Titele:

A novel diagnostic method targeting genomic instability in intracystic tumors of the breast.

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ABSTRACT

Background: Even after needle biopsy, intracystic tumors of the breast are challenging differentially diagnose preoperatively because of their nonspecific radiological characteristics and subtle cytological and histological appearance. The aim of this study is to investigate a novel diagnostic method, targeting genomic instability (GIN) in intracystic tumors of the breast, using tumor DNA from samples obtained by fine-needle aspiration biopsy (FNAB).

Methods: Thirteen consecutive intracystic tumors of the breast, including five cancers and eight benign tumors, were studied. Three FNAB passages per tumor were used for array comparative genomic hybridization (aCGH) analysis to quantify GIN in each tumor. Tumor DNA from the main tumor, taken from formalin-fixed, paraffin-embedded (FFPE) blocks corresponding to FNAB samples, was also analyzed to compare cytogenetic profiles between these sample types.

Results: After three FNAB passages, an average of 7.09 μg (0.24–25.0 μg) of DNA was obtained. The quality of the DNA and the aCGH data was excellent, as judged by the mean derivative log ratio spread (DLRSpread) of 0.22 (0.15–0.29). The cytogenetic profiles of paired FNAB and main tumor FFPE samples were highly similar, with an average concordance rate of 97.7% (81.2–100%). aCGH analysis from FNAB samples showed significantly more GIN in cancers than in benign tumors, with mean

frequencies of aberrant chromosomal regions of 17.5% and 0.34%, respectively (Wilcoxon’s rank sum test, P = 0.0016).

Conclusions: Our novel diagnostic method, which targets GIN, can clearly distinguish cancers from benign tumors of breast intracystic lesions with minimal invasion, thereby avoiding the need for surgical excisional biopsy.

Introduction

Intracystic tumors of the breast include benign papillomas, which account for approximately 10% of benign breast tumors, as well as carcinomas in situ and carcinomas with invasion, which together account for less than 1% of malignant breast tumors [1–7]. Intracystic papillary carcinoma develops with a predilection for elderly women, who often present with a palpable mass and/or bloody nipple discharge [1, 2, 4]. Although this type of carcinoma has a good prognosis regardless of whether the tumor is diagnosed as in situ or invasive, there have been reports of metastasis to lymph nodes or distant organs [5, 8, 9].

In breast lesions, indication for surgery is usually determined by pathological diagnosis together with radiological findings. However, preoperative differential diagnosis between intracystic papillary carcinomas vs. papillomas is very challenging even after needle biopsy, because of their nonspecific radiological characteristics, and subtle cytological and histological appearance. Hence, surgical excisional biopsy is recommended in the clinical management of these lesions, especially when associated with risk factors for malignancy, such as age (≥ 50 years) and the presence of microcalcifications [6–8, 10]. A few papers have reported immunohistochemical techniques to be effective, but only in combination with other diagnostic techniques [11–13]. Although Shamonki et al. [14] recently reported that core needle biopsy (CNB) tissue samples consisting of at least seven cores, obtained by using a 12-gauge or larger

needle, had a negative predictive value for atypia/malignancy of 100 %, a less invasive diagnostic method is required.

Meanwhile, genomic instability (GIN) is an important hallmark of cancer [15], which may be useful for distinguishing cancers from benign tumors. There are many reports indicating the presence of genomic alterations in intracystic papillary carcinoma of the breast [16–20]. We recently reported that genome-wide copy number aberrations could easily be detected by array comparative genomic hybridization (aCGH), which could then be used to quantify GIN, and that intracystic papillary carcinoma harbors significant GIN compared with intracystic papilloma [21]. However, a cytogenetic method for preoperative diagnosis of breast intracystic tumors has not been reported thus far.

The aim of this study is to investigate a novel diagnostic method, targeting GIN in intracystic tumors of the breast, using tumor DNA from samples obtained by fine-needle aspiration biopsy (FNAB).

Materials and methods

Tumor samples and pathological diagnosis

Thirteen consecutive intracystic tumors of the breast were resected at Nagasaki University Hospital between August 2010 and March 2012, which included five cancers (four invasive ductal carcinoma and one non-invasive carcinoma) and eight benign tumors (five papilloma, two ductal adenoma and one sclerosing papilloma), were enrolled. Preoperative diagnosis by CNB and FNAB, and postoperative final diagnosis was independently determined by two pathologists (Table 1).

