK2NA* genes in pairs, comparing tumor tissue and the cfDNA in a total of 51 samples. Remaining cases were not possible to evaluate in pairs because of the limited amount of FFPE material, or cfDNA degradation. No concordance in mutations was found in the paired samples.

A total of 37 mutations were found overall, being 8/58 (14%), 18/58 (31%), and 11/58 (19%) for the *PIK3CA*, *TP53*, and *CDKN2A* genes, respectively. The distribution of mutations per gene and specimen evaluated is shown in Table 2.

Among the 8 mutations found in the *PIK3CA* gene, 4/8 (50%), 3/8 (37.5%), and 1/8 (12.5%) were synonymous, missense, and frameshift, respectively. Four of these mutations have been described in the Catalogue of Somatic Mutations in Cancer (COSMIC) database. The mutation distribution per assessed tissue and the nomenclature are shown in Table 3.

For the *TP53* gene, 18 mutations were detected: 9/18 (50%), synonymous; 7/18 (39%), missense; 1/18 (5.5%), stop codon; and 1/18 (5.5%), frameshift. Some synonymous mutations were repeated, with 3/9 (33%), p.G279G; 3/9 (33%), p.Q100Q; and 2/9 (22%), p.R249R. These positions correspond to the hotspot regions. Of the 18 mutations detected, 10 mutations have been described in COSMIC and 15 cataloged in the database of the International Agency for Research on Cancer (IARC). Mutation details, as well as nomenclature, are shown in Table 4. The polymorphisms detected in the samples evaluated for *TP53*, excluding codon 72 polymorphism, can be seen in Supplementary Table 1.

In the *CDKN2A* gene, 11 mutations were detected: 1/12 (8%), synonymous; 8/12 (67%), missense; 1/12 (8%), stop codon; and 1/12 (8%), frameshift. One mutation has been described in COSMIC. The polymorphism p.V28 L was detected in one case and is detailed along with the mutations in Table 5.

The lymph node involvement and mutations in the *PIK3CA* for malignant lesions ($P = 0.001$), and the relationship between mutations in *PIK3CA*, comparing ductal tumors with benign lesions ($P = 0.05$), were statistically significant. The other statistical analysis showed no significant association between mutational findings and correlated clinical pathological variables. The significant *P* value found was above 0.05 (*P* values for each variable are shown in Table 6).

4. Discussion

The screening program expansion and the imaging technology advancement have allowed the detection of early stage breast cancers. In this context, impalpable lesions have become a challenge for the medical field because they are small, and when biopsied represent an unstable tumor phenotype, where lesions in situ and malignant share the same microenvironment [39]. For this reason, many of these lesions are re-biopsied for the tumor cell phenotype confirmation. The discovery of circulating biomarkers would avoid complementary and invasive examinations, in addition to assessing the potential for metastasis and the tumor ability to acquire resistance to treatment.

The data presented here requires careful interpretation. In general, the *TP53* gene was the most mutated, but when observed in the distribution data, the genes showed relatively close mutational rates (see Table 2). The relationship between the mutations and the specimens evaluated were not significant, except for the *PIK3CA* gene in malignant lesions ($P = 0.05$). The mutational frequencies in this study for cfDNA tended to increase in malignant tumors (see Results), but this statement

Table 2
Mutations detected in each gene.

	Benign lesions (*N* = 9) (%)			Malignant lesions (*N* = 49) (%)		
	PIK3CA	*TP53*	*CDKN2A*	*PIK3CA*	*TP53*	*CDKN2A*
Breast lesion	4/9 (44%)	3/9 (33%)	3/9 (33%)	3/49 (6%)	7/49 (14%)	6/49 (12%)
cfDNA	0/9 (0%)	1/9 (11%)	1/9 (11%)	1/49 (2%)	7/49 (14%)	1/49 (2%)

Table 3
Mutations found in the *PIK3CA* gene in breast lesions and cfDNA.

