FGD5 amplification in breast cancer patients is associated with tumour proliferation and a poorer prognosis

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Purpose: Proliferation is a hallmark of cancer. Using a combined genomic approach, *FGD5* amplification has been identified as a driver of proliferation in Luminal breast cancer. We aimed to describe FGD5 copy number change in breast cancer, and to assess a possible association with tumour proliferation and prognosis. Methods: We used fluorescence *in situ* hybridization targeting *FGD5* and chromosome 3 centromere (CEP3) on formalin-fixed, paraffin-embedded tissue from 430 primary breast cancers and 108 lymph node metastases, from a cohort of Norwegian breast cancer patients. We tested the association between *FGD5* copy number status and proliferation (assessed by Ki67 levels and mitotic count) using Pearson's Chi-square test, and assessed the prognostic impact of *FGD5* copy number change by estimating cumulative risks of death and hazard ratios. Results: We identified *FGD5* amplification (defined as FGD5/CEP3 ratio ≥2 or mean FGD5/tumour cell ≥4) in 9.5% of tumours. Mitotic count and Ki67 levels were higher in tumours with *FGD5* copy number increase, compared to tumours with no copy number change. After 10 years of follow-up, cumulative risk of death from breast cancer was higher among cases with *FGD5* amplification (48.1% (95% CI 33.8-64.7)), compared to non-amplified cases (27.7% (95% CI 23.4-32.6)). Conclusions: *FGD5* is a new prognostic marker in breast cancer, and increased copy number is associated with higher tumour proliferation and poorer long-term prognosis.

Key words: Breast cancer, *FGD5*, FISH, gene amplification, proliferation, prognosis

Introduction

Sustaining proliferative signaling is a hallmark of cancer [1], and the proliferation marker Ki67 is included in current treatment guidelines for breast cancer patients [2]. Given the crucial role of proliferation, identification of essential proliferation-associated genes could be important for prognostication and development of targeted treatment.

Using data from two independent datasets [3, 4], and by combining gene expression and copy number analysis data with data from a genome-wide RNA-mediated interference screen on breast cancer cell lines, Gatza et al identified eight essential genes (*FGD5, METTL6, CPT1A, DTX3, MRPS23, EIF2S2, EIF6* and *SLC2A10*) uniquely amplified in highly proliferative luminal (non-basal) breast tumours [5]. Amplification of four of these genes (*FGD5, METTL6, DTX3* and *MRPS23*) was associated with poorer prognosis. *FGD5 (*Facio-Genital Dysplasia 5), located on the short arm of chromosome 3(3p25.1)[6], is a member of the FGD family, and mutations in *FGD1* results in Faciogenital Dysplasia[7]. Genetic and epigenetic changes on the short arm of chromosome 3 have been found in epithelial tumours, including breast cancer [8-10]. *FGD5* methylations and deletions have been identified in cervical [11], lung [12] and renal cell carcinomas [13]. Molecular mechanisms explaining an association between *FGD5* amplification and tumour cell proliferation in breast cancer are unknown. Furthermore, an *in situ* assessment of *FGD5* copy number change in breast cancer tissue has to our knowledge, not previously been performed.

Using a cohort of Norwegian breast cancer patients, the aims of this study were threefold. First, to characterize *FGD5* copy number change using fluorescence *in situ* hybridization (FISH) on formalin-fixed, paraffin-embedded primary tumour tissue and lymph node metastases; second, to assess the association of *FGD5* copy number change with

proliferation and known prognostic factors such as histologic grade and molecular subtype, and third, to evaluate the association of *FGD5* copy number status with prognosis.

Materials and methods

Study population

A population-based survey for early detection of breast cancer was performed in Nord-Trøndelag County, Norway in 1956-59, and 25,727 women born 1886-1928 were invited. These women were followed for breast cancer occurrence from 1961-2008 through the Cancer Registry of Norway. Information on date and cause of death was obtained from the Norwegian Cause of Death Registry. The cohort has previously been described in detail [14]. Briefly, 1379 incident breast cancers occurred from 1961-2008, and 909 were reclassified into molecular subtypes. For the present study, FISH was carried out on cases diagnosed after 1985 (n=453). Of these, five were excluded due to missing or insufficient tumour tissue, and 18 were excluded due to unsuccessful FISH. Thus, 430 cases were suitable for assessment of *FGD5* and chromosome 3 centromere (CEP3) copy number in primary tumours.

Of the 430 cases, 146 were lymph node positive at diagnosis, and tissue was available for 115 of these. Two cases were later excluded due to insufficient tumour tissue, and five were excluded due to unsuccessful FISH. Thus, 108 cases were suitable for assessment of *FGD5* and CEP3 copy number in lymph node metastases.

Specimen characteristics

All cases were previously classified according to histopathological type and grade [14], and tissue microarrays (TMA) were constructed using the Tissue Arrayer Minicore® 3 with TMA Designer2 software (Alphelys, 78370 Plaisir, France). Three 1 mm tissue cores from the tumour periphery were assembled in recipient blocks. Tumours were reclassified into molecular subtypes using immunohistochemistry (IHC) and chromogenic *in situ* hybridization (CISH) as surrogates for gene expression analysis (Supplementary Figure 1). The following antibodies were used: oestrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), Ki67, and basal markers cytokeratin 5 (CK5) and epidermal growth factor receptor (EGFR) (Supplementary Table 1) [14-16]. HER2 gene status was assessed using CISH.

TMAs were constructed from lymph node metastases, and 4 µm sections were mounted on Superfrost+ glass slides, dried at 37°C overnight, and stored in the freezer (-20°C). Slides were stained with HES. For immunohistochemistry, slides were heated at 60°C for 2 hours, and pre-treatment was carried out in a PT Link, Pre-Treatment Module for Tissue Specimens (Dako Denmark A/S, 2600 Glostrup, DK) with buffer (Low pH Target Retrieval Solution K8005) at 97°C for 20 minutes. Immunostaining for Ki67 was carried out in a DakoCytomationAutostainer Plus (Dako) (Supplementary Table 1). Dako REAL™EnVision™ Detection System with Peroxidase/DAB+, Rabbit/Mouse, code K5007, was used for visualization.

FGD5 and CEP3 FISH was done according to the manufacturer's guidelines with some modifications, using Dako Histology Accessory Kit K5799. After de-waxing and rehydration, slides from primary tumours and lymph node metastases were boiled in a microwave oven (10 minutes) in Pre-treatment Solution, cooled (15 minutes), and washed in Wash Buffer (3 minutes x 2). Protein digestion of tissue samples was performed with Dako Pepsin Solution

at 37 ˚C (7 minutes), and then rinsed in Dako Wash Buffer (3 minutes x 2). Dehydration was done in ethanol (70%, 85%, 100%), for 2 minutes at each concentration, and slides were air-dried at room temperature (10 minutes).

FISH custom probeFGD5 (2 µL) (code G110996R-8, Agilent Technologies) and SureFISHChr3 CEP (2 µL) (G101065G-8, Agilent Technologies) were mixed in IQFISH Fast Hybridization buffer (18µL) (code G9415A, Agilent Technologies) and applied to TMA slides. Coverslips were applied and sealed with Dako Coverslip Sealant. Denaturation was performed at 80 ˚C (10 minutes) and hybridization was done at 45 ˚C for 120 minutes using Dako Hybridizer. Post-hybridization wash was done with Dako Stringent Wash Buffer at 62.5˚C (10 minutes), and with Dako Wash Buffer (3 minutes x 2). Slides were air-dried at 37 ˚C (30 minutes), mounted with Dako Fluorescence Mounting Medium, and coverslipped.

The REMARK criteria for reporting tumour marker studies were followed [17].