All experimental procedures for this study were approved by the Committee for Ethical Issues on the Human Genome and Gene Analysis of Nagasaki University (#08073134) and all patients provided informed consent for voluntary participation.

Sampling and tumor DNA extraction

An experienced surgeon performed FNABs using a 22-gauge aspiration needle. Three FNAB passages per tumor were performed. All samples were suspended in 380 μL PBS and tumor DNA was immediately extracted using the QIAmp DNA Mini Kit (Qiagen, Dusseldorf, Germany) following the manufacturer's instructions. Formalin-fixed, paraffin-embedded (FFPE) blocks of the resected tumors were processed to extract tumor DNA, as previously reported [21–23]. Briefly, samples were macrodissected using 10–20 10-μm-thick sections. Tumor tissue areas containing more

than 70% tumor cells, as identified by a guide slide stained with hematoxylin, were selected and manually dissected using surgical scalpels. Paraffin removal was performed in 80% xylene after which samples were washed twice with absolute ethanol. Deparaffinized tissue pieces were spun down, after which the dried up pellet was resuspended in 360 μL buffer ATL and incubated at 95 °C for 15 min. Samples were allowed to cool down to room temperature and subsequently digested with proteinase K for 3 days at 56 °C in a rotation oven with periodic mixing and fresh addition of proteinase K every 24 h. DNA was extracted using the QIAmp DNA Mini Kit according to the manufacturer's instructions with some modifications. Briefly, 400 μL buffer AL was added to the sample, which was then incubated at 70 °C for 10 min followed by the addition of 400 μL absolute ethanol and mixing by vortex. The sample solution was applied to the spin column followed by centrifugation for 1 min at 8000 ×g. The spin column was washed twice with 500 μL buffer AW1 by centrifugation at 8000 ×g for 1 min and washed with 80% ethanol by centrifugation at 14,000 ×g for 3 min. Finally, the DNA was eluted with 55 μL nuclease free water.

DNA was quantitated using the ratio of absorbance at 260 and 280 nm (A260/A280), and 260 and 230 nm (A260/A230) on a NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). The double-strand DNA concentration in each sample was quantitated on a Qubit fluorometer with the Qubit dsDNA HS Assay Kit (Life Technologies, Carlsbad, CA) following the manufacturer's

instructions as a quality indicator of DNA for aCGH analysis [25].

aCGH analysis

The Genomic DNA ULS Labeling Kit (Agilent Technologies, Palo Alto, CA) was used to chemically label 500 ng of DNA from each sample and 250 ng of reference female genomic DNA (Promega, Madison, WI) with Cy5 or Cy3 dye, respectively, for 30 min at 85 °C, followed by purification using Agilent-KREApure™ columns. The amount of input DNA was determined on a Qubit fluorometer using the Qubit dsDNA HS Assay Kit. The degree of Cy5 labeling (absorbance at 650 nm) was calculated using a NanoDrop ND-2000 spectrophotometer. Purified, labeled samples were then combined and mixed with human Cot-1 DNA (Invitrogen, Carlsbad, CA), Agilent 10× Blocking Agent and Agilent 2× Hybridization Solution. Prior to array hybridization, hybridization mixtures were denatured at 95 °C for 3 min and incubated at 37 °C for 30 min. Agilent CGHblock was added and samples were hybridized to SurePrint G3 Human CGH 8×60K Microarrays, which contain eight identical arrays consisting of ~63,000 in situ synthesized 60-mer oligonucleotide probes that span coding and noncoding sequences with an average spatial resolution of ~54 kb. Hybridization was carried out at 65 °C for 40 h before washing with Agilent Oligo aCGH Wash Buffer 1 at room temperature for 5 min, followed by washing with Agilent Oligo aCGH Wash Buffer 2 at 37 °C for 1 min. Scanning and image analysis were done on an Agilent DNA

Microarray Scanner. Agilent Feature Extraction Software (version 9.5) was used for data extraction from raw microarray image files. Agilent Genomic Workbench (version 5.0) was used to visualize, detect and analyze chromosomal patterns using the Aberration Detection Method 2 (ADM-2) algorithm with the default settings. The derivative log ratio spread (DLRSpread) of each sample, which estimates the log ratio noise by calculating the spread of log ratio differences between consecutive probes along all chromosomes, and is an indicator of the quality of aCGH analysis, was also calculated using Agilent Genomic Workbench (version 5.0). A copy number gain or loss was defined as a log2 ratio > 0.25 or < -0.25, respectively.