Sample type	Histopathologic grade	TNM	IHC ER/PR/HER	Ki67(%)	Lymph node involvement	Gene *PIK3CA* (Exon)	Codon	Protein	Cosmic
4 – BL	DE/AM	–	–	–	–	9	c.1700A > G	p.N515D	1716810
6 – BL	DE/AM	–	–	–	–	9	c.1700A > G	p.N515D	1716810
16-BL	HCWA	–	–	–	–	20	c.3114A > G	p.K986R	–
							c.3250 T > C	p.T1031 T	
3 – BL	IDC grade II	T1N1M0	NI	NI	Yes	20	c.3313A > G	p.T1052 T	4778879
12 – BL	IDC grade II	T1N1M0	+/+/−	10%	Yes	20	c.3250 T > C	p.T1031 T	–
21 – BL	DCIS grade I	TisN0M0	NI	NI	No	9	c.1702_1703insA	p.R1_K1inA	–
17 – cfDNA	IDC grade II	T1N0M0	+/+/+	20%	No	20	c.3232C > T	p.T1025 T	21451[a]
47 – cfDNA	IDC grade I	T1N0M0	+/+/−	5%	No	9	c.1808C > T	p.L551 L	308546

BL = breast lesion; cfDNA = circulating free DNA; DE = Ductal ectasia; AM = apocrine metaplasia; HCWA = Hyperplasia of columnar cells with and without atypia; IHC = immunohistochemistry; COSMIC = Catalogue Of Somatic Mutations In Cancer; ER = estrogen receptor; PR = progesterone receptor; HER = Human Epidermal Receptor; IDC = infiltrative ductal carcinoma; ILC = infiltrative lobular carcinoma; NI = not informative.
[a] SNP (single nucleotide polymorphism) reference rs1784907.

is possible whether applied to a larger study or a different cohort.

The *PIK3CA* gene evaluation in cfDNA from ER+ breast cancer patients, initial ($N = 17$) and metastatic ($N = 69$), revealed that 25% of MBCs (metastatic breast cancers) were mutated [13]. With regard to impalpable lesions, we detected a much lower rate (6%). When cfDNA was evaluated in pairs with metastatic biopsies from 18 advance-stage tumors (breast, ovary, lung, anal, endometrium, gastric, esophagus, pancreas etc.), 28 mutations (97%) were identical. Of the five mammary tumors available in this study, one case treated with paclitaxel presented the p.E545K mutation in *PIK3CA* [18]. Interestingly, in another study, the same mutation was detected in the cfDNA in one of the mammary tumors also treated by paclitaxel [22].

The data presented here for the *PIK3CA* gene do not match mutations between tumor and cfDNA; two mutations (p.N515D and p.T1031T) were found in more than one tumor tissue, confirming the mutational hotspot regions of the gene. In relation to Brazilian data, Mangone et al. [34] found the *PIK3CA* gene mutated in 27% ($N = 23/86$) of the initial mammary tumors. The rates presented here were lower for malignant tumors (6%) and higher for benign lesions (33%).

The relationships between mutations in the *PIK3CA* gene with lymph node involvement ($P = 0.001$) and ductal subtype ($P = 0.05$) were significant in our study. It is known that mutation presence in exon 20 of the *PIK3CA* gene has a relation with worse survival ($P = 0.026$) and disease-free survival (DSF) ($P = 0.079$) [34], but more studies should be conducted to confirm this association.

The *TP53* gene showed a mutation rate of 14% for cases with malignant lesions (breast lesion and cfDNA). This same frequency was reported by Kim et al. [40] in breast tumors of South Korean women with pathological characteristics similar to those evaluated here. Three mutations in our study (p.Q100Q, p.R249R, and p.G279G) were detected more than once (see Results). Among these mutations, hotspot 249 has already been described as mutant in concomitance in a lung adenocarcinoma and the respective cfDNA of the case [41]. Further, p.R249R mutation was detected in 28% of mammary tumors of different histological grades [42], and in hypothesis, it is more frequent in cases exposed to tobacco, pollution, and ingestion of alcohol [43]. The cases detected here with this mutation were not tobacco smokers or those who ingested alcohol, but they were exposed to a pollution environment, a scenario common to large urban centers. The relationship between this mutation and the exposure to these agents can only be affirmed in a populational study.