Scoring and reporting

FGD5 and CEP3 copy number were assessed in a fluorescence microscope (Nikon Eclipse 90i). For each case, all available TMA spots were examined, and the proportion of tumour cells with >2 FGD5 copies/cell was recorded. *FGD5* and CEP3 copy number in 20 non-overlapping, well-preserved tumour cells was then recorded, and if present, tumour cells with *FGD5* copy number increase (>2) were selected. The observer was blinded for other tumour data. For each case, a gene to chromosome ratio was estimated, dividing the sum of *FGD5* copies by the sum of CEP3 copies in 20 tumour cells.

To assess the impact of *FGD5*/CEP3 ratio, cases were divided into three categories: 1a) Cases with ≤2 *FGD5* copies/nucleus in all tumour cells; 1b) Cases with >2 *FGD5* copies/nucleus in some tumour cells, and *FGD5*/CEP3 ratio <2; and 1c) Cases with >2 *FGD5* copies/nucleus in some tumour cells, and *FGD5*/CEP3 ratio ≥2.

To assess the impact of *FGD5* copy number change regardless of *FGD5*/CEP3 ratio, mean *FGD5* copy number was estimated for each case. Cases were then divided into three categories: 2a) Cases with mean *FGD5* copies/nucleus ≤2; 2b) cases with mean *FGD5* copies/nucleus >2<4; and 2c) Cases with mean *FGD5* copies/nucleus ≥4.

Finally, tumours were defined as amplified when *FGD5*/CEP3 ≥2 and/or mean *FGD5* ≥4 (Category 3b), and as non-amplied when *FGD5*/CEP3 <2 and mean *FGD5* <4 (Category 3a).

Statistical analyses

We used Pearson's Chi-square test to compare proportions of patient and tumour characteristics across categories of *FGD5* copy number status in primary tumours and lymph node metastases. For each category of *FGD*5 status in primary tumours, cumulative incidence of death from breast cancer was estimated, considering death from other causes a competing event. Gray's test was used to test for equality between cumulative incidence curves. We used Cox proportional hazards models to estimate hazard ratios (HRs) of death from breast cancer (with 95% confidence intervals (CIs)) according to *FGD5* status in primary tumours, censoring at time of death from other causes. Category 1a was used as the reference in the assessment of prognosis according to *FGD5*/CEP3 ratio, category 2a in analyses according to mean *FGD5* copy number/nucleus, and category 3a in assessment of

prognosis according to *FGD5* amplification status. Adjustments were made for other prognostic factors at baseline, including age (≤49, 50-59, 60-64, 65-69, 70-74, ≥75 years), stage (I, II, III, IV), histological grade, Ki67 (</≥ 15%), and molecular subtype. Adjustments were made for each variable separately, and for all variables combined. No clear violations of proportionality were observed in log-minus-log plots. Stata version 13.1 (Stata Corp., College Station, TX, USA) was used for the statistical analyses.

Results

Mean age at diagnosis was 76.0 years, and mean follow-up after diagnosis was 8.0 years (Table 1).

FGD5 in the primary tumours

FISH analysis of breast cancer tumours revealed three distinct patterns, denoted a, b and c (Figure 1): Cases with a maximum of 2 *FGD5* and CEP3 copies in all nuclei (a); cases with copy number >2 for both *FGD5* and CEP3 (b), and cases with copy number >2 for *FGD5,* but not for CEP3 (c). We identified tumour cells with >2 copies of *FGD5* in 308 cases (72%, Table 1). A total of 26 cases (6%) had *FGD5*/CEP3 ratio ≥2, 229 cases (53%) had a mean *FGD5* copy number >2<4, and 32 cases (7%) had a mean copy number ≥4. By defining *FGD5* amplification as *FGD5*/CEP3 ratio ≥2 and/or mean copy number ≥4, 41/430 cases (9.5%) were found to be amplified.

The distribution of cells with increased *FGD5* copy number was focal in many cases, either as dispersed single cells, or as small clusters of cells. Among all cases with *FGD5* copy number increase, the median proportion of tumour cells with >2 *FGD5* copies/cell was 10% (IQR 4-20%). Among amplified cases (FGD5/CEP3 ratio≥2 and/or mean FGD5≥4), the median proportion was higher (20%, IQR 15-40).

FGD5 copy number increase was identified within all molecular subtypes, and amplified cases were found in all molecular subtypes except the 5 negative phenotype (Table 1).

FGD5 in lymph node metastases

FISH analysis showed that the three phenotypic patterns (a, b, c) described above were also present in the lymph node metastases.

We identified tumour cells with >2 copies of *FGD5* in 91/108 cases (84%, Table 2) with lymph node metastases. *FGD5* copy number increase was identified in lymph node metastases in 25 of the 34 cases (74%) that showed no evidence of *FGD5* copy number increase in the primary tumour (Table 2). Of the 74 cases that had *FGD5* copy number increase in the primary tumour, 66 (89%) also had cells with >2 *FGD5* copies in the lymph node metastases. Only two cases (2%) had *FGD5*/CEP3 ratio ≥2 in the lymph node metastases. When cases were categorized according to mean *FGD5*/tumour cell irrespective of CEP3 copy number, 65 cases (60%) had mean *FGD5*/tumour cell >2<4, and 5 cases (5%) had mean *FGD5*/tumour cell ≥4. *FGD5* amplification (*FGD5*/CEP3>2 and/or mean *FGD5*≥4) was found in 6 cases (5.5%).

The distribution of cells with *FGD5* copy number increase was often focal and dispersed. Among all cases with *FGD5* copy number change in the lymph nodes, the median proportion of tumour cells with >2 copies of *FGD5* was 9% (IQR 4-20). Among amplified cases, the median proportion was higher (25%, IQR 20-30).

FGD5, proliferation and histological grade

Mitotic count was higher in amplified, compared to non-amplified tumours (29% vs. 21% in highest quartile, p<0.001, Table 1). Ki67 levels were also higher in amplified tumours (54% vs. 36% had Ki67≥15%, p= 0.026). Cases with *FGD5* amplification had a higher proportion of grade 3 tumours, compared to non-amplified cases (59% vs. 28%, p<0.001).

We found no clear association between *FGD5* copy number increase and Ki67 levels in lymph node metastases (Supplementary Table 2).

FGD5 and prognosis

Prognosis according to *FGD5*/CEP3 ratio

For cases without *FGD5* copy number increase (Category 1a), cumulative risk of death from breast cancer after 10 years of follow-up was 23.7% (95% CI 16.9-32.7) (Table 3, Figure 2). For Category 1b and 1c, the corresponding cumulative risks of death were higher (30.5% (95% CI 25.4-36.5) and 47.0% (95% CI 29.9-67.8), respectively).

Comparing rates of death between categories, we found higher rates among patients in Category 1b and 1c, compared to Category 1a (age-adjusted HRs of 1.4 (95% CI 0.9-2.1)

and 2.6 (95% CI 1.3-4.9), respectively). Adjustments for grade, stage, Ki67, and molecular subtypes gave similar results, regardless of whether adjustments were made for each variable separately, or for all variables combined.

Prognosis according to mean *FGD5* copy number/tumour cell

Cumulative risk of death from breast cancer increased with increasing mean *FGD5* copies/nucleus (Table 3, Figure 2). For cases with mean *FGD5*/cell ≤2 (Category 2a), cumulative risk after 10 years of follow-up was 22.6% (95% CI 16.8-29.9). For cases with mean *FGD5/*cell >2<4 (Category 2b), the corresponding risk was 32.1% (95% CI 26.3-38.8), and for patients with mean FGD5/tumour cell ≥4 (Category 2c), risk of death was 49.6% (95% CI 33.3-68.5).

Comparing rates of death, we found higher rates among patients in Category 2b and 2c, compared to Category 2a (age-adjusted HRs of 1.6 (95% CI 1.1-2.4) and 2.6 (95% CI 1.4-4.6), respectively). Adjustments for grade, stage, Ki67, and molecular subtype gave similar results for category 2b, both after adjustment for each variable separately, and for all variables combined. For category 2c, the HR after adjustment for all factors combined was attenuated to 1.6 (95% CI 0.8-3.0).

