Characterization of FGD5 expression in primary breast cancers and lymph node metastases

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FGD5 amplification drives tumour cell proliferation, and is present in 9.5% of breast cancers. We describe FGD5 expression; assess associations between *FGD5* amplification and FGD5 expression; and assess FGD5 expression in relation to proliferation and prognosis. FGD5 immunohistochemistry was done on primary tumours (n=829) and lymph node metastases (n=231) from a cohort of Norwegian patients. We explored associations between *FGD5* amplification, FGD5 expression, and proliferation, and analyzed the prognostic value of FGD5 expression by estimating cumulative risks of death and hazard ratios (HR). We identified nuclear and cytoplasmic expression in 64% and 73% of primary tumours, respectively, and found an association between gene amplification and nuclear expression (p=0.02). The proportion of cases with FGD5 expression was higher in lymph node metastases, compared to primary tumours (p=0.004 for nuclear and p=0.001 for cytoplasmic staining). Neither proliferation nor prognosis was associated with FGD5 expression (age-adjusted HR 1.12 (95% CI 0.89-1.41) for nuclear expression; and 0.88 (95% CI 0.70-1.12) for cytoplasmic expression). FGD5 is expressed in a high proportion of breast cancers and lymph node metastases. There was a correlation between *FGD5* amplification and nuclear expression, but no association between FGD5 expression and proliferation or prognosis.

Key words: Cohort study, breast neoplasms, prognosis

INTRODUCTION

Sustained proliferation is one of the hallmarks of cancer (1), and identification of genes that are essential for tumour cell proliferation could be important for prognostication and development of targeted treatment of cancer patients. A combined genomic approach has identified *FGD5* (Faciogenital dysplasia 5) amplification as a driver of proliferation in breast

cancer (2). Using fluorescence *in situ* hybridization (FISH), we previously identified *FGD5* amplification in 9.5% of breast cancers, and found that *FGD5* amplification was associated with higher tumour proliferation and a poorer prognosis (3). *FGD5* is located on the short arm of chromosome 3 (4), and in our study of *FGD5*, we defined amplification as *FGD5*/CEP3 ratio ≥2 and/or mean *FGD5* copy number/tumour cell ≥4 (3).

FGD5 is a Rho guanine nucleotide exchange factor (Rho GEF). Rho GEFs activate Rho GTPases through replacement of GDP by GTP (5). Rho GTPases regulate the cytoskeleton (6, 7) and are involved in cellular processes such as cell cycle progression (8), gene expression (9, 10) and cell movement (7). Furthermore, their activity has been linked to tumorigenesis (11), and overexpression has been demonstrated in breast cancer (12), with higher levels in high grade and highly proliferative tumours (13, 14). Some genes encoding Rho GEFs are classified as oncogenes (15, 16), and although rare, mutations in Rho GEF encoding genes have been identified in cancer (17-19). Upregulation of Rho GEFs may be present in a large proportion of breast cancers (20-22), and high expression is associated with poor differentiation (21) and poor outcome (23). Due to their role in cancer progression, Rho GEFs and Rho GTPases may be targets for therapy (23, 24).

In the present study, we used tissue microarrays (TMA) from 829 primary breast cancers from a cohort of Norwegian breast cancer patients.(25) The aims of the study were: to describe FGD5 expression by immunohistochemistry (IHC) in primary breast cancers and lymph node metastases; to assess a possible association between *FGD5* amplification and FGD5 IHC expression; and to assess a possible association between FGD5 expression, and proliferation and prognosis.

MATERIALS AND METHODS

Ethical considerations

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

Study population

Between 1956 and 1959, a population-based survey for the early detection of breast cancer was carried out in three counties in Norway (26). We have studied breast cancers occurring among women from one county (Trøndelag), between 1961 and 2008 (25). The women were born between 1886 and 1928. The Cancer Registry of Norway (27) provided information on incident cancer, and the Norwegian Cause of Death Registry supplied information on date and cause of death. During follow-up, 1379 breast cancers were diagnosed, and 909 were previously reclassified into molecular subtypes by means of IHC and in situ hybridization (ISH) (25).

The majority of subtyped tumours (867/909) were included in TMAs, and in the present study, these were stained with FGD5 antibody. A total of 38 cases were excluded, due to insufficient amount (n=32) or poor quality (n=6) of tumour tissue. Thus, 829 tumours remained.