Although we have not found mutations coincident among the specimens evaluated for the *TP53* gene, it has emerged as the most

Table 4
Mutations found in the *TP53* gene in breast lesions and cfDNA.

Sample type	Grade histopathologic	TNM	IHC ER/PR/HER	Ki67 (%)	Lymph node involvement	Gene *TP53* (Exon)	Codon	Protein	Cosmic	IARC
6 – BL	DE/AM	–	–	–	–	4	c.159A > G	p.W53STOP	–	No
7 – BL	HCWA	–	–	–	–	4	c.326 T > A	p.F109 L	–	Yes
1 - BL	IDC grade II	T1N0M0	+/+/−	20%	No	6	c.604C > A	p.R202S	44174	Yes
19 – BL	IDC grade I	T1N0M0	+/+/−	7%	No	4	c.300G > A	p.Q100Q	5028207	Yes
22 – BL	IDC grade II	T1N0M0	+/−/−	20%	No	7	c.710 T > A	p.M237K	43952	Yes[d]
26[a] – BL	IDC grade II	T1N0M0	+/+/−	10%	No	4	c.229C > T	p.P77S	5991554	Yes
30 – BL	ILC grade II/LCIS	T1N0M0	+/+/−	5%	No	8	c.837G > T	p.G279G	c	Yes
39 – BL	IDC grade I	T1N0M0	+/+/−	10%	No	8	c.837G > T	p.G279G	c	Yes
42 – BL	IDC grade II	T1N0M0	NI	NI	No	8	c.837G > T	p.G279G	c	Yes
54 – BL	IDC grade II	T1N0M0	+/+/−	NI	No	4	c.235G > T	p.A79S	–	Yes
8 – cfDNA	DE/AM	–	–	–	–	7	c.232_233insA	p.T1_H1ins	–	No
12 – cfDNA	IDC grade II	T1N1M0	+/+/−	10%	Yes	7	c.763A > C	p.I255L	–	Yes
14 – cfDNA	DCIS grade I	T1N0M0	+/+/NI	NI	No	7	c.747G > A	p.R249R	44625	Yes
15 – cfDNA	IDC grade I	T1N1M0	+/+/−	5%	Yes	7	c.747G > A	p.R249R	44625	Yes
17 – cfDNA	IDC grade II	T1N0M0	+/+/+	20%	No	4	c.300G > A	p.Q100Q	5028207	Yes
20 – cfDNA	IDC grade I	T1N0M0	+/−/−	5%	No	7	c.732C > A	p.G244G	44787	Yes
21 – cfDNA	DCIS grade I	TisN0M0	NI	NI	No	4	c.300G > A	p.Q100Q	5028207	Yes
27[b] – cfDNA	IDC grade III	T1N0M0	+/+/NI	NI	No	7	c.710 T > A	p.M237K	43952	No

BL = breast lesion; cfDNA = circulating free DNA; DE = Ductal ectasia; AM = apocrine metaplasia; HCWA = Hyperplasia of columnar cells with and without atypia; IHC = immunohistochemistry; ER = estrogen receptor; PR = progesterone receptor; HER = Human Epidermal Receptor; COSMIC = Catalogue Of Somatic Mutations In Cancer; IARC = International Agency for Research on Cancer; IDC = infiltrative ductal carcinoma; ILC = infiltrative lobular carcinoma; NI = not informative.
[a] cfDNA not tested.
[b] Breast lesion not tested.
[c] Described in COSMIC, number 46284, but with exchange G > A.
[d] SNP (single nucleotide polymorphism) no validated (no. 765848205).