Prognosis according to *FGD5* amplification status

For non-amplified cases, cumulative risk of death from breast cancer after 10 years of follow-up was 27.7% (95% CI 23.4-32.6) (Table 4, Figure 2). For amplified cases (*FGD5*/CEP3

ratio ≥2 and/or mean *FGD5* ≥4), the corresponding cumulative risk of death was higher (48.1% (95% CI 33.8-64.7)).

We found higher rates of death from breast cancer among amplified cases, compared to non-amplified cases (age-adjusted HR 2.0 (95% CI 1.2-3.2). After adjustments for age, grade, stage, Ki67, and molecular subtype, the HR was attenuated to 1.4 (95% CI 0.8-2.3).

FGD5 and prognosis within molecular subtypes of breast cancer

For Luminal A cases, cumulative risk of death 10 years after diagnosis was higher for cases with *FGD5* copy number increase, compared to cases without (Supplementary Table 3, Supplementary Figure 2). For category 1a, cumulative risk of death 10 years after diagnosis was 20% (95% CI 12.1-32.1), and for category 1c, the corresponding risk was 27.7% (95% CI 9.9-63.7). Category 1b had a better prognosis than category 1a five years after diagnosis. For category 2a, cumulative risk after 10 years was 16.1% (95% CI 10.0-25.4), and for category 2c, the corresponding risk was 34.8% (95% CI 16.1-64.9).

FGD5 amplified Luminal A cases had a higher risk of death from breast cancer than non-amplified cases. The cumulative risk of death estimates 10 years after diagnosis were 34.3% (95% CI 17.0-61.2) and 21% (95% CI 15.9-27.4), respectively (Supplementary Table 4).

Comparing rates of death between categories, we found a poorer prognosis with increasing *FGD5*/CEP3 ratio and with increasing mean *FGD5*, and for amplified cases compared to non-amplified cases (Supplementary Table 3 and 4). For the remaining subtypes, statistical power was considered too limited for separate survival analyses.

Discussion

We identified *FGD5* copy number increase in primary tumours and lymph node metastases in a large proportion of breast cancer patients. *FGD5* amplification in the primary tumour was associated with higher proliferation, and poorer survival.

Using *FGD5*/CEP3 ratio ≥2 and/or *FGD5* copy number ≥4 as a definition of gene amplification, 41 cases (9.5%) were amplified. We are not aware of other studies where *FGD5* gene amplification status in breast cancer has been assessed by FISH. However, in the TCGA data set, 15% of cases were found to be *FGD5* amplified by copy number analysis, whereas in the METABRIC dataset 3% were amplified [5].

In accordance with Gatza et al, we found that *FGD5* amplification was associated with higher proliferation and poorer prognosis. In our study, *FGD5* amplified cases had a higher mitotic count and higher Ki67 levels than non-amplified cases. The prognosis of patients with *FGD5* copy number increase was poorer both when analyses were based on *FGD5*/CEP3 ratio and on mean *FGD5* copy number. Comparing rates of death between amplified and non-amplified cases, we found that associations were attenuated and less clear after adjustments for grade, Ki67 levels, stage and molecular subtype. The question is whether adjustments for these factors are justified. If *FGD5* amplification is a driver of proliferation, as suggested by Gatza *et al* [5], then consequences of *FGD5* amplification could, at least in part, be mediated through grade, stage and Ki67 levels. Although valuable from a prognostication perspective, adjustments for these factors could mask the overall role of *FGD5* as a driver of breast cancer progression [18].

In the study by Gatza et al, *FGD5* was uniquely amplified in highly proliferative luminal tumours. Subtyping was based on gene expression analysis, and luminal tumours

were defined as all tumours that were not Basal [5, 19]. We categorized tumours into six different subtypes based on IHC and ISH as surrogates for gene expression analysis, and found amplifications in all subtypes, except the 5 negative phenotype. Even though studies have shown good correlation between subtyping by gene expression and surrogate markers, classification by these two methods are not identical [20-22]. Furthermore, focal *FGD5* copy number increase may be easier to identify with an *in situ* technique such as FISH, than with copy number analysis. This could explain why we, contrary to Gatza, identified *FGD5* copy number increase in all molecular subtypes.

We found some Luminal A tumours with *FGD5* copy number increase. According to our subtyping algorithm, Luminal A tumours have Ki67 levels <15%, and are thus not highly proliferative. In this cohort, we have found that Ki67 levels in TMAs are generally lower than in the corresponding whole sections (unpublished data), a finding that is in accordance with others [23]. It is therefore possible that some of our Luminal A cases are misclassified Luminal B tumours. Furthermore, 5/18 (28%) of the *FGD5* amplified Luminal A tumours were histological grade 3. According to the recent St. Gallen Expert Consensus, histological grade 3 Luminal A tumours could represent misclassified Luminal B tumours [2].

Using FISH, we were able to study *FGD5* gene- and CEP3 copy number status while observing the morphology of breast cancer tumours. Thus, only invasive epithelial tumour cells were assessed, and the distribution and proportion of amplified cells could be evaluated. Amplification has been defined as a copy number increase in a segment of the genome [24], however there is no established gold standard as to how gene amplification should be defined. According to current HER2 treatment guidelines [25], both *HER2*/centromere 17 (CEP17) ratio and *HER2* copy number (regardless of ratio), are taken

into account [26, 27]. *FGD5* is a new marker with no available guidelines for assessment. By using a centromere probe in addition to the gene probe, we could assess the prognostic value of both *FGD5*/CEP3 ratio and of mean *FGD5* copy number. When present, cells with *FGD5* copy number increase were selected for assessment, even when such changes were seen only focally. One could argue that the overall mean copy numbers of *FGD5* and CEP3 would be a better way to report each tumour. However, this approach could mask the potential prognostic impact of the focal changes identified in this study.

FGD5 amplification status in primary tumours and lymph node metastases was assessed in TMAs, comprising three 1 mm tissue cores from each case. When present, amplifications were only identified in a proportion of tumour cells. Previous studies have shown good correlation between TMA and whole sections for other markers [28-30], but it is likely that intratumoural heterogeneity may have led to an underestimation of cases with gene amplification in the present study. The presence of *FGD5* amplified cells in the lymph nodes of some patients without findings in the primary tumour support this hypothesis. Thus, the observed associations of *FGD5* amplification with tumour characteristics and prognosis may underestimate the true effect of *FGD5* copy number increase. Still, a high number of comparisons were made, and with a limited number of amplified cases, the CIs were relatively wide.

Molecular mechanisms explaining the association between *FGD5* amplification and proliferation and prognosis are unknown. However, *FGD5* is expressed in hematopoietic stem cells [31] and in vascular endothelial cells, and it has been found to regulate the proangiogenic effect of vascular endothelial growth factor (VEGF), including network

formation, cell-matrix interaction, endothelial cell permeability, movement, proliferation and adhesion [32, 33].

We were able to reproduce the main findings from Gatza's study in our cohort of breast cancer patients, with a mean age at diagnosis of 76 years. It has been suggested that proliferation is a stronger prognostic factor in younger breast cancer patients [34]. Therefore, amplification of *FGD5*, a gene associated with proliferation, could be of greater relevance in prognostication of younger breast cancer patients. It is necessary to validate our findings in a cohort of younger breast cancer patients.

In conclusion, *FGD5* has been identified as an essential gene in breast cancer proliferation, making it valuable as a prognostic marker, and a potential target for treatment. Using FISH in a large, well-described cohort of breast cancer patients, we have demonstrated that *FGD5* amplification is associated with higher proliferation and a poorer prognosis.

Ethical standards

The study was approved by the Regional Committee for Medical and Health Sciences Research Ethics (REK, Midt-Norge, Norway, reference number 836/2009).

Conflicts of interest

The authors declare that they have no conflicts of interest.