Statistical analysis

The total rate of a copy number altered (CNA) region, defined as the sum of each segment gained or lost and divided by 2,829 Mb, which is the total Mb in the genome excluding heterochromatic, centromeric and telomeric regions not covered by probes, was used as an indicator of GIN. Wilcoxon's rank sum test was performed to compare the rate of a CNA region between cancer and benign tumor samples.

To compare the cytogenetic profiles of FNAB samples and their corresponding FFPE blocks, we used the concordance rate (CR), which was defined as the ratio of the copy number length of a given segment within pairs.

The analyses above were done with EZR (Saitama Medical Center, Jichi

Medical University; http://www.jichi.ac.jp/saitama-sct/SaitamaHP.files/statmedEN.html; Kanda, 2012), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria, version 2.13.0) [24]. More precisely, it is a modified version of R commander (version 1.6-3) that was designed to add statistical functions frequently used in biostatistics. The results were considered statistically significant when the p-value was < 0.05.

Results

Preoperative FNAB was conducted in 12 of 13 cases, of which four cases (33.3 %) could be diagnosed as malignant, preoperatively. In four of 13 cases, preoperative CNB was conducted, of which three cases (75%) could be diagnosed as malignant, preoperatively. Notably, one case of ductal adenoma (case 3) was diagnosed as malignant by FNAB and as undetermined by CNB.

After three FNAB passages, sufficient amounts of DNA were obtained with an average of 7.09 μg (0.24–25.0 μg). The mean ratio of double-strand to total DNA (dsDNA ratio) of the samples, which is a DNA quality indicator for aCGH analysis, with a higher ratio indicating better quality [25], was 0.70 (0.31–0.95). The quality of the aCGH data were excellent, with a DLRSpread of less than 0.30, with mean of 0.22 (0.15–0.29) for all cases (Table 1).

The aCGH results using DNA from paired FNAB and main tumor FFPE samples, produced highly similar cytogenetic profiles within pairs, with an average concordance rate, defined as the ratio of copy number length of a given segment within pairs, of 97.7% (81.2–100%) (Table 1, Figure 1).

aCGH analysis of FNAB samples showed that cancers harbored significantly more GIN than benign tumors, with mean frequencies of aberrant chromosomal regions of 17.5% and 0.34%, respectively (Wilcoxon’s rank sum test, P = 0.0016) (Figure 1, Figure 2). In

five malignant tumors, four cases (80%) showed 16q loss and three cases (60%) showed 1q gain (Figure 3).

Discussion

Preoperative differential diagnosis of intracystic tumors of the breast is challenging because of their nonspecific radiological characteristics, and subtle cytological and histological appearance. Only 33 % and 75 % of intracystic cancers of the breast were diagnosed as malignant by FNAB and CNB, respectively in this study, which is comparable to the other reports [6]. In the present study, we therefore used intra-cystic tumors of the breast to validate a new diagnostic method targeting GIN in intracystic tumors of the breast, using tumor DNA from samples obtained by FNAB.

By performing three passages of FNAB, sufficient amounts and quality of DNA were obtained for aCGH analysis, which requires only 250 ng of tumor DNA per sample, which resulted in sufficient aCGH quality with a mean DLRSpread of 0.22. In this study, we did not assess the purity of tumor cells. However, Symmans et al. [26] reported that compared with CNB, FNAB yields a similar quality of tumor RNA, with less contamination with normal stromal cells. Considering the clear cytogenetic profile of our cancer samples, the purity of tumor cells was assumed to be acceptable.

The cytogenetic profiles of paired FNAB and main tumor FFPE samples were highly similar, with an average concordance rate of 97.7 %. Although intra-tumoral heterogeneity may result in discordant cytogenetic profiles, this minor effect may be considered negligible when distinguishing malignant from benign samples by our

method. Moreover, cytogenetic profiles of five cancer cases showed 16q loss and 1q gain as the most frequently altered region, which is a typical cytogenetic profile of papillary breast carcinomas, as reported previously [16–20].