Table 5
Mutations found in the *CDKN2A* gene in breast lesions and cfDNA.

Sample Type	Histopathologic Grade	TNM	IHC ER/PR/HER	Ki67(%)	Lymph node involvement	Gene *CDKN2A* (Exon)	Codon[a]	Protein[b]	Cosmic
4 – BL	DE/AM	–	–	–	–	2	c.724_725insT (NM_000077.4)	p.S1_STOP (CCQ43815.1)	–
6 – BL	DE/AM	–	–	–	–	1	c.336G > C (NM_000077.4)	p.E10D (NP_00068.1)	–
7 – BL	HCWA	–	–	–	–	1	c.388G > T (NM_000077.4)	p.V28 L (NP_478104.2)	b
8 – BL	DE/AM	–	–	–	–	2	c.611G > A (NM_000077.4)	p.A54T (CCQ43815.1)	–
1 - BL	IDC grade II	T1N0M0	+/+/–	20%	No	2	c.615G > A (NM_000077.4)	p.G74D (CCQ43815)	–
2 – BL	IDC grade II	T1N0M0	+/+/–	10%	No	2	c.634C > T (NM_000077.4)	p.A61A (CCQ43815.1)	–
10 – BL	DCIS grade II	T1N0M0	+/+/NI	NI	No	2	c.678G > A/c.680G > A (NM_000077.4)	p.R76H/p.D77N (CCQ43815.1)	–
12 – BL	IDC grade II	T1N1M0	+/+/–	10%	Yes	2	C.648C.T (NM_000077.4)	p.P66L (CCQ43815)	–
21 – BL	DCIS grade I	TisN0M0	NI	NI	No	2	c.602G > A (NM_000077.4)	p.R99Q (NP_00068.1)	13618
38 – cfDNA	Fibroadenoma	–	–	–	–	1	c.166G > A (NM_058197.4)	p.A56S (NP_478104.2)	–
41 – cfDNA	IDC grade I	T1N0M0	+/+/–	7%	No	1	c.311_312insA (NM_000077.4)	p.E2insE3 (NP_000068.1)	–

BL = breast lesion; cfDNA, circulating free DNA; DE = Ductal ectasia; AM = apocrine metaplasia; HCWA = Hyperplasia of columnar cells with and without atypia; IHC = immunohistochemistry; COSMIC = Catalogue Of Somatic Mutations In Cancer; IARC = International Agency for Research on Cancer; ER = estrogen receptor; PR = progesterone receptor; HER = Human Epidermal Receptor; IDC = infiltrative ductal carcinoma; ILC = infiltrative lobular carcinoma; NI, not informative.

[a] Access number of the database (Genbank).

[b] Single Nucleotide Polymorphim (SNP), rs876658895 (Genbank).

Table 6
Statistical evaluation between the variables for each gene[a].

		PIK3CA	*TP53*	*CDKN2A*
Breast lesion	Age	0.296	0.355	0.800
cfDNA		0.073	0.491	0.565
Breast lesion	Histologic Grade	0.728	0.345	0.641
cfDNA		0.583	0.229	0.315
Breast lesion	Lymph node involvement	**0.001**	0.266	0.740
cfDNA		0.577	0.168	0.697
Breast lesion	Ki67	0.387	0.946	0.500
cfDNA		0.706	0.460	0.672
Breast lesion	Ductal vs Micropappilar	0.675	0.461	0.825
cfDNA		0.687	0.421	0.778
Breast lesion	Ductal vs Lobular	0.508	0.933	0.931
cfDNA		0.604	0.301	0.717
Breast lesion	Benign lesions vs Ductal	**0.050**	0.968	0.104
cfDNA		0.487	0.620	0.247
Breast lesion	Molecular subtype	0.208	0.662	0.829
cfDNA		0.819	0.604	0.830

[a] Numbers represent p value, $p < 0.05$ is significative.