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References

1. Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. Cell 144:646-674. doi: 10.1016/j.cell.2011.02.013
2. Coates AS, Winer EP, Goldhirsch A, Gelber RD, Gnant M, Piccart-Gebhart M, Thurlimann B, Senn HJ, Panel M (2015) -Tailoring therapies-improving the management of early breast cancer: St Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2015. Ann. Oncol. 26:1533-1546. doi: 10.1093/annonc/mdv221
3. Cancer Genome Atlas N (2012) Comprehensive molecular portraits of human breast tumours. Nature 490:61-70. doi: 10.1038/nature11412
4. Curtis C, Shah SP, Chin SF, Turashvili G, Rueda OM, Dunning MJ, Speed D, Lynch AG, Samarajiwa S, Yuan Y, Graf S, Ha G, Haffari G, Bashashati A, Russell R, McKinney S, Group M, Langerod A, Green A, Provenzano E, Wishart G, Pinder S, Watson P, Markowetz F, Murphy L, Ellis I, Purushotham A, Borresen-Dale AL, Brenton JD, Tavare S, Caldas C, Aparicio S (2012) The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. Nature 486:346-352. doi: 10.1038/nature10983
5. Gatza ML, Silva GO, Parker JS, Fan C, Perou CM (2014) An integrated genomics approach identifies drivers of proliferation in luminal-subtype human breast cancer. Nat. Genet. 46:1051-1059. doi: 10.1038/ng.3073
6. (2016) Genecards. The Human Gene Database. In:
7. Pasteris NG, Cadle A, Logie LJ, Porteous ME, Schwartz CE, Stevenson RE, Glover TW, Wilroy RS, Gorski JL (1994) Isolation and characterization of the faciogenital dysplasia (Aarskog-Scott syndrome) gene: a putative Rho/Rac guanine nucleotide exchange factor. Cell 79:669-678.
8. Braga E, Senchenko V, Bazov I, Loginov W, Liu J, Ermilova V, Kazubskaya T, Garkavtseva R, Mazurenko N, Kisseljov F, Lerman MI, Klein G, Kisselev L, Zabarovsky ER (2002) Critical tumor-suppressor gene regions on chromosome 3P in major human epithelial malignancies:

allelotyping and quantitative real-time PCR. Int. J. Cancer 100:534-541. doi: 10.1002/ijc.10511
9. Braga E, Pugacheva E, Bazov I, Ermilova V, Kazubskaya T, Mazurenko N, Kisseljov F, Liu J, Garkavtseva R, Zabarovsky E, Kisselev L (1999) Comparative allelotyping of the short arm of human chromosome 3 in epithelial tumors of four different types. FEBS Lett. 454:215-219.
10. Kok K, Naylor SL, Buys CH (1997) Deletions of the short arm of chromosome 3 in solid tumors and the search for suppressor genes. Adv. Cancer Res. 71:27-92.
11. Senchenko VN, Kisseljova NP, Ivanova TA, Dmitriev AA, Krasnov GS, Kudryavtseva AV, Panasenko GV, Tsitrin EB, Lerman MI, Kisseljov FL, Kashuba VI, Zabarovsky ER (2013) Novel tumor suppressor candidates on chromosome 3 revealed by NotI-microarrays in cervical cancer. Epigenetics : official journal of the DNA Methylation Society 8:409-420. doi: 10.4161/epi.24233
12. Dmitriev AA, Kashuba VI, Haraldson K, Senchenko VN, Pavlova TV, Kudryavtseva AV, Anedchenko EA, Krasnov GS, Pronina IV, Loginov VI, Kondratieva TT, Kazubskaya TP, Braga EA, Yenamandra SP, Ignatjev I, Ernberg I, Klein G, Lerman MI, Zabarovsky ER (2012) Genetic and epigenetic analysis of non-small cell lung cancer with NotI-microarrays. Epigenetics : official journal of the DNA Methylation Society 7:502-513. doi: 10.4161/epi.19801
13. Dmitriev AA, Rudenko EE, Kudryavtseva AV, Krasnov GS, Gordiyuk VV, Melnikova NV, Stakhovsky EO, Kononenko OA, Pavlova LS, Kondratieva TT, Alekseev BY, Braga EA, Senchenko VN, Kashuba VI (2014) Epigenetic alterations of chromosome 3 revealed by NotI-microarrays in clear cell renal cell carcinoma. BioMed research international 2014:735292. doi: 10.1155/2014/735292
14. Engstrom MJ, Opdahl S, Hagen AI, Romundstad PR, Akslen LA, Haugen OA, Vatten LJ, Bofin AM (2013) Molecular subtypes, histopathological grade and survival in a historic cohort of breast cancer patients. Breast Cancer Res. Treat. 140:463-473. doi: 10.1007/s10549-013-2647-2
15. Blows FM, Driver KE, Schmidt MK, Broeks A, van Leeuwen FE, Wesseling J, Cheang MC, Gelmon K, Nielsen TO, Blomqvist C, Heikkila P, Heikkinen T, Nevanlinna H, Akslen LA, Begin LR, Foulkes WD, Couch FJ, Wang X, Cafourek V, Olson JE, Baglietto L, Giles GG, Severi G, McLean CA, Southey MC, Rakha E, Green AR, Ellis IO, Sherman ME, Lissowska J, Anderson WF, Cox A, Cross SS, Reed MW, Provenzano E, Dawson SJ, Dunning AM, Humphreys M, Easton DF, Garcia-Closas M, Caldas C, Pharoah PD, Huntsman D (2010) Subtyping of breast cancer by immunohistochemistry to investigate a relationship between subtype and short and long term survival: a collaborative analysis of data for 10,159 cases from 12 studies. PLoS Med. 7:e1000279. doi: 10.1371/journal.pmed.1000279
16. Cheang MC, Chia SK, Voduc D, Gao D, Leung S, Snider J, Watson M, Davies S, Bernard PS, Parker JS, Perou CM, Ellis MJ, Nielsen TO (2009) Ki67 index, HER2 status, and prognosis of patients with luminal B breast cancer. J. Natl. Cancer Inst. 101:736-750. doi: 10.1093/jnci/djp082
17. McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM, Statistics Subcommittee of NCIEWGoCD (2006) REporting recommendations for tumor MARKer prognostic studies (REMARK). Breast Cancer Res. Treat. 100:229-235. doi: 10.1007/s10549-006-9242-8
18. Cole SR, Hernan MA (2002) Fallibility in estimating direct effects. Int. J. Epidemiol. 31:163-165.
19. Hoadley KA, Yau C, Wolf DM, Cherniack AD, Tamborero D, Ng S, Leiserson MD, Niu B, McLellan MD, Uzunangelov V, Zhang J, Kandoth C, Akbani R, Shen H, Omberg L, Chu A, Margolin AA, Van't Veer LJ, Lopez-Bigas N, Laird PW, Raphael BJ, Ding L, Robertson AG, Byers LA, Mills GB, Weinstein JN, Van Waes C, Chen Z, Collisson EA, Cancer Genome Atlas Research N, Benz CC, Perou CM, Stuart JM (2014) Multiplatform analysis of 12 cancer types reveals molecular classification within and across tissues of origin. Cell 158:929-944. doi: 10.1016/j.cell.2014.06.049