The results of aCGH analysis from FNAB samples showed that cancers harbored significantly more GIN than benign tumors, with mean frequencies of aberrant chromosomal regions of 17.5% and 0.34%, respectively, which was concordant with our data published previously using FFPE samples of breast intracystic tumors [21]. If we endorse 3 % of frequencies of aberrant chromosomal regions as a cut-off value to distinguish malignant from benign samples, our data set was completely segregated. However, to decide on an accurate cut-off value, prospective analysis of additional samples with receiver operating characteristic (ROC) analysis is required.

In conclusion, our novel diagnostic method, which targets GIN, can clearly distinguish cancers from benign tumors of breast intracystic lesions with minimum invasion, thereby avoiding the need for surgical excisional biopsy. This methodology may be extrapolated to other breast lesions where it is difficult to distinguish malignant from benign tumors preoperatively.

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Conflict of interest

No financial or other potential conflicts of interest exist for any of the authors.

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FIGURE LEGENDS

Figure 1. Graphic display of whole genome copy number changes in carcinoma samples using FNAB (a), FFPE samples of the main tumor (b) and benign lesion samples using FNAB (c).

Log2 ratio values for all oligonucleotide probes are plotted as a function of their chromosomal position. Aberration calls identified by the ADM-2 algorithm are shown as upper and lower horizontal bars, respectively. The number of each column represents each chromosome.

Figure 2. The mean ratio of copy number alteration (CNA) was compared between carcinomas and benign lesions.

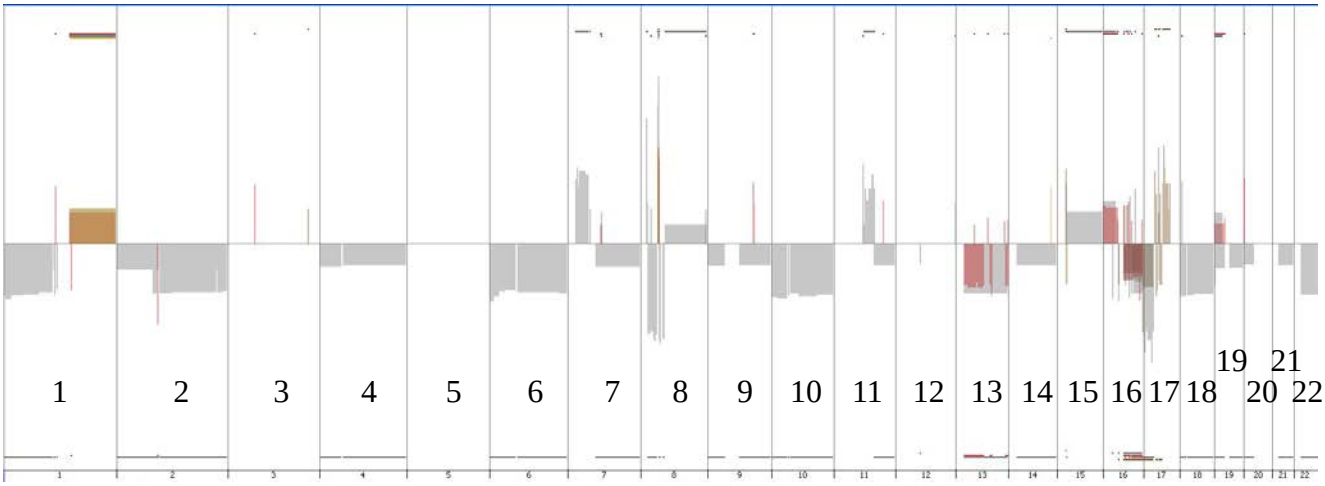
The central rectangle spans the first to the third quartile. The segment inside the rectangle indicates the median and "whiskers" above and below the box indicate the locations of the minimum and maximum.

Figure 3. Graphic display of whole genome aberrations in intracystic carcinoma of the breast.

