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Original Research

Cytogenetic Equivalents of Satellite DNA Amplification are Detectable in Advanced Breast Cancers and Seem to Lead to Satellite RNA Expression

Mariam Mahmoud [1], Katrin Schindler [1], Stefanie Kankel [1], Prahlad Balakrishnan [1], Bernhard Theis [2], Bashdar Mahmud Hussen [3, 4], Mohammad Taheri [5], Thomas Liehr [1, *]

1. Jena University Hospital, Friedrich Schiller University, Institute of Human Genetics, Am Klinikum 1, Jena, Germany; E-Mails: mariammhassan1996@gmail.com; Katrin.Schindler@med.uni-jena.de; Stefanie.Kankel@med.uni-jena.de; bprahlad@gmail.com; Thomas.Liehr@med.uni-jena.de
2. Jena University Hospital, Friedrich Schiller University, Institute of Forensic Medicine, Section Pathology, Jena, Germany; E-Mail: Bernhard.Theis@med.uni-jena.de
3. Department of Clinical Analysis, College of Pharmacy, Hawler Medical University, Kurdistan Region, Erbil, Iraq; E-Mail: bashdar.hussen@hmu.edu.krd
4. Department of Medical Laboratory Science, College of Health Sciences, Lebanese French University, Kurdistan Region, Erbil, Iraq
5. Urology and Nephrology Research Center, Research Institute for Urology and Nephrology, Shahid Beheshti University of Medical Sciences, Tehran, Iran; E-Mail: mohammad_823@yahoo.com

* **Correspondence:** Thomas Liehr; E-Mail: Thomas.Liehr@med.uni-jena.de

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Abstract

The cytogenetic equivalents of oncogene amplification are well-known in cancer cells as homogeneously staining regions (HSR) or double minutes (DM). We previously described similar amplifications of satellite-DNA (Sat-DNA) in advanced prostate cancer. In this study, we systematically screened for Sat-DNA amplification in 47 breast cancer (BCa) samples, which is a prevalent malignancy in women. Interphase fluorescence in situ hybridization (iFISH)

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was applied using 23 Sat-DNA probes and a probe for *ErbB2*/*Her2* gene amplification. *ErbB2*/*Her2* gene amplification was identified in 20 of the studied BCA samples; 13 showed an HSR pattern and seven showed a DM pattern by iFISH. Additionally, the application of 23 Sat-DNA probes revealed massive chromosome instability (CIN) in all samples studied. Notably, 15 out of 47 BCa samples displayed amplification of one or two Sat-DNAs. Each was detectable as HSR-like structures in 13 out of 15 cases. In two cases, Sat-DNA was amplified as DMs. Sat-III-DNA (1q12/HSat3 and D9Z3) was involved in nine cases, and α-Sat-DNA derived from chromosomes 9, 11, 17 and 18 showed amplification in nine cases. Sat-DNA amplification was positively aligned with advance in tumor grade, Ki67 concentration and positive *ErbB2*/*Her2* immunohistochemistry status and/or gene amplification. Four selected cases with α-Sat-DNA amplification showed enhanced α-Sat-RNA-expression between 1.4- and 9.7-fold, while two cases without α-Sat-DNA amplification showed such RNA-presence only at very low levels. Overall, Sat-DNA amplification may be an overlooked feature of advanced solid tumors, as demonstrated here for BCa and in previous studies for prostate cancer and liposarcoma.

Keywords
Satellite DNA (Sat-DNA); amplification; breast cancer (BCa); heterochromatin; fluorescence in situ hybridization (FISH)

1. Introduction

The desire to understand tumor initiation and progression to help affected patients is a main driver of cancer research [1]. For solid tumors, the Vogelstein model [2] is a model that summarizes our current understanding of (a) tumor initiation in a single cell by collecting growth-promoting oncogene and tumor-suppressor gene mutations leading to a pre-tumor, via (b) tumor progression and (c) metastasis by different mechanisms. For over a decade, the Vogelstein model focused on the idea that accumulating gene mutations was the main element of tumor progression. However, in 2002, chromosome instability (CIN) was added as an additional hallmark of cancer development [3].

CIN can easily be characterized by (molecular) cytogenetics, including interphase fluorescence in situ hybridization (iFISH) on formalin-fixed paraffin-embedded (FFPE) sections [4]. Both numerical and structural aberrations, such as deletions of tumor suppressors or translocations and amplifications involving oncogenes, are routinely assessed using iFISH and have predictive value [5]. As early as the 1960s and 1970s, cytogenetic equivalents of gene amplification were described as double minutes (DMs) or homogeneously staining regions (HSRs) [6].