20. Nielsen TO, Parker JS, Leung S, Voduc D, Ebbert M, Vickery T, Davies SR, Snider J, Stijleman IJ, Reed J, Cheang MC, Mardis ER, Perou CM, Bernard PS, Ellis MJ (2010) A comparison of PAM50 intrinsic subtyping with immunohistochemistry and clinical prognostic factors in tamoxifen-treated estrogen receptor-positive breast cancer. Clin. Cancer Res. 16:5222-5232. doi: 10.1158/1078-0432.CCR-10-1282
21. Viale G, Slaets L, Bogaerts J, Rutgers E, van't Veer L, Piccart-Gebhart MJ, de Snoo FA, Stork-Sloots L, Russo L, Dell'Orto P, van den Akker J, Glas A, Cardoso F, Consortium T, the MI (2014) High concordance of protein (by IHC), gene (by FISH; HER2 only), and microarray readout (by TargetPrint) of ER, PgR, and HER2: results from the EORTC 10041/BIG 03-04 MINDACT trial. Ann. Oncol. 25:816-823. doi: 10.1093/annonc/mdu026
22. Nielsen TO, Hsu FD, Jensen K, Cheang M, Karaca G, Hu Z, Hernandez-Boussard T, Livasy C, Cowan D, Dressler L, Akslen LA, Ragaz J, Gown AM, Gilks CB, van de Rijn M, Perou CM (2004) Immunohistochemical and clinical characterization of the basal-like subtype of invasive breast carcinoma. Clin. Cancer Res. 10:5367-5374. doi: 10.1158/1078-0432.CCR-04-0220
23. Knutsvik G, Stefansson IM, Aziz S, Arnes J, Eide J, Collett K, Akslen LA (2014) Evaluation of Ki67 expression across distinct categories of breast cancer specimens: a population-based study of matched surgical specimens, core needle biopsies and tissue microarrays. PLoS One 9:e112121. doi: 10.1371/journal.pone.0112121
24. Albertson DG (2006) Gene amplification in cancer. Trends Genet. 22:447-455. doi: 10.1016/j.tig.2006.06.007
25. Goldhirsch A, Winer EP, Coates AS, Gelber RD, Piccart-Gebhart M, Thurlimann B, Senn HJ, Panel m (2013) Personalizing the treatment of women with early breast cancer: highlights of the St Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2013. Ann. Oncol. 24:2206-2223. doi: 10.1093/annonc/mdt303
26. Wolff AC, Hammond ME, Hicks DG, Dowsett M, McShane LM, Allison KH, Allred DC, Bartlett JM, Bilous M, Fitzgibbons P, Hanna W, Jenkins RB, Mangu PB, Paik S, Perez EA, Press MF, Spears PA, Vance GH, Viale G, Hayes DF, American Society of Clinical O, College of American P (2013) Recommendations for human epidermal growth factor receptor 2 testing in breast cancer: American Society of Clinical Oncology/College of American Pathologists clinical practice guideline update. J. Clin. Oncol. 31:3997-4013. doi: 10.1200/JCO.2013.50.9984
27. Rakha EA, Pinder SE, Bartlett JM, Ibrahim M, Starczynski J, Carder PJ, Provenzano E, Hanby A, Hales S, Lee AH, Ellis IO, National Coordinating Committee for Breast P (2015) Updated UK Recommendations for HER2 assessment in breast cancer. J. Clin. Pathol. 68:93-99. doi: 10.1136/jclinpath-2014-202571
28. Batistatou A, Televantou D, Bobos M, Eleftheraki AG, Kouvaras E, Chrisafi S, Koukoulis GK, Malamou-Mitsi V, Fountzilas G (2013) Evaluation of current prognostic and predictive markers in breast cancer: a validation study of tissue microarrays. Anticancer Res. 33:2139-2145.
29. Dekker TJ, Borg ST, Hooijer GK, Meijer SL, Wesseling J, Boers JE, Schuuring E, Bart J, van Gorp J, Mesker WE, Kroep JR, Smit VT, van de Vijver MJ (2012) Determining sensitivity and specificity of HER2 testing in breast cancer using a tissue micro-array approach. Breast Cancer Res. 14:R93. doi: 10.1186/bcr3208
30. Camp RL, Charette LA, Rimm DL (2000) Validation of tissue microarray technology in breast carcinoma. Lab. Invest. 80:1943-1949.
31. Gazit R, Mandal PK, Ebina W, Ben-Zvi A, Nombela-Arrieta C, Silberstein LE, Rossi DJ (2014) Fgd5 identifies hematopoietic stem cells in the murine bone marrow. J. Exp. Med. 211:1315-1331. doi: 10.1084/jem.20130428
32. Kurogane Y, Miyata M, Kubo Y, Nagamatsu Y, Kundu RK, Uemura A, Ishida T, Quertermous T, Hirata K, Rikitake Y (2012) FGD5 mediates proangiogenic action of vascular endothelial growth factor in human vascular endothelial cells. Arterioscler. Thromb. Vasc. Biol. 32:988-996. doi: 10.1161/ATVBAHA.111.244004

33. Nakhaei-Nejad M, Haddad G, Zhang QX, Murray AG (2012) Facio-genital dysplasia-5 regulates matrix adhesion and survival of human endothelial cells. Arterioscler. Thromb. Vasc. Biol. 32:2694-2701. doi: 10.1161/ATVBAHA.112.300074
34. Baak JP, van Diest PJ, Voorhorst FJ, van der Wall E, Beex LV, Vermorken JB, Janssen EA, Gudlaugsson E, other collaborators of the Multicenter Morphometric Mammary Carcinoma P (2007) The prognostic value of proliferation in lymph-node-negative breast cancer patients is age dependent. Eur. J. Cancer 43:527-535. doi: 10.1016/j.ejca.2006.10.001

Figure legends

Fig. 1 a) Breast cancer cell without increased numbers of *FGD5* or CEP3 signals. b) Breast cancer cell with increased numbers of *FGD5* and CEP3 signals. c) Breast cancer cell with increased numbers of *FGD5* signals

Fig. 2 Cumulative incidence of death from breast cancer according to *FGD5* copy number status. a) *FGD5* copy number status based on *FGD5*/CEP3 ratio (Gray's test: p=0.018). b) *FGD5* copy number status based on mean *FGD5* (Gray's test: p=0.0013). c) *FGD5* amplification status (Gray's test: p=0.0027)

Table 1. Characteristics of the study population.

		Categories defined by *FGD5*/CEP3 ratio				Categories defined by mean *FGD5*				Categories defined by amplification status		
	Study population	**Max. 2 *FGD5* copies/cell (1a)**	**<2[a] (1b)**	**≥2[b] (1c)**	**Chi²**	**≤2 (2a)**	**>2<4 (2b)**	**≥4 (2c)**	**Chi²**	***FGD5*/CEP3< 2 and mean *FGD5*<4**	***FGD5*/CEP3≥ 2 and/or mean *FGD5*≥4**	**Chi²**
N (%)	430 (100)	122 (28)	282 (66)	26 (6)		169 (39)	229 (53)	32 (7)		389	41	
Mean age at diagnosis (SD)	76.0 (7.7)	75.4 (7.4)	76.2 (7.8)	76.2 (8.2)		75.3 (7.3)	76.2 (8.0)	77.8 (7.5)		75.9 (7.7)	76.7 (8.0)	
Mean follow-up after diagnosis (SD)	8.0 (5.8)	8.4 (5.9)	8.1 (5.9)	6.2 (4.6)		9.0 (6.1)	7.6 (5.8)	6.1 (4.0)		8.2 (5.9)	6.1 (4.4)	
Deaths from BC (%)	139 (32)	30 (25)	96 (34)	13 (50)		41 (24)	82 (36)	16 (50)		119 (31)	20 (49)	
Deaths from other causes (%)	179 (42)	53 (43)	118 (42)	8 (42)		77 (46)	93 (41)	9 (28)		167 (43)	12 (29)	
Grade (%)												
I	53 (12)	17 (14)	34 (12)	2 (8)	p=0.003	25 (15)	26 (11)	2 (6)	p=0.001	50 (13)	3 (7)	P<0.001
II	243 (57)	82 (67)	151 (54)	10 (38)		107 (63)	125 (55)	11 (34)		229 (59)	14 (34)	
III	134 (31)	23 (19)	97 (34)	14 (54)		37 (22)	78 (34)	19 (59)		110 (28)	24 (59)	
Lymph node metastasis												
Yes	146 (34)	45 (37)	91 (32)	10 (38)	p=0.859	57 (34)	75 (33)	14 (44)	p=0.579	129 (33)	17 (41)	P=0.348
No	198 (46)	57 (47)	129 (46)	12 (46)		78 (46)	107 (47)	13 (41)		181 (47)	17 (41)	
Unknown histopathology[c]	86 (20)	20 (16)	62 (22)	4 (15)		34 (20)	47 (21)	5 (16)		79 (20)	7 (17)	
Tumour size												
≤2 cm	226 (53)	68 (56)	144 (51)	14 (54)	p=0.977	101 (60)	109 (48)	16 (50)	p=0.515	206 (53)	20 (49)	P=0.623
>2 cm, ≤5 cm	86 (20)	26 (21)	55 (20)	5 (19)		33 (20)	46 (20)	7 (22)		77 (20)	9 (22)	
>5 cm	10 (2)	3 (2)	7 (2)	0		4 (2)	6 (3)	0		10 (3)	0	
Uncertain, but >2 cm	48 (11)	12 (10)	33 (12)	3 (12)		14 (8)	30 (13)	4 (13)		42 (11)	6 (15)	
Uncertain	60 (14)	13 (11)	43 (15)	4 (15)		17 (10)	38 (17)	5 (16)		54 (14)	6 (15)	
Stage												
1	195 (45)	56 (46)	126 (45)	13 (50)	p=0.868	84 (50)	98 (43)	13 (41)	p=0.639	177 (46)	18 (44)	P=0.332
2	195 (45)	55 (45)	130 (46)	10 (38)		72 (43)	109 (48)	14 (44)		179 (46)	16 (39)	
3	24 (6)	8 (7)	15 (5)	1 (4)		9 (5)	12 (5)	3 (9)		20 (5)	4 (10)	