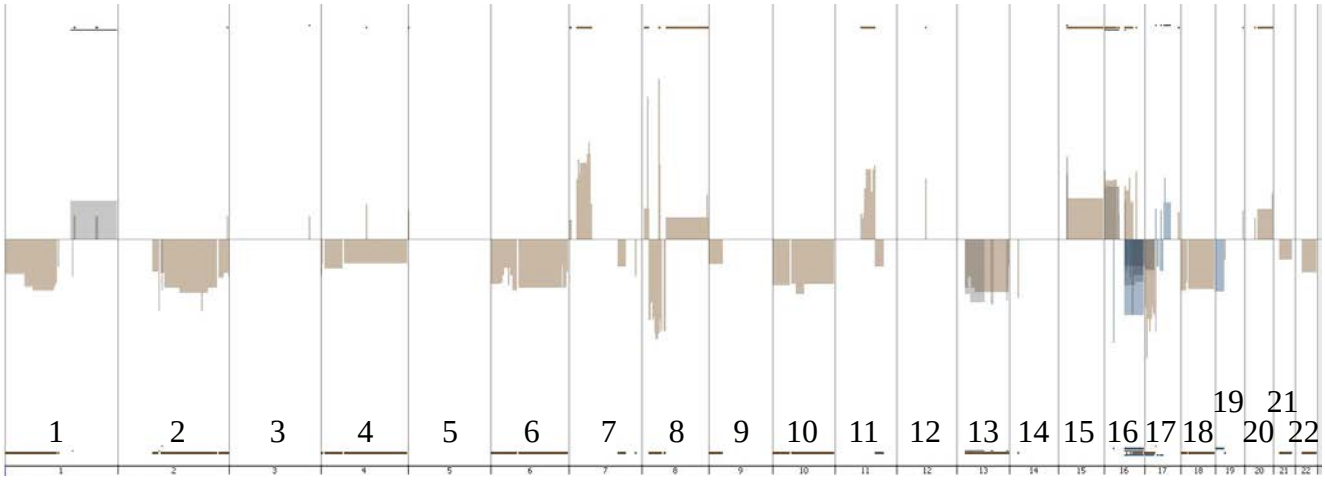
The panels to the right of each chromosome show the frequency of gains (red bars) and

losses (green bars), each ranging from 0 % to 100 %.

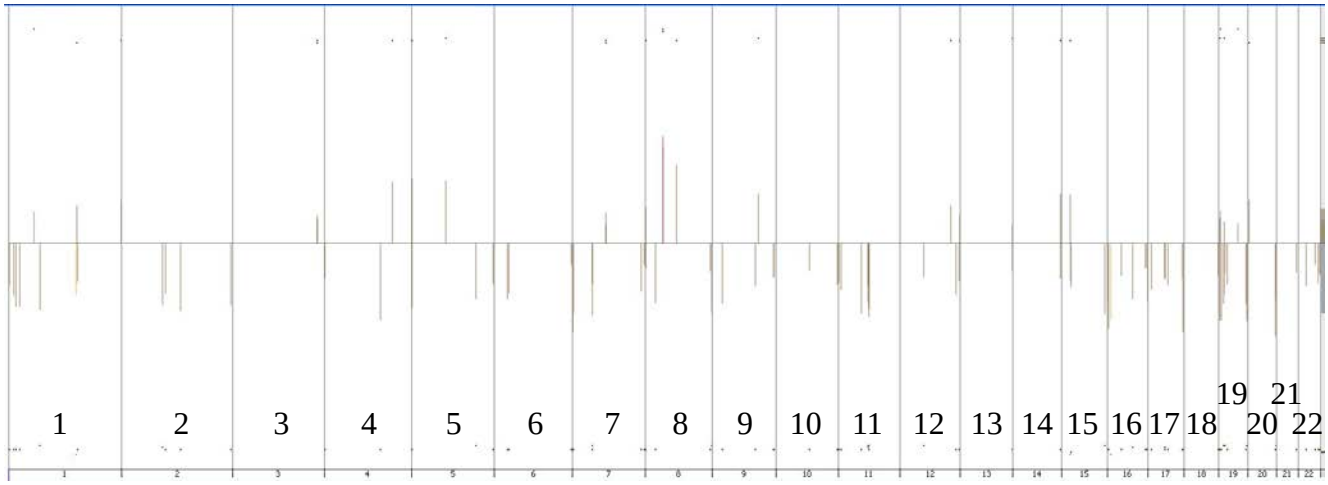
(a). FNAB-aCGH, carcinoma (n=5)



(b). FFPE-aCGH, carcinoma (n=5)



(c). FNAB-aCGH, benign (n=8)