Breast cancer (BCa) is a good example of the diagnostic impact of iFISH when screening for oncogene amplification. iFISH is commonly used to assess *ErbB2/Her2* gene amplification [7]. *ErbB2*/*Her2* gene amplification was originally associated with an adverse prognosis; however, the development of anti-Her2 therapies has improved outcomes for affected women [8]. Nonetheless, BCa is the most prevalent cancer in women [9], and 15% of treated patients still develop metastases [10].

ErbB2/*Her2* gene amplification diagnostics are performed by applying a probe specific to the *ErbB2*/*Her2* gene region in 17q12, as well as a control probe. The latter is an α-satellite-DNA (α-Sat-DNA) probe for chromosome 17 (=D17Z1) [7, 11]. Sat-DNAs form different subgroups (α-satellites, β-satellites, satellite III, and others) and map to one, two, or three human chromosomes at or near the centromere [12, 13] (Table S1). Previously, we reported α-Sat-DNA D17Z1 amplification in 1 of 414 (=0.24%) BCa cases [14]. Additionally, in a systematic study in prostate cancer, we reported Sat-DNA amplification predominantly in advanced stages [15].

Here we did the first systematic molecular cytogenetic study in 47 BCa cases to screen for Sat-DNA amplification involving all commercially available 23 satellite-DNA sequences in the female human genome. Sat-RNA expression was also exclusively proven in cases with α-Sat-DNA amplification.

2. Materials and Methods

2.1 Samples

Salahaddin University-Erbil provided 43 FFPE BCa samples. The study was approved by the Research Ethics Committee Office (RECO) of the Kurdistan Regional Government-Iraq under registration number 429/06. 4 samples were acquired during routine diagnostics at the Jena University Hospital; in those, only *ErbB2*/*Her2* status and centromere 17 were tested (cases 28, 45, 46 and 47). All patients were female.

More detailed information regarding grading, age, staging, minimal information on pathological findings, Ki67 status, lymph node status, *ErbB2*/*Her2* status, and patient ages is shown in Table 1.

Table 1 Clinical and laboratory findings of the 47 breast cancer (BCa) cases studied, together with iFISH results from this study in the last two columns.

Patient #	Grade	Stage	Age [y]	Pathological findings – minimal details	Ki67 status [%]	Tumor size [mm]	Lymph node	HER2 immuno	HER2 FISH ampl.	Sat-DNA ampl.
1	1	IIB	56	IDC	10	25	+	-	DM	-
2	1	IIIA	40	multifocal IDC	10	10	+	-	-	-
3	2	n.a.	61	low-grade DCIS of cribriform type	n.a.	18	+	-	-	-
4	2	I	n.a.	n.a.	90	n.a.	-	-	-	-
5	2	IA	63	IDC with extensive high-grade DCIS	10	8	-	-	-	-
6	2	IC	n.a.	n.a.	30	n.a.	n.a.	++	-	-
7	2	IIA	62	IDC	15	23	+	-	-	-
8	2	IIA	38	IDC	10	24	+	-	-	-
9	2	IIA	63	multifocal IDC	n.a.	28	-	-	-	-
10	2	IIA	n.a.	n.a.	20	42	-	-	-	-
11	2	IIA	n.a.	n.a.	20	28	-	++	DM	HSR 1q12/HSat3 30%
12	2	IIA	49	ILC (with extensive LCIS and focal DCIS)	10	48	+	-	-	HSR 1q12/HSat3 30%
13	2	IIB	63	IDC	20	22	+	-	-	-
14	2	IIB	50	IDC with DCIS	10	21	+	-	-	-
15	2	IIB	48	IDC with multifocal high-grade DCIS	15	35	+	-	HSR	-
16	2	IIB	63	IDC	20	35	+	-	-	-
17	2	IIB	n.a.	n.a.	10	18	+	-	-	-
18	2	IIIA	45	IDC	25	30	+	+++	DM	-