Of the 829 cases, 293 had known lymph node metastases at diagnosis, and 233 of these were available in TMAs. Two cases were excluded due to insufficient amounts of tumour tissue. Thus, 231 cases were suitable for assessment of FGD5 expression in lymph node metastases.

Specimen characteristics

All tumours were previously classified according to histopathological type and grade using current guidelines (28, 29) and molecular subtype (25). Briefly, TMAs were constructed using the Tissue Arrayer Minicore® 3 with TMA Designer2 software (Alphelys, 78370 Plaisir, France). Three 1 mm-in-diameter tissue cores from the periphery of the primary tumour and three cores from the lymph node metastases were assembled in recipient blocks. Immunohistochemical markers used for molecular subtyping included: oestrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), Ki67, cytokeratin 5 (CK5) and epidermal growth factor receptor (EGFR) (Table 1) (25). *HER2* status was assessed using chromogenic *in situ* hybridization (CISH). A detailed description of marker assessment used in molecular subtyping is given in previous publications by our group (25, 30). With regard to Ki67, assessment was done in hotspots, counting 500 tumour cells. Nuclear Ki67 staining was considered positive, regardless of staining intensity (31).

From the TMAs, 4-µm thick sections were cut and mounted on Superfrost+ glass slides. Sections were dried at 37°C overnight, and stored in the freezer at -20°C until use. FGD5 IHC was performed according to the manufacturer's guidelines: Slides were heated at 60°C for 1.5 hours, and pre-treated in a PT Link, Pre-Treatment Module for Tissue Specimens (Dako Denmark A/S, 2600 Glostrup, DK) with EnVision FLEX Target Retrieval Solution Low pH, DAKO DM829) at 97°C for 20 minutes. IHC using rabbit polyclonal FGD5 antibody (Table 2) was done in a DakoCytomation Autostainer Plus (Dako). Dako REAL™EnVision™ Detection System with Peroxidase/DAB+, Rabbit/Mouse, code K5007 was used for visualization.

Immunohistochemical controls

Negative and positive controls were included. Normal breast tissue was used as a positive tissue control (Supplementary Figure 1). For negative controls, we omitted the primary antibody in a section from normal breast and in a TMA section containing breast cancer tissue from our study cohort (Supplementary Figure 1 and 2). For isotype controls, we used a section from normal breast, and a TMA section containing breast cancer tissue from our study cohort (Supplementary Figure 1 and 3). Isotype control sections were compared to the corresponding FGD5 stained sections. Cytoplasmic staining was not present in the isotype control sections, however some cases had sporadic cells with faint nuclear staining. The isotype control (rabbit IgG, polyclonal, Table 2) was diluted in order to obtain the same protein concentration as the primary FGD5 antibody (32).

Cell culture

MCF-7 (human breast adenocarcinoma) cell line obtained from ATCC (Manassas, USA) were grown in EMEM (Life Technologies, Carlsbad, California, USA) with 0.01 mg/mL insulin (Life Technologies), 10% FCS (Life Technologies) and 1% penicillin/streptomycin (Life Technologies), in 5% CO2 atmosphere at 37°C.

Immunoblotting

For validation of FGD5 antibody specificity, we performed immunoblot analysis of MCF-7 whole cell extracts (WCE) prepared according to a previously published protocol (33). 20 μg of WCE was separated on NuPAGE 4-12% gradient Bis-Tris polyacrylamide gels (Invitrogen) and transferred to an Immobilon-FL membrane (Millipore) for subsequent immunoblotting. Upon blocking the membrane was incubated with primary antibodies targeting FGD5 and

tubulin. Primary antibodies were detected using infrared (IR) Dye-conjugated secondary antibodies IRDye® 800CW Goat anti-Mouse IgG and IRDye® 680RD Goat anti-Rabbit IgG (Table 2). The signal was visualized using direct IR fluorescence via the Odyssey Scanner, LI-COR Biosciences.