4	16 (4)	3 (2)	11 (4)	2 (8)		4 (2)	10 (4)	2 (6)		13 (3)	3 (7)	
Molecular subtype (%)												
Luminal A	238 (55)	72 (59)	155 (55)	11 (42)	p=0.098	101 (60)	122 (53)	15 (47)	p=0.241	220 (57)	18 (44)	P=0.250
Luminal B (HER2-)	102 (24)	27 (22)	68 (24)	7 (27)		39 (23)	54 ((24)	9 (28)		27 (7)	3 (7)	
Luminal B (HER2+)	30 (7)	6 (5)	22 (8)	2 (8)		8 (5)	20 (9)	2 (6)		91 (23)	11 (27)	
HER2 type	20 (5)	3 (2)	14 (5)	3 (12)		4 (2)	14 (6)	2 (6)		16 (4)	4 (10)	
5NP	10 (2)	7 (6)	3 (1)	0		7 (4)	3 (1)	0		10 (3)	0	
BP	30 (7)	7 (6)	20 (7)	3 (12)		10 (6)	16 (7)	4 (13)		25 (6)	5 (12)	
Ki67 high/low (%)												
Ki67<15%	268 (62)	84 (69)	173 (61)	11 (42)	p= 0.03	114 (67)	138 (60)	16 (50)	p=0.112	249 (64)	19 (46)	P=0.026
Ki67 ≥15%	162 (38)	38 (31)	109 (39)	15 (58)		55 (33)	91 (40)	16 (50)		140 (36)	22 (54)	
Mitoses/10 HPF, median (IQR p25, p75)	4.5 (1, 12)	2 (0, 8)	5 (1, 12)	9.5 (5, 17)		3 (1, 9)	5 (1, 12)	9 (5, 14)		4 (1, 11)	9 (5, 16)	
Mitoses/10HPF, quartiles (%)												
≤ 1	125 (29)	48 (39)	73 (26)	4 (15)	p=0.012	60 (36)	61 (27)	4 (13)	p=0.001	119 (31)	6 (15)	P<0.001
>1, ≤ 4.5	90 (21)	26 (21)	62 (22)	2 (8)		36 (21)	52 (23)	2 (6)		88 (23)	2 (5)	
>4.5, ≤12	121 (28)	28 (23)	82 (29)	11 (42)		44 (26)	59 (26)	18 (56)		100 (26)	21 (51)	
>12	94 (22)	20 (16)	65 (23)	9 (35)		29 (17)	57 (25)	8 (25)		82 (21)	12 (29)	

Abbreviations: Max.= maximum, SD= standard deviation, BC= breast cancer, HER2= human epidermal growth factor receptor 2, 5NP= 5 negative phenotype, BP= Basal phenotype, HPF= high power fields, IQR= interquartile range.

[a]Cells with >2 *FGD5* copies present, and *FGD5*/CEP3 ratio<2. [b]Cells with >2 *FGD5* copies present, and *FGD5*/CEP3 ratio≥2. [c] Includes cases where histopathological examination was done, but reports were not available, and cases where no axillary lymph nodes were removed.

Table 2. FGD5 status in primary tumours and lymph node metastases according to *FGD5*/CEP3 ratio and mean *FGD5*					
	FGD5/CEP3 ratio, primary tumours				
	Max. 2 *FGD5* copies/cell (1a)	**FGD5/CEP3<2[a] (1b)**	**FGD5/CEP3≥2[b] (1c)**	**Total**	**Chi$_2$**
FGD5/CEP3 ratio, lymph nodes					
Max. 2 *FGD5* copies/cell	9 (26)	7 (11)	1 (11)	17	p= 0.06
FGD5/CEP3<2[a]	25 (74)	57 (88)	7 (78)	89	
FGD5/CEP3≥2[b]	0	1 (2)	1 (11)	2	
Total	34	65	9	108	
	Mean FGD5/tumour cell, primary tumours				
	Mean FGD5≤2 (2a)	**Mean FGD5>2<4 (2b)**	**Mean FGD5≥4 (2c)**	**Total**	**Chi$_2$**
Mean FGD5/tumour cell, lymph nodes					
Mean *FGD5*≤2	26 (62)	11 (21)	1 (8)	38	p<0.001
Mean *FGD5*>2<4	16 (38)	41 (77)	8 (62)	65	
Mean *FGD5*≥4	0	1 (2)	4 (31)	5	
Total	42	53	13	108	

Abbreviation: Max.= maximum.

[a]Cells with >2 *FGD5* copies present, and *FGD5*/CEP3 ratio<2. [b]Cells with >2 *FGD5* copies present, and *FGD5*/CEP3 ratio≥2.

Table 3. Absolute and relative risks of death from breast cancer according to *FGD5*/CEP3 ratio and mean *FGD5*/ tumor cell						
	***FGD5*/CEP3 ratio, primary tumors**			**Mean *FGD5*/tumor cell, primary tumors**		
	Max. 2 *FGD5* copies/cell (1a)	**<2[a] (1b)**	**≥2[b] (1c)**	**≤2 (2a)**	**>2<4 (2b)**	**≥4 (2c)**
Cum. risk after 5 years (%) (95% CI)	15.3 (9.9-23.2)	22.0 (17.5-27.3)	30.8 (16.7-52.2)	14.6 (10.0-21.0)	23.0 (18.0-29.0)	35.3 (21.3-54.6)
Cum. risk after 10 years (%) (95% CI)	23.7 (16.9-32.7)	30.5 (25.4-36.5)	47.0 (29.9-67.8)	22.6 (16.8-29.9)	32.1 (26.3-38.8)	49.6 (33.3-68.5)
HR[c] adjusted for age (95% CI)	1.0	1.4 (0.9-2.1)	2.6 (1.3-4.9)	1.0	1.6 (1.1-2.4)	2.6 (1.4-4.6)
HR[c] adjusted for age, grade, stage, Ki67 (95% CI)	1.0	1.4 (0.9-2.1)	2.5 (1.3-4.9)	1.0	1.5 (1.0-2.1)	1.7 (0.9-3.1)
HR[c] adjusted for age, grade, stage, Ki67, and molecular subtype (95% CI)	1.0	1.4 (0.9-2.1)	2.5 (1.2-4.9)	1.0	1.4 (1.0-2.1)	1.6 (0.8-3.0)

Abbreviations: Cum.=Cumulative, CI= Confidence interval, HR=Hazard ratio

[a]Cells with >2 *FGD5* copies present, and *FGD5*/CEP3 ratio<2. [b]Cells with >2 *FGD5* copies present, and *FGD5*/CEP3 ratio≥2. [c]Hazard ratios (HR) from Cox regression analyses for the entire observation period.