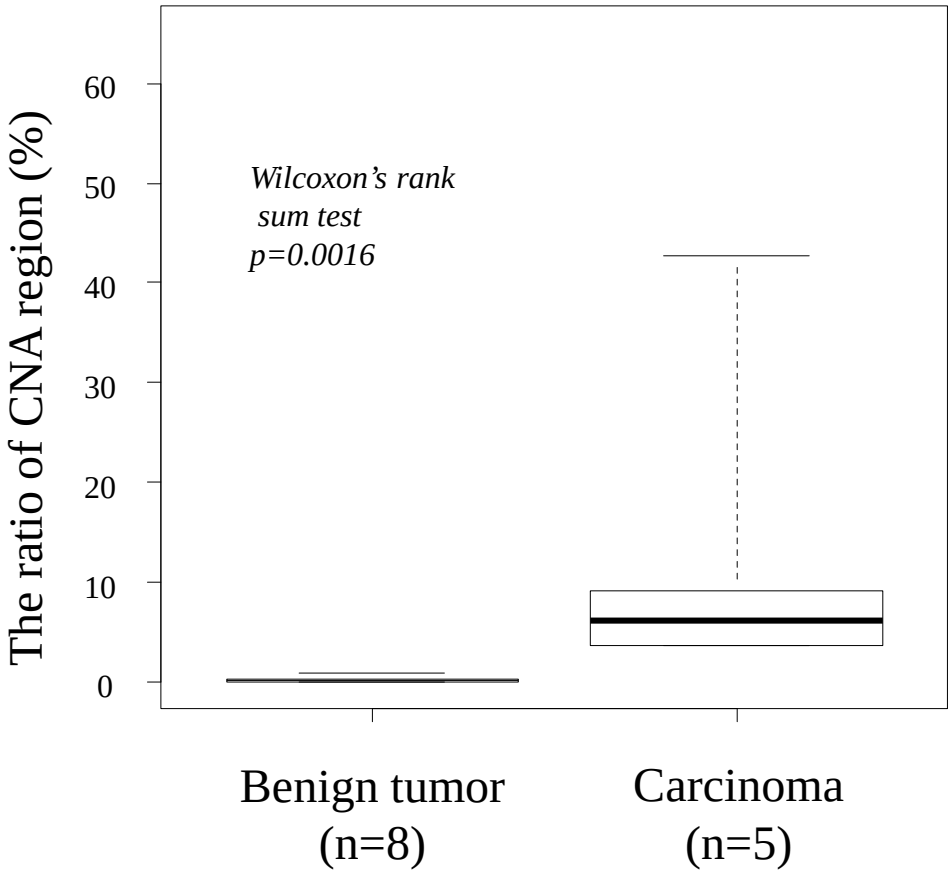

The ratio of CNA region (%)
60
50
40
30
20
10
0
Wilcoxon's rank sum test
p=0.0016
Benign tumor (n=8)
Carcinoma (n=5)