Scoring, reporting and classification of tumours

Slides were assessed in a bright-field microscope, and for each case, the reported score was an average of all available TMA cylinders. Cytoplasmic FGD5 staining intensity (0 =no staining, 1 =weak, 2 =moderate, and 3 =strong staining) and the proportion of cells with cytoplasmic staining (0: no staining, 1: <10%, 2: 10-50%, and 3: >50%) were recorded. A staining index (SI) was calculated, multiplying intensity by proportion. SI 0-1 was considered negative, and SI ≥2 was considered positive. The proportion of tumour cells with positive nuclear staining was also recorded, irrespective of staining intensity. All IHC stains were assessed by two pathologists (MV and AMB or PGM) independently. Discrepant results were discussed, and consensus was reached. The REMARK criteria for tumour marker prognostic studies were followed.(34)

Statistical analyses

We used Pearson's Chi-square test to assess differences in clinical and tumour characteristics across categories of FGD5 IHC staining; to compare proportions of FGD5 IHC staining across categories of *FGD5* copy number status; and to compare FGD5 IHC staining in primary tumours and lymph node metastases. We also performed multivariate logistic regression to adjust for other tumour characteristics. Cumulative incidence of death from

breast cancer was estimated according to categories of FGD5 staining. In these analyses, death from other causes was considered a competing event, and 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 staining, censoring at time of death from other causes. Negative staining was used as the reference. Adjustments were made for other prognostic factors at baseline: age (≤49, 50-59, 60-64, 65-69, 70-74, ≥75 years), histological grade (1-3), and stage (I-IV). Adjustments were made for each factor separately, and all 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 statistical analyses.

RESULTS

FGD5 expression and *FGD5* copy number status in primary tumours

Cytoplasmic FGD5 staining was present in a large proportion of tumours, and a SI ≥2 was identified in 73% of cases (Table 3, Fig. 1B). We found no clear associations between *FGD5* copy number status and cytoplasmic staining in the primary tumours (Table 4). With cut-offs of 1% and 10%, we identified positive nuclear FGD5 staining in 64% and 26% of cases, respectively (Table 3, Fig. 1A). There was an association between *FGD5* copy number status and nuclear staining (1% cut-off), with a higher proportion of positive nuclei among amplified, compared to non-amplified cases (85% vs. 67%, p=0.02). With a cut-off of 10%, however, there was no association between *FGD5* copy number status and nuclear staining. Positive cytoplasmic and nuclear staining of FGD5 was found in a high proportion of cases within all molecular subtypes (Table 3). For cytoplasmic staining, SI>2 was found in 74% of Luminal A, 75% of Luminal B (HER2-), 70% of Luminal B (HER2+), 61% of HER2 type, 50% of 5NP, and 81% of BP tumours, respectively. After adjustment for other characteristics, the odds of positive cytoplasmic staining (SI>2) was highest for BP, and lowest for 5NP. For nuclear staining, the odds of FGD5 expression (≥1% or ≥10%) was highest in Luminal A tumours. Positive FGD5 staining in endothelial cells was observed (Fig. 1C).

FGD5 expression in lymph node metastases

A SI ≥2 was identified in 80 % of lymph node metastases (Table 5). Of all cases with SI <2 in the primary tumour, 68% had SI ≥2 in the corresponding lymph node metastases (p=0.001). Nuclear FGD5 staining was present in 92 % (1% cut-off) and 45 % (10% cut-off) of lymph node metastases. Among cases with negative nuclear staining (1% cut-off) in the primary

tumours, 85% had positive nuclear staining in the corresponding lymph node metastases (p=0.004). Correspondingly, with a 10 % cut-off, 38% of cases with negative nuclear staining in the primary tumours, had positive staining in the lymph node metastases (p<0.001).

Immunoblotting

Antibody specificity was validated by immunoblot analysis of three human breast cancer cell lines (MCF-7, T47-D and HCC1806), in which the most prominent band (61 kDa, Fig. 1D) corresponded to isoform 2 of FGD5 (Figure 1D) (35, 36).

FGD5 and proliferation

The distribution of Ki67 low/high (<15% vs. ≥15%) tumours was similar when FGD5 staining of primary tumours was categorized based on SI, or based on nuclear staining with a 1% cut-off (p=0.27 and p=0.15, respectively, Table 3). The proportion of Ki67-high tumours was lower among cases with ≥10% FGD5-positive nuclei, compared to cases with <10% positive nuclei (37% vs. 47%, p=0.007). We found no clear differences in the number of mitoses in the primary tumours across categories of cytoplasmic or nuclear FGD5 staining.

FGD5 IHC and prognosis

We found similar cumulative risks of death from breast cancer when cases were subdivided based on SI status (SI <2 vs. ≥2, Gray's test: p=0.36) and nuclear staining (1% cut-off, Gray's test: p=0.62; 10% cut-off, Gray's test: p=0.83) (Table 6). Cox regression analyses using negative staining as a reference confirmed this finding, and showed age-adjusted HR of 0.88 (95% CI 0.70-1.12) for SI, 1.12 (95% CI 0.89-1.41) for nuclear staining at 1% cut-off, and 0.96 (95% CI 0.74-1.24) for nuclear staining at 10% cut-off. Adjustments for stage and histological

grade at diagnosis did not significantly change these estimates. We also performed separate analyses for each molecular subtype and found no clear differences in prognosis across categories of cytoplasmic and nuclear staining (data not shown).