Table 4. Absolute and relative risks of death from breast cancer according to *FGD5* amplification status.		
	***FGD5* amplification status, primary tumors**	
	***FGD5*/CEP3<2 and mean *FGD5*<4**	**FGD5/CEP3≥2 and/or mean *FGD5*≥4**
Cum. risk after 5 years (%) (95% CI)	18.8 (15.3-23.1)	37.2 (24.4-54.0)
Cum. risk after 10 years (%) (95% CI)	27.7 (23.4-32.6)	48.1 (33.8-64.7)
HR[a] adjusted for age (95% CI)	1.0	2.0 (1.2-3.2)
HR[a] adjusted for age, grade, stage, Ki67 (95% CI)	1.0	1.4 (0.9-2.3)
HR[a] adjusted for age, grade, stage, Ki67, and molecular subtype (95% CI)	1.0	1.4 (0.8-2.3)
Abbreviations: Cum.=Cumulative, CI= Confidence interval, HR=Hazard ratio. [a]Hazard ratios (HR) from Cox regression analyses for the entire observation period.		

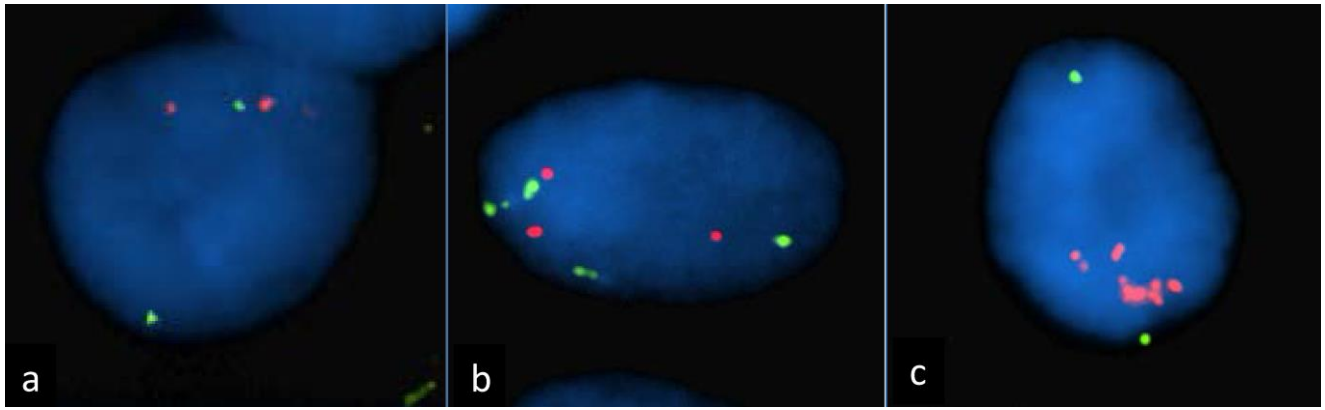

Figure 1

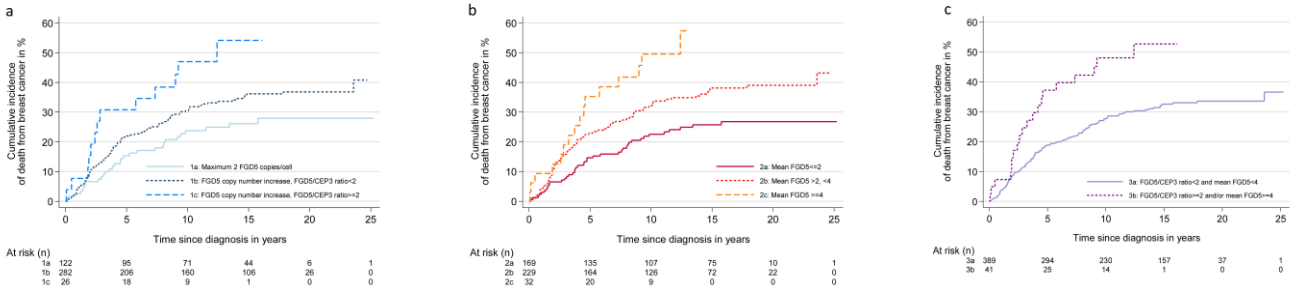
a
Cumulative incidence of death from breast cancer in %
0
10
20
30
40
50
60
0
5
10
15
20
25
Time since diagnosis in years
1a: Maximum 2 FGD5 copies/cell
1b: FGD5 copy number increase, FGD5/CEP3 ratio<2
1c: FGD5 copy number increase, FGD5/CEP3 ratio>=2
At risk (n)
1a 122 95 71 44 6 1
1b 282 206 160 106 26 0
1c 26 18 9 1 0 0
b
Cumulative incidence of death from breast cancer in %
0
10
20
30
40
50
60
0
5
10
15
20
25
Time since diagnosis in years
2a: Mean FGD5<=2
2b: Mean FGD5 >2, <4
2c: Mean FGD5 >=4
At risk (n)
2a 169 135 107 75 10 1
2b 229 164 126 72 22 0
2c 32 20 9 0 0 0
c
Cumulative incidence of death from breast cancer in %
0
10
20
30
40
50
60
0
5
10
15
20
25
Time since diagnosis in years
3a: FGD5/CEP3 ratio<2 and mean FGD5<4
3b: FGD5/CEP3 ratio>=2 and/or mean FGD5>=4
At risk (n)
3a 389 294 230 157 37 1
3b 41 25 14 1 0 0

Figure 2

Supplementary Table 1: Primary antibodies used in molecular subtyping of breast cancer				
Antibody	**Clone**	**Manufacturer[a]**	**Concentration of antibody**	**Dilution**
ER	SP1	Cell marque	33 mg/ml	1:100
PR	16	Novocastra	360 mg/l	1:400
HER2	CB11	Novocastra	3.9 g/l	1:640
Ki67	MIB1	Dako	35 mg/l	1:100
CK5	XM26	Novocastra	50 mg/l	1:100
EGFR	2-18C9	Dako	Ready to use	No dilution

Abbreviations: ER= Oestrogen receptor, PR= Progesterone receptor, HER2= Human epidermal growth factor receptor, CK5= Cytokeratin 5, EGFR= Epidermal growth factor receptor
[a]Full name and address of manufacturers: Cell Marque, Rocklin, United States. NovoCastra Laboratories, Newcastle Upon Tyne, UK. Dako Denmark A/S, Glostrup, Denmark.

Supplementary Table 2. *FGD5* status and Ki67 levels in lymph nodes										
	FGD5/CEP3 ratio, lymph nodes					**Mean FGD5/tumor cell, lymph nodes**				
	Max. 2 *FGD5* copies/cell (1a)	**<2[a] (1b)**	**≥2[b] (1c)**	**Total**	**Chi²**	**≤2 (2a)**	**>2<4 (2b)**	**≥4 (2c)**	**Total**	**Chi²**
Ki67, lymph nodes (%)										
Ki67<15%	9 (53)	47 (53)	0 (0)	56	p=0.334	22 (58)	33 (51)	1 (20)	56	p= 0.27
Ki67≥15%	8 (47)	42 (47)	2 (100)	52		16 (42)	32 (49)	4 (80)	52	
Total	17	89	2	108		38	65	5	108	
Abbreviations: Max.= maximum [a]Cells with >2 *FGD5* copies present, and *FGD5*/CEP3 ratio<2. [b]Cells with >2 *FGD5* copies present, and *FGD5*/CEP3 ratio≥2.										