19	2	IIIA	n.a.	n.a.	25	32	n.a.	++	HSR	-
20	2	IIIA	60	IDC	n.a.	30	+	-	-	-
21	2	IIIA	n.a.	n.a.	n.a.	20	+	n.a.	-	-
22	2	IIIA	64	IDC with high grade DCIS	10	21	-	-	-	-
23	2	IIIA	43	IDC	60	29	+	+++	HSR	-
24	2	IIIA	66	IDC with intermediate grade DCIS	10	80	+	++	-	-
25	2	IIIA	n.a.	n.a.	n.a.	12	+	n.a.	DM	HSR 1q12/HSat3 20%
26	2	IIIC	46	DCIS	n.a.	50	+	+++	HSR	-
27	2	IIIC	59	ILC	15	26	+	++	-	-
28	3	IA	51	IDC	29	n.a.	n.a.	+	HSR	HSR D17Z1 66%
29	3	IIA	64	IDC with high grade DCIS	20	38	-	-	-	-
30	3	IIA	n.a.	n.a.	20	40	-	++	-	-
31	3	IIA	48	IDC and high-grade DCIS with Paget's disease	30	23	+	+++	HSR	HSR 1q12/HSat3/D18Z1 10%
32	3	IIA	52	IDC with extensive high-grade DCIS	40	25	+	+++	HSR	HSR D17Z1/D18Z1 100%
33	3	IIA	43	IDC	35	28	-	-	HSR	HSR D9Z4 40%
34	3	IIA	n.a.	n.a.	n.a.	12	-	n.a.	HSR	HSR 1q12/HSat3 20%

35	3	IIA	n.a.	n.a.	70	n.a.	-	++	DM	HSR 1q12/HSat3 100%
36	3	IIA	n.a.	n.a.	70	n.a.	-	-	-	HSR 1q12/HSat3/D9Z3 5%
37	3	IIB	35	IDC	25	33	+	++	HSR	-
38	3	IIIA	48	IDC	25	25	+	++	DM	HSR 1q12/HSat3 40%
39	3	IIIA	55	IDC with Paget's disease	50	30	+	+++	HSR	HSR D11Z1 100%
40	3	IIIB	43	multiple foci with residual extensive DCIS and IDC	n.a.	55	+	-	DM	-
41	3	IIIC	44	IDC	25	21	+	-	-	-
42	3	IIIC	47	IDC	40	15	+	++	HSR	-
43	3	IIIC	43	IDC (inflammatory carcinoma)	35	40	+	-	-	-
44	3	IIIC	43	IDC (lateral aspect with high grade DCIS)	20	30	+	-	-	-
45	3	IV	80	resected specimen after neoadjuvant therapy	26	25	+	+	HSR	HSR D17Z1 40%
46	n.a.	n.a.	65	n.a.	14	n.a.	n.a.	++	-	DM D17Z1 98%
47	n.a.	IA	48	inguinal lymph node metastasis	26	52	-	+	-	DM D17Z1 70%

Abbreviations: ampl. = amplification; DCIS = ductal carcinoma in situ; DM = double minutes; HSR = homogeneously staining region; IDC = invasive ductal carcinoma; ILC = invasive lobular carcinoma; LCIS = lobular carcinoma in situ; n.a. = data not available.

2.2 Sample Workup and FISH

We processed FFPE tissues as previously reported [15]. Commercially available centromere-specific FISH probes for all human chromosomes (1-22 and X) and heterochromatic regions 1q12, 9q12, 15p11.2, and 16q11.2 were applied per sample (Table S1) – excluding cases 28, 45, 46 and 47. We did not use Y-chromosome probes, as we tested only females. *ErbB2*/*Her2* amplification was tested by a corresponding specific probe from Zytovision (Bremerhaven, Germany; Table S1). We combined the probes into 2 to 4-color FISH probe sets; FISH and evaluation were done as previously described [15].

Sat-DNA amplification was recorded as either an HSR-typical 'laddering' signal (Figures 1A-1C) or as a cell with more than ten centromeric signals distributed over the cell, which were interpreted as DMs (Figure 1D). Besides, the number of Sat-DNA signals was counted in each of the 50 analzed cells. The results obtained were typical of a solid tumor with CIN: almost all of the tested probes showed, besides the expected signals typical for a diploid genome, as well as the losses and gains of signals, which are typical for monosomic and polysomic cells. In Table S2, the corresponding results are summarized.