DISCUSSION

We identified FGD5 staining in the cytoplasm and nuclei in a large proportion of primary breast cancers and lymph node metastases. The proportion of cases with positive FGD5 staining in lymph node metastases was markedly higher compared to the corresponding primary tumours. There was a positive association between *FGD5* amplification status and nuclear staining at 1% cut-off in the primary tumours. However, neither proliferation nor prognosis was found to be associated with FGD5 expression.

A strength of our study is the large number of cases with long-term follow-up data acquired from high quality national registries (27). Assessment of IHC was done by two pathologists independently, using predetermined criteria for evaluation. Discrepant results were discussed, and consensus was reached.

Breast cancers included in this study were diagnosed between 1961 and 2008, and it is likely that preanalytical conditions have varied during the inclusion period, possibly influencing our results (37). However it has been shown in other studies that valuable results can be obtained using archival material (37, 38).

According to the Human Protein Atlas, a cytoplasmic staining pattern was expected (39). FGD5 is a new marker with no available guidelines for assessment, and for cytoplasmic staining, we used a staining index, where the intensity of staining was multiplied with the proportion of stained cells. This approach has been used for other markers (40-42). We regarded SI ≥2 as positive, similar to previous studies of other markers (25, 30, 40, 43). In addition, we observed nuclear staining in a large proportion of cases, and the proportion of stained nuclei, irrespective of staining intensity was recorded. Not including staining intensity in the assessment of nuclear markers is routine both for hormone receptors and

Ki67 in breast cancer (31, 44). For nuclear staining, two different cut-off levels were used in the analyses.

Gatza et al. found that FGD5 was amplified uniquely in highly proliferative luminal tumours, and that amplification of the gene was prognostic in luminal tumours (2). Their molecular subtyping was done by gene expression analysis, and luminal tumours were defined as all tumours that were not basal. We reclassified breast cancer tumours into six different molecular subtypes using surrogate IHC and ISH markers (25, 30). It has been shown that molecular subtyping by gene expression analysis is similar, but not identical to subtyping using surrogate markers (45-48). In our previous study, *FGD5* copy number increase was identified within all molecular subtypes, and amplifications were found in all molecular subtypes except the 5NP. In the present study, we identified positive FGD5 staining in a high proportion of cases within all molecular subtypes, with the highest proportion observed in the BP. Despite some differences in cytoplasmic and nuclear staining, we found no clear differences in prognostic value of FGD5 across subtypes.

There was a positive association between *FGD5* amplification and nuclear FGD5 staining. For most cases in which nuclear FGD5 staining was identified, the percentage of positive nuclei was low (median value 4%).

In breast cancer, amplification of the *HER2* gene is strongly associated with overexpression of the HER2 protein (49). Therefore, in order to select patients for targeted anti-HER2 treatment, both ISH and IHC can be used (50, 51). However, studies of many other genes have demonstrated a poor correlation between gene copy number or mRNA expression, and protein level (14, 49, 52).

In this study, lack of correlation between *FGD5* copy number and cytoplasmic staining was observed. While a correlation between gene copy number and mRNA levels has been indicated previously (2), for the samples included in this study this correlation has not been demonstrated so far. Lack of correlation between *FGD5* copy number and cytoplasmic staining suggests that the amplified gene copy is potentially silenced. The amplified gene copy could have been translocated to a new region in the genome, and potentially silenced through mechanisms such as methylation. Further, since within *FGD5* is encoded non-coding RNA (LOC105376963), the copy number increase does not only affect FGD5 expression, but could also result in altered expression of the non-coding RNA, potentially influencing other genes and/or other pathways.

The lack of correlation between copy number and protein levels could reflect underlying biology, and our findings suggest that *FGD5* affects proliferation in other ways than through its protein product. FGD5 protein has been described to exist in two isoforms, the longer 160kDa isoform 1 and shorter 61kDa isoform 2. Interestingly, the immunoblot analysis indicated presence of only shorter isoform 2 in the tested breast cancer cell line (Figure 1D). It is however not possible to exclude that another isoform not detected by the antibody is present and potentially play a role in breast cancer cells. Since this is the first study of FGD5 expression in a cohort of breast cancer patients, our findings need to be validated in other cohorts. Further studies using other FGD5 antibodies or a proteomic approach would be of added value.