Supplementary Table 3. Absolute and relative risks of death from breast cancer according to *FGD5/*CEP3 ratio and mean *FGD5/* tumor cell in Luminal A cases.						
	***FGD5*/CEP3 ratio, primary tumours**			**Mean *FGD5,* primary tumours**		
	Max. 2 *FGD5* copies/cell (1a)	**<2[a] (1b)**	**≥2[b] (1c)**	**≤2 (2a)**	**>2<4 (2b)**	**≥4 (2c)**
Cum. risk after 5 years (%) (95% CI)	13.2 (7.1-23.8)	11.2 (7.1-17.4)	18.2 (4.9-55.3)	10.3 (5.6-18.2)	11.7 (7.1-18.9)	26.8 (11.0-56.6)
Cum. risk after 10 years (%) (95% CI)	20.0 (12.1-32.1)	22.6 (16.5-30.5)	27.7 (9.9-63.7)	16.1 (10.0-25.4)	25.4 (18.2-34.7)	34.8 (16.1-64.9)
HR[c] adjusted for age (95% CI)	1.0	1.3 (0.7-2.3)	2.0 (0.7-6.2)	1.0	1.9 (1.1-3.4)	3.2 (1.2-8.3)
HR[c] adjusted for age, grade, stage, Ki67 (95% CI)	1.0	1.2 (0.7-2.3)	2.2 (0.7-6.7)	1.0	1.7 (0.9-3.1)	2.9 (1.1-7.6)
Abbreviations: Cum.=Cumulative, CI= Confidence interval, HR= hazard ratio. [a]Cells with >2 *FGD5* copies present, and *FGD5*/CEP3 ratio<2. [b]Cells with >2 *FGD5* copies present, and *FGD5*/CEP3 ratio≥2. [c]Hazard ratios (HR) from Cox regression for the entire follow-up period.						

Supplementary Table 4. Absolute and relative risks of death from breast cancer according to *FGD5* amplification status in Luminal A cases.		
	***FGD5* amplification status, primary tumours**	
	***FGD5*/CEP3<2 and mean *FGD5*<4**	**FGD5/CEP3≥2 and/or mean *FGD5*≥4**
Cum. risk after 5 years (%) (95% CI)	10.7 (7.3-15.7)	27.8 (12.7-54.5)
Cum. risk after 10 years (%) (95% CI)	21 (15.9-27.4)	34.3 (17.0-61.2)
HR[a] adjusted for age (95% CI)	1.0	2.1 (0.9-4.7)
HR[a] adjusted for age, grade, stage, Ki67 (95% CI)	1.0	2.1 (0.9-4.8)
Abbreviations: Cum.=Cumulative, CI= Confidence interval, HR=Hazard ratio. [a]Hazard ratios (HR) from Cox regression analyses for the entire observation period.		

Supplementary material

Breast Cancer Research and Treatment

FGD5 amplification in breast cancer patients is associated with tumour proliferation and a poorer prognosis

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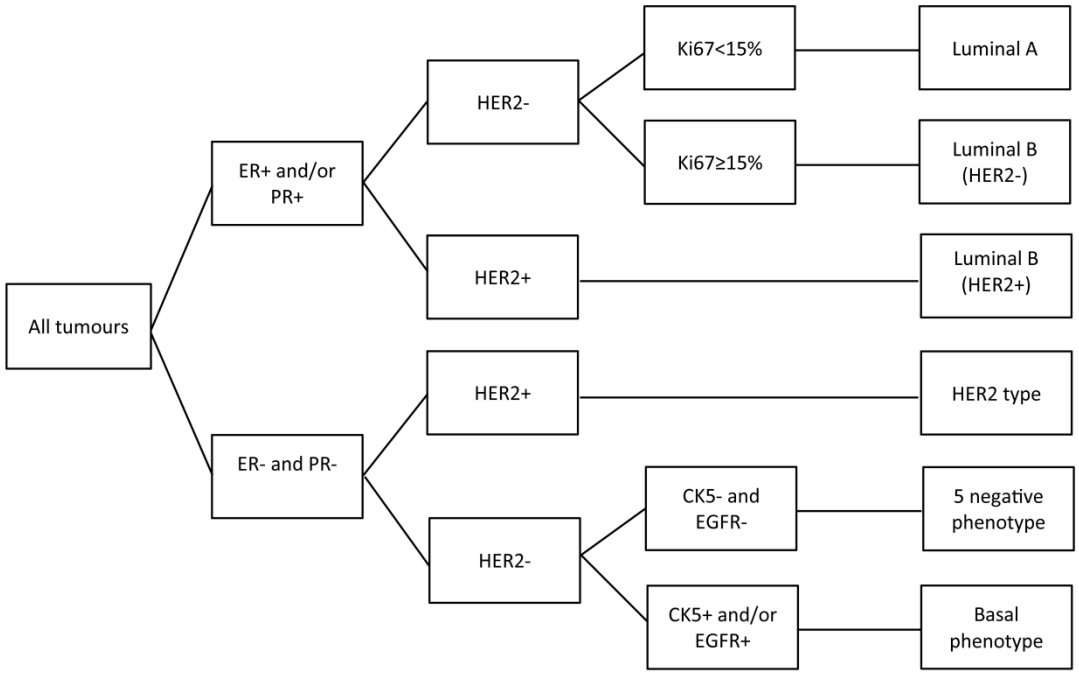
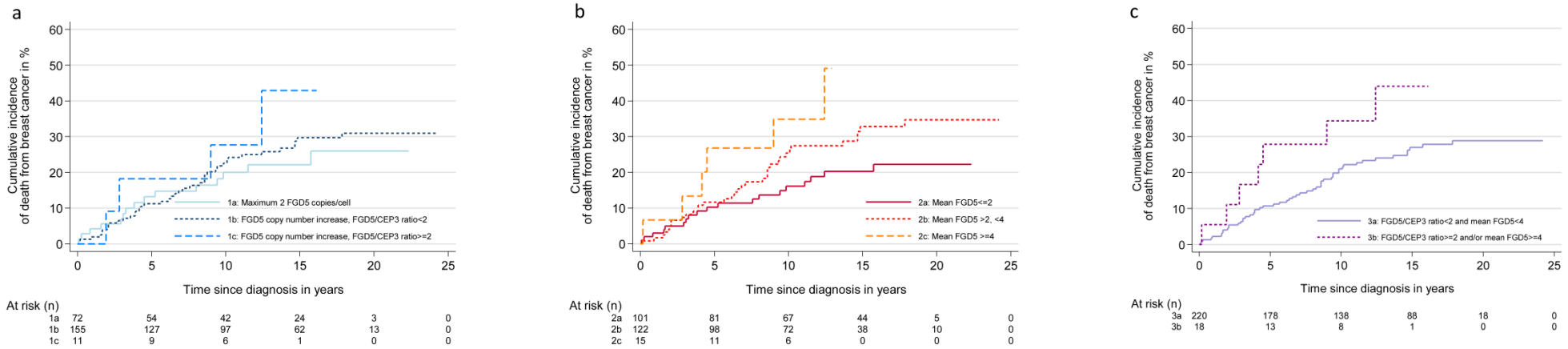
Supplementary Figure 1



Supplementary Fig. 1 Algorithm for molecular subtyping. Abbreviations: ER= Ostrogen receptor, PR= Progesterone receptor, HER2= Human epidermal growth factor receptor 2, CK5= Cytokeratin 5, EGFR= Epidermal growth factor receptor

Supplementary Figure 2



Supplementary Fig. 2 Cumulative incidence of death from Luminal A breast cancer according to *FGD5* copy number status. a) *FGD5* copy number status based on *FGD5*/CEP3 ratio (Gray's test: p=0.57). b) *FGD5* copy number status based on mean *FGD5* (Gray's test: p=0.064). c) *FGD5* amplification status (Gray's test: p=0.079)