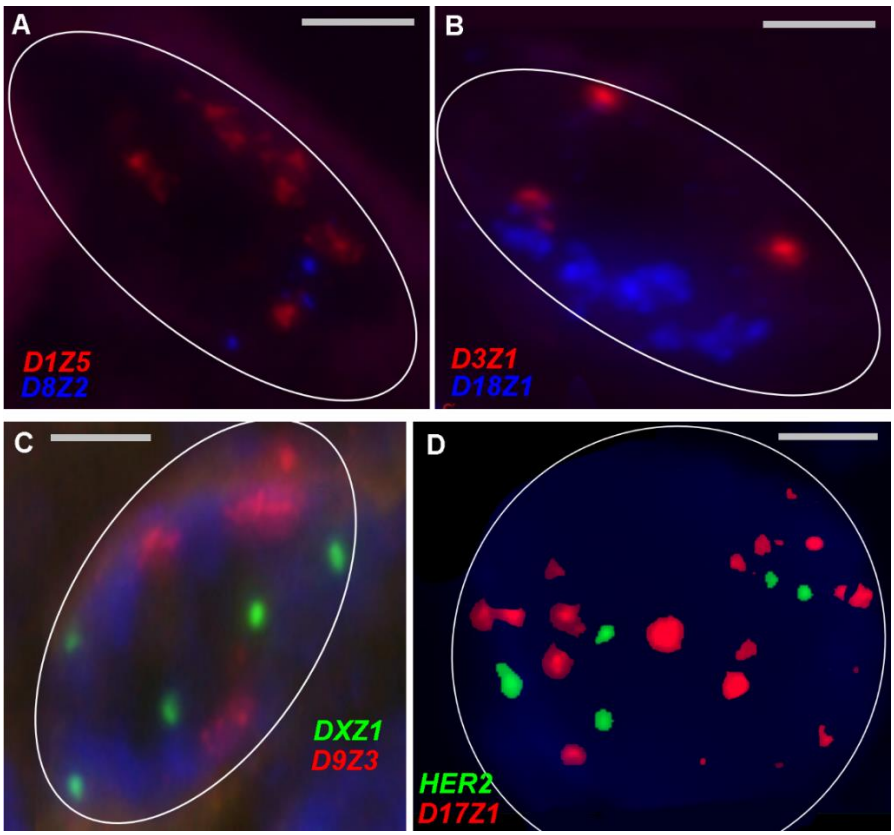


Figure 1 These are typical interphase fluorescence in situ hybridization (iFISH) images of Sat-DNA amplifications detected in four samples from this study. In panels A-C, the Sat-DNA amplification is stained as homogeneously staining regions (HSRs), and in panel D, it is stained as double minute (DM) structures. One nucleus from samples #11 (A), #32 (B), #36 (C), and #46 (D) is depicted in each image. The applied probes are labeled in the corresponding colors (SpectrumOrange, SpectrumAqua, or SpectrumGreen). The shapes of the nuclei are highlighted with artificial white lines and ovals. Scale bars (grey): 5 µm.

2.3 Sat-RNA Expression Studies

As previously reported by Ljubić et al. [16] for prostate cancer samples, we studied Sat-RNA expression in 11 selected BCa samples. Cases 2-3 and 19 were chosen as negative controls, and cases 12, 25, 32-35, and 38-39 as such, with different grades of α-Sat-DNA amplification. 5-15 FFPE tissue samples (according to tumor size) were cut into 4 µm sections and collected in a tube. We extracted RNA and transcribed it into cDNA, as described in [16]. Quantitative PCR was done with primers specific for α-Sat-RNA as described in [16], as ALPHAfw: CACTCTTTTTGTAGAATCTGC and ALPHrev: AATGCACACATCACAAAGAAG, under conditions described in [16], with housekeeping gene controls Glucuronidase β (GUSB) specific primers GUSfw GAAAATATGTGGTTGGAGAGAGCATT and GUSrev CCGAGTGAA-GATCCCCTTTTTA [16].

2.4 Ethics Statement

The study was conducted in accordance with the Declaration of Helsinki and approved by the Research Ethics Committee Office (RECO) of the Kurdistan Regional Government-Iraq under registration number 429/06. Informed consent was obtained from all subjects involved in the study.

3. Results

In 20 of the 47 studied samples (~43%) *ErbB2*/*Her2* gene amplification was present. 13 cases showed an HSR pattern and seven cases showed a DM pattern (Figure 2).