promising molecular biomarker for investigations of tumor-matching mutations in cfDNA [28,43,44]. The *TP53* gene evaluation by NGS from nine different advanced tumor types (14 lung cancer, 3 ovarian, 2 endometrial, 2 thyroid, 2 hepatocellular, 2 unknown primary, 1 cholangiocarcinoma, 1 gastroesophageal junction adenocarcinoma, and 1 peritoneal adenocarcinoma), when compared to their respective cfDNA, revealed a mutation concordance with sensitivity and specificity of 80% and 87.5%, respectively [28]. Moreover, 20 metastatic colorectal tumors when compared to primary tumor and cfDNA, resulted in a concordance of 39% between the cfDNA and the primary tumor, and 55% between the cfDNA and the metastases [44].

The *CDK2NA* gene evaluation by NGS in 44 cases with MBC (24 triple negative breast cancer, 16 estrogen receptor positive, and 4 human epidermal growth factor receptor 2 positive patients) revealed 3 mutated cases (8%) (2 triple negatives and 1 ER+/HER-) [27]. Considering only the malignant lesion data, our frequency was higher (12%). Further, all our mutated cases for *CDK2N2A* were estrogen and progesterone-positive (see Results).

Recently, the *CDKN2A* gene has been tested as a predictive biomarker of relapse in triple negative breast cancer with residual disease after neoadjuvant chemotherapy. Among the 38 early-stage triple-negative breast cancer patients with matched tumor, blood, and plasma, 33 patients had a mutation identified in their primary tumor, but just one matched mutation in cfDNA (3%) [31]. For the impalpable lesions evaluated here, the frequency was similar (2%). In another study, the frequency was higher (8%), but the mutations were identified in the cfDNA of one lung tumor and one ovary, both at advanced stages [28].

Although we did not find identical mutations in the pairs (cfDNA and lesion), the mutational frequencies in cfDNA were similar to those found in the mammary lesions, demonstrating the possibility of using cfDNA for monitoring and exploring the heterogeneity of the lesion. Beije et al. [43] reported that mutations at estrogen receptor 1 (ESR1) and splicing variants occur more frequently in cfDNA, than in CTC from metastatic breast cancer (MBC) patients progressing on endocrine treatment. The mutation percentages before and after treatment, respectively, were 11% and 5% (cfDNA), and 42% and 8% (CTCs). These same conclusions were observed by Bettegowda et al. [44]. Among the 640 advanced tumors evaluated from different histological subtypes (breast, ovary, hepatocellular, head and neck cancer, among others), the mutational profile of cfDNA was more informative than CTC. ctDNA was often present in patients without detectable CTC, suggesting that these two biomarkers are distinct entities.

In this study, we detected diversified mutations in the impalpable lesions and in corresponding cfDNA. These data demonstrate that non-invasive tests are likely to be successful in evaluating tumor heterogeneity. Although we found representative mutational rates in benign lesions, there are intrinsic factors for each tumor microenvironment that can be

influenced by environmental and behavioral variables that deserve to be studied individually. Further, in some cases, individual genetic alterations are not capable of leading to tissue malignancy [45].

The mutation evaluation in cfDNA by NGS and digital PCR has shown higher sensitivity values, when compared to other conventional methods, as performed here [18-22,27,28,31,43,44]. However, the impalpable breast lesion molecular characteristics are still research objectives and deserve research amplification.

This study is original and detected different mutations in *PIK3CA*, *TP53*, and *CDKN2A* genes, which represent, in part, the heterogeneity from impalpable lesions. We believe that the search for mutations in cfDNA, in parallel with the lesion, may minimize unnecessary biopsies and complement imaging tests, which are not always informative to describe the risk of invasion of the lesion, or to follow the tumor response to treatment.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.anndiagpath.2018.12.008.

Acknowledgments

The authors thank everyone at the Americas Medical City and at Dr. Mauricio Magalhães da Costa clinics for the assistance and patient data collection. Delmonico L. is a recipient of a graduate fellowship from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

Author disclosure statement

No competing financial interests exist.

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