Figure 3

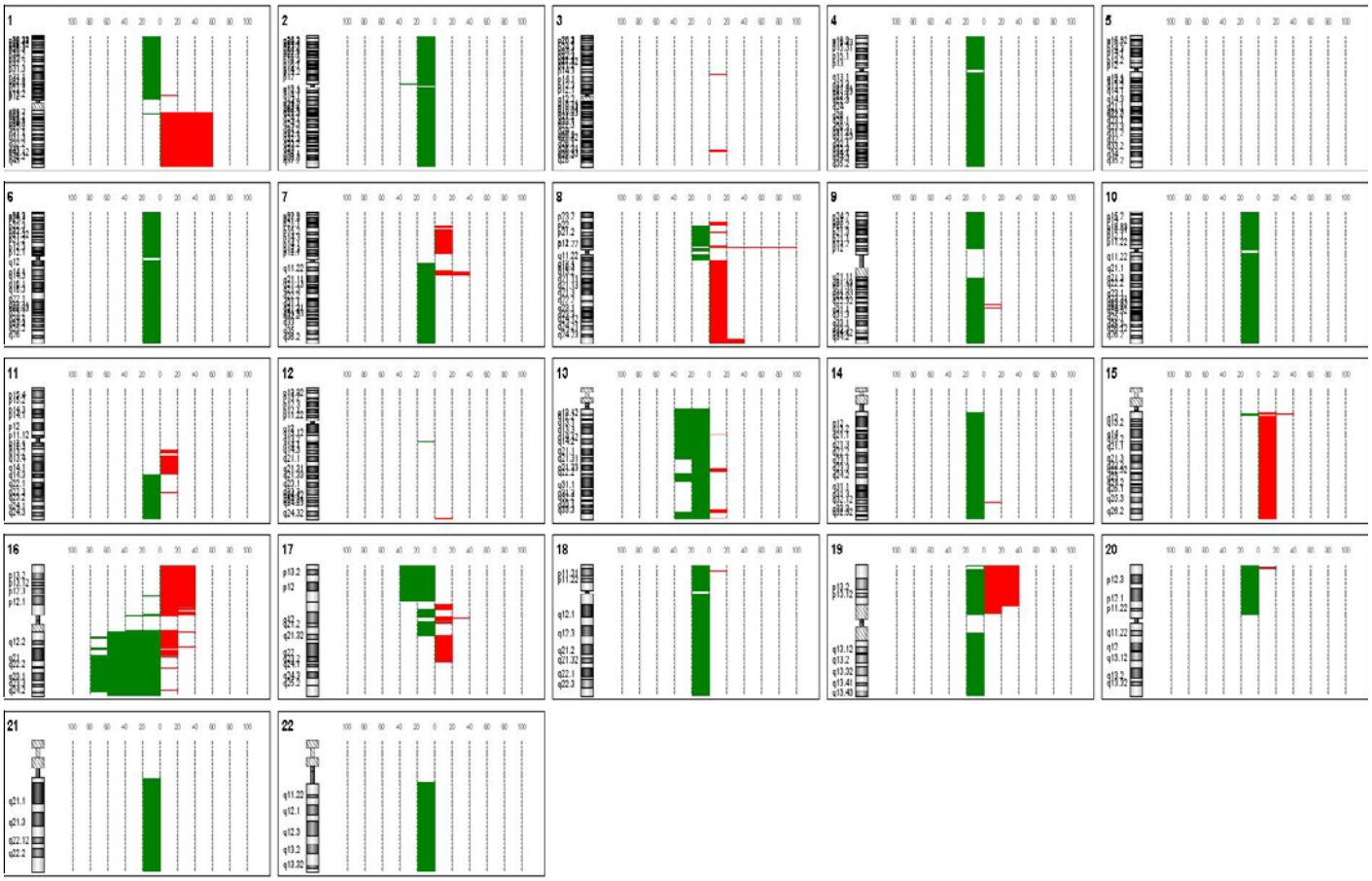


Table 1. Characteristics of cases in the present study.

Case	Age	Final diagnosis	Cytology	CNB	Total amount of DNA (μg)	dsDNA ratio	Ratio of CNA region (%)	Number of CNA region	Overlap of FNAB-aCGH and FFPE-aGGH (%)
1	58	IDC	V	Malignancy	8.6	0.74	3.57	20	99.7
2	40	Papilloma	III	NA	1.8	0.76	0.07	3	99.9
3	63	Ductal adenoma	V	Indeterminate	4.1	0.81	0.004	1	100
4	41	Papilloma	III	NA	5.9	0.74	0.05	2	99.9
5	67	Sclerosing Papilloma	III	NA	0.24	0.48	0.17	10	NA
6	57	Papilloma	III	NA	25.0	0.93	0.007	1	100
7	74	Papilloma	III	NA	0.71	0.57	1.90	42	98.1
8	75	IDC	V	NA	18.0	0.88	65.1	79	81.2
9	49	IDC	III	NA	16.1	0.95	9.12	16	99.1
10	68	Papilloma	NA	NA	1.21	0.63	0.36	15	99.7
11	67	IDC	V	Malignancy	5.81	0.51	6.20	15	98.8
12	67	DCIS	V	Malignancy	4.34	0.79	3.65	4	96.3
13	89	Ductal adenoma	III	NA	0.41	0.31	0.15	17	99.8

IDC: invasive ductal carcinoma, CNB: core needle biopsy, dsDNA: double-stranded DNA, CNA: copy number alteration, FNAB: fine needle aspiration biopsy, aCGH: array-comparative genomic hybridization, FFPE: formalin-fixed paraffin-embedded, NA: not analyzed.