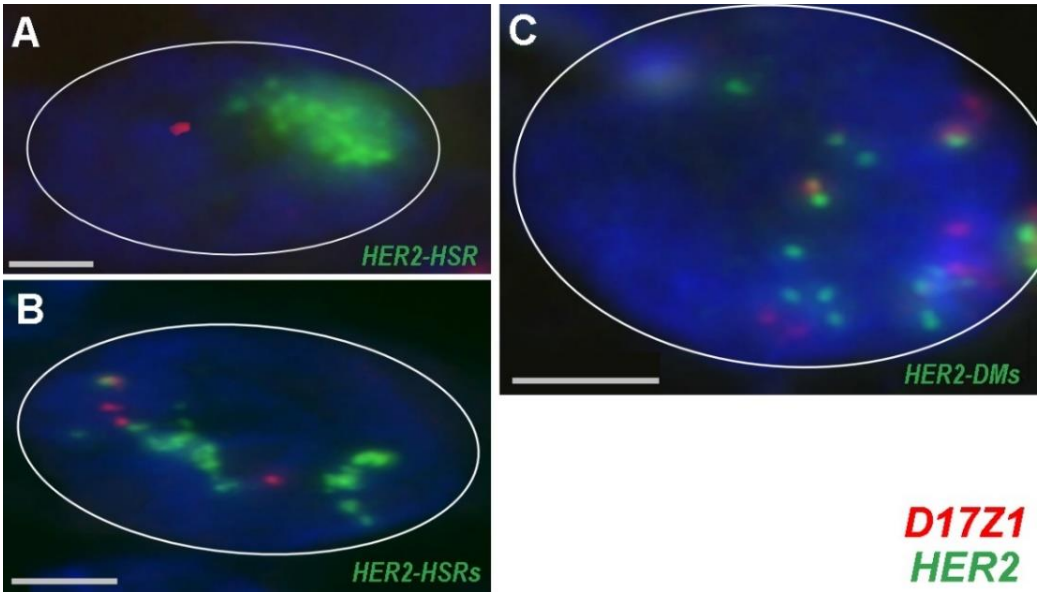


Figure 2 iFISH results of *ErbB2*/*Her2* gene amplification (green signals) from three cases are shown as in Figure 1 for Sat-DNA amplification. The red signals represent centromere 17 (D17Z1). In panels A (case #32) and B (case #39) *ErbB2*/*Her2* gene amplification is present as homogeneously-staining regions (HSRs), and in panel C (case #47) as double minute (DM) structures. Scale bars (grey): 5 µm.

Table S2 and Figure S1 show that all cases displayed massive CIN for most of the Sat-DNA probes applied.

15 of 47 BCa samples displayed Sat-DNA amplification; three of 15 cases showed two different Sat-DNA stretches derived from different chromosomes in the same percentage of the cells, each. Sat-DNA amplification was observed in 5 to 100% of the studied cells.

Sat-DNA amplification was observed as HSR-like structures in 13 cases and as DMs in two cases. Sat-III-DNA derived from chromosomes 1 (1q12/HSat3) and 9 (D9Z3) was amplified in eight and one case, respectively; α-Sat-DNA D17Z1 and D18Z1 were amplified in five and two cases, respectively; α-Sat-DNA, derived from chromosomes 9 and 11, was amplified in one case each (Figure 1).

Figure 3 shows the relationship between Sat-DNA amplification and various clinical parameters, including grading (Figure 3A), staging (Figure 3B), Ki67 status (Figure 3C), age of the patients (Figure 3D), lymph node status/metastases (Figure 3E), tumor size (Figure 3F), and *ErbB2*/*Her2* status (Figure 3G). The latter is depicted as immuno-status [Her2 immuno], *ErbB2*/*Her2* gene amplification status [Her2 FISH] and the presence of both: positive *ErbB2*/*Her2* immuno and/or amplification status versus negative for both tests. Sat-DNA amplification showed positive correlation with grading, Ki67 status and Her2 status. No clear interrelation was found between Sat-DNA amplification and staging, patient age, lymph node status, or tumor size.

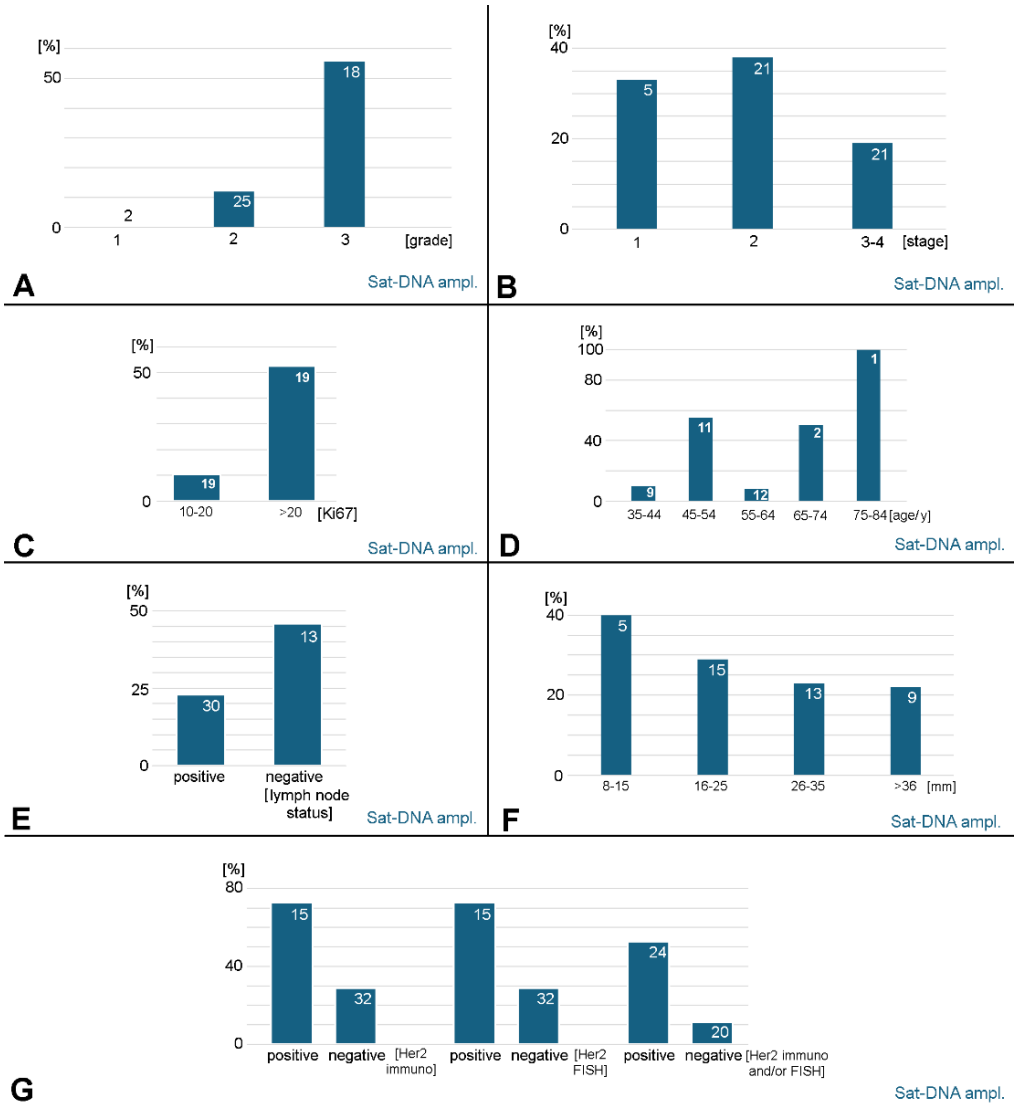


Figure 3 The relationship between Sat-DNA amplification and clinical parameters. (A) grading, (B) staging, (C) Ki67 status, (D) patient age, (E) lymph node status/metastases,

(F) tumor size, and (G) *ErbB2*/*Her2* status are shown. The white figures in the blue bars show the number of cases included from Table 1 for each. The y-axes show the percentage of cases with the corresponding features mentioned in Figures A through G. The x-axes show in (A) grades 1-3, in (B) stages 1-4, in (C) Ki67 values below or above 20%, in (D-G) the sat-DNA-amplification according to the patient's age (D), the lymph node status (E), the tumor size (F) and the Her2- immuno-, -FISH- and combined immuno/FISH status.

The same alignments were performed for *ErbB2*/*Her2* gene amplification status by iFISH for comparison. The results are shown in Figure S2. There were positive correlations with staging, Ki67 status, lymph node status and Her2 immuno-status. No clear interrelation was found with grading, patient age, or tumor size. This section may be divided by subheadings. It should provide a concise and precise description of the experimental results, their interpretation, as well as the experimental conclusions that can be drawn.

Sat-RNA expression studies were performed in eleven samples (Table 2). In four of the samples, no result could be obtained, most likely due to low RNA quality (cases 19, 32, 33 and 38). Results for the remaining cases are shown in Figure 4. Cases 2 and 3 gave the expected results as negative controls: no relevant α-Sat-RNA expression was found compared to housekeeping gene expression. Cases 12, 34, 35 and 39 showed enhanced α-Sat-RNA expression. These cases had between 30 and 100% of cells with different α-Sat-DNA amplifications. In case 25, only minimal α-Sat-RNA expression was found, which may be because only 20% of the studied cells showed 1q12/HSat3 amplification. Case 39 also showed comparatively low α-Sat-RNA expression, even though all studied cells in FISH showed D11Z1 amplification. This may reflect tumor heterogeneity between samples at the DNA and RNA levels.

Table 2 Laboratory findings of the 11 selected breast cancer (BCa) studied cases together with iFISH and qPCR results from this study in the last three columns.

Patient #	Grade	Stage	*ErbB2*/*Her2* FISH-amplification	Sat-DNA amplification	Sat-RNA amplification x-fold in comparison to GUSB
2	1	IIIA	-	-	0.1
3	2	n.a.	-	-	0.1
12	2	IIA	HSR	1q12/HSat3 30%	2.8
19	2	IIIA	HSR	-	Did not work
25	2	IIIA	HSR	1q12/HSat3 20%	0.1
32	3	IIA	HSR	D17Z1/D18Z1 100%	Did not work
33	3	IIA	HSR	D9Z4 40%	Did not work
34	3	IIA	HSR	1q12/HSat3 20%	9.7
35	3	IIA	HSR	1q12/HSat3 100%	5.7
38	3	IIIA	HSR	1q12/HSat3 40%	Did not work
39	3	IIIA	HSR	D11Z1 100%	1.4

Abbreviations: DM = double minutes; HSR = homogeneously staining region; n.a. = data not available.

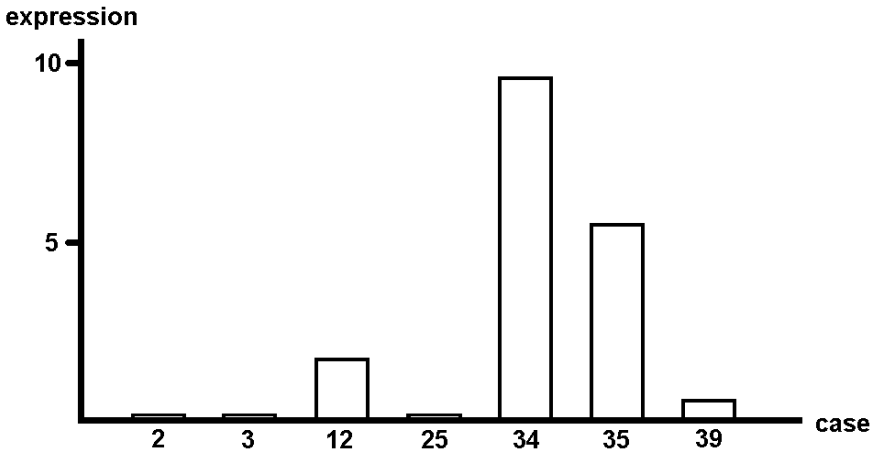


Figure 4 Results of α-Sat-RNA expression in the seven informative cases. The expression levels of α-Sat-RNA are compared to those of the housekeeping gene control. I.e. Glucuronidase β (GUSB)-expression was set as x1 and corresponding expression levels of α-Sat-RNA were comparatively plotted here (see also Table 1).

4. Discussion

In the present study, the presence of chromosomal imbalances was assessed using iFISH with 23 commercially available Sat-DNA probes for all 22 autosomes and the female gonosomes, plus a probe for *ErbB2*/*Her2* gene (Table S1).

At first, the *ErbB2*/*Her2* gene amplification status was determined using iFISH. The well-known positive correlations between *ErbB2*/*Her2* gene amplification and staging, Ki67 status, lymph node status and Her2 immuno-status as reported by Joensuu et al. [17], were also observed in our samples. The absence of a clear positive correlation with grading and tumor size, as reported in the literature [17], may be indicative of a small sample size. This finding highlights the fact that, apart from two cases classified as grade I, all cases included in this study were grade II or III. As expected [17], age of the patient had no correlation with *ErbB2*/*Her2* gene amplification status.

Furthermore, we determined chromosome loss and gains (Table S2, Figure S1) and found the expected high CIN rate. There are no chromosome-specific tendencies of gains and losses for the centromeric/Sat-DNA regions studied. This is not consistent with data on euchromatic imbalances in BCa. These comparative genomic hybridization-based studies [18] did not assess the regions studied here. However, these studies primarily observed gains or losses of chromosomal arms and whole chromosomes. For example, loss of the short arm of chromosome 8 combined with gain of its long arm. This suggests that centromeres, such as that of chromosome 8, may be lost or gained according to the underlying type of tumor associated aberration and/or breakpoints [18].

This study tested the hypothesis, based on previous studies of prostate cancer [15] and liposarcoma [14], that Sat-DNA amplification can be observed in many, if not all, advanced solid tumors [14]. This idea was checked in 47 BCa samples, and Sat-DNA amplification was found in 15 of the 47 tested cases (32%). This rate is higher than that originally suggested for solid tumors in general (~20%) [14], and found in prostate cancer (16%) [15]. Sat-DNA amplification was found as HSRs in 13 of the 15 cases (85%) and as DMs in the remaining two cases. All corresponding cases were stage II to IV (except for two cases), grade 2 or 3, and had Ki67 levels >20 (except for two cases). Figure 3 summarizes the correlations observed for Sat-DNA amplification and all parameters also

checked for *ErbB2*/*Her2* gene amplification (Figure S2). Sat-DNA amplification was positively correlated with advanced tumor grade, positive Ki67- and *ErbB2*/*Her2* immunohistochemistry status and/or gene amplification. Patient age showed no correlation with Sat-DNA amplification. However, staging and lymph node status seemed to be negatively correlated with Sat-DNA amplification. This may be attributed to the small sample size, which is relevant as substantiated by the above-discussed *ErbB2*/*Her2* amplification data. Conversely, this could suggest that Sat-DNA amplification is a step in the development of advanced BCa tumors that occurs independently of *ErbB2*/*Her2* amplification.

The studies described in this paper are the first to provide evidence that α-Sat-DNA amplification leads to increased α-Sat-RNA expression. Previously, this could only be suggested based on our own FISH studies in advanced prostate cancer cases showing α-Sat-DNA amplification [15], and on the studies of others in different prostate cancer samples showing α-Sat-RNA overexpression [16]. In parallel, we have also demonstrated the presence of α-Sat-RNA overexpression in our own cohort of advanced prostate cancer cases (unpublished data).

5. Conclusions

Overall, this study and two previous ones [15, 16] indicate that sat-DNA amplification may be an overlooked feature of advanced solid tumors. This oversight is partially due to limitations of standard high-throughput approaches over the past few decades, which could access only euchromatic genomic regions rather than heterochromatic ones [19]. These regions were seemingly falsely attributed as meaningless for disease development [13, 14]. However, T2T-sequencing approaches recently closed this technical gap [19]. Further research may reveal that Sat-DNA amplification is a biomarker worthy of inclusion in the Vogelstein model of cancer development.

Author Contributions

Conceptualization, T.L. and M.T.; methodology, M.M., K.S., S.K., P.B., and B.T.; software, T.L.; validation, M.M., K.S., S.K., P.B., and T.L.; formal analysis, M.M., K.S., and T.L.; investigation, M.M., S.K., P.B., B.T. and B.M.H.; resources, B.M.H., M.T. and T.L.; data curation, T.L.; writing—original draft preparation, T.L.; writing—review and editing, M.M., K.S., S.K., P.B., B.T., B.M.H., M.T. and T.L.; visualization, M.M. and T.L.; supervision, T.L.; project administration, T.L. and B.M.H. All authors have read and agreed to the published version of the manuscript.

Competing Interests

The authors have declared that no competing interests exist.

Data Availability Statement

All data is included in this paper, its supplements and corresponding references.

AI-Assisted Technologies Statement

No generative artificial intelligence or any AI-assisted tools has been used.

Additional Materials

The following additional materials are uploaded at the page of this paper.

1. Figure S1: Distribution of iFISH signals per Sat-DNA probes used in the tested cases (Table 1). The percentage of cases that showed no copy number variation (CNV), loss or gain of copy numbers (CN), or chromosome instability without a clear tendency toward loss or gain.
2. Figure S2: The relationship between *ErbB2/Her2* gene amplification and clinical parameters. (A) grading, (B) staging, (C) Ki67 status, (D) patient age, (E) lymph node status/metastases, (F) tumor size, and (G) *ErbB2/Her2* immuno-status are shown. The white figures in the blue bars of the charts provide the number of cases included from Table 1, each.
3. Table S1: Commercially available DNA-probes used in this study for heterochromatic regions of human genome and for one locus known to be altered in breast cancer.
4. Table S2: Copy number changes of satellite DNA regions tested and in *ErbB2*/*Her2* region, given for every BCa sample.

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