The antibody used in the present study was a polyclonal Protein Epitope Signature Tag antibody (PrEST) (53) selected from the Human Protein Atlas (54, 55). PrESTs have similar specificity to monoclonal antibodies (53).

We found no clear association between FGD5 expression and prognosis in this study. However, we previously found strong associations between *FGD5* amplification status and prognosis in the same cohort of breast cancer patients. Evidently, the role of *FGD5* in breast cancer proliferation and prognosis is not yet clarified. FGD5 is classified as a RhoGEF, and these molecules are known to activate Rho GTPases, whose activity has been linked to cancer progression (8, 11-14). FGD5 is expressed in endothelial cells (56-60), and studies have demonstrated its presence in the plasma membrane (58, 60) and in the perinuclear region (58) of human umbilical vein endothelial cells (HUVECs). FGD5 can affect endothelial cell barriers through interactions with the Rho GTPase Cdc42 (60), and the latter has been identified as the selective binding partner for FGD5 (57). In a recent study, Cdc42 protein expression was identified in a large proportion of breast cancers, and cytoplasmic expression was associated with higher grade and higher Ki67 levels (61). A possible link between *FGD5* and angiogenesis has been suggested (57-59), and Rho GTPases such as Cdc42 are involved in angiogenesis through regulation of the cytoskeleton and organization of endothelial cells (62, 63). Interestingly, positive FGD5 staining in blood vessels was identified in our study, and thus, assessing whether *FGD5* copy number status or FGD5 expression may be associated with tumour angiogenesis could provide valuable information on the role of *FGD5* in breast cancer development.

In conclusion, we have demonstrated cytoplasmic and nuclear FGD5 staining in a large proportion of primary breast cancers and lymph node metastases. The proportion of FGD5 positive lymph nodes was higher compared to the corresponding primary tumours. There was an association between *FGD5* amplification status and nuclear FGD5 staining,

however neither proliferation nor prognosis was found to be associated with FGD5 expression.

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COMPETING INTERESTS

The authors declare they have no competing interests.

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AUTHOR CONTRIBUTIONS

All authors have contributed to this paper. More specifically:

MV: Study design and writing the article. Annotation of FGD5 IHC. Statistical analyses. Interpretation of results.

PGM: Writing the article. Annotation of FGD5 IHC.

MJE: Writing the article. Molecular subtyping of the patient cohort.

BY: Writing the article. Laboratory work during molecular subtyping and FGD5 IHC.

DLB: Writing the article. Immunoblot. Interpretation of results.

BvL: Writing the article. Immunoblot. Interpretation of results.

LAA: Writing the article. Interpretation of results.

LJV: Writing the article. Interpretation of results.

SO: Writing the article. Interpretation of results.

AMB: Study design and writing the article. Annotation of FGD5 IHC. Interpretation of results.

Molecular subtyping of the patient cohort.

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FIGURE LEGENDS

Figure 1. FGD5 immunohistochemical staining in the nuclei (A) and cytoplasm (B) of tumour cells, and in blood vessels (arrows) (C). Scale bar = 50 µm. D) Immunoblot analysis using anti-FGD5 rabbit polyclonal antibody (HPA019191, Sigma-Aldrich) on breast cancer cell lines (from the left; MCF7, T47-D, and HCC1806) showing a prominent band consistent with isotype 2 (61 kDa).

Table 1. Reclassification into molecular subtypes (25, 30).

Molecular subtype	Criteria for molecular subtype
Luminal A	ER and/or PR positive, HER2 negative, Ki67<15%
Luminal B (HER2-)	ER and/or PR positive, HER2 negative, Ki67≥15%
Luminal B (HER2+)	ER and/or PR positive, HER2 positive
HER2 type	ER and PR negative, HER2 positive
Basal phenotype	ER, PR and HER2 negative, CK5 and/or EGFR positive
5 negative phenotype	ER, PR, HER2, CK5, and EGFR negative

Abbreviations: ER= oestrogen receptor, PR= progesterone receptor, HER2= human epidermal growth factor receptor 2, CK5= cytokeratin 5, EGFR= epidermal growth factor receptor

Table 2. Antibodies used for immunohistochemistry and immunoblotting

Antibody	Clone/ Product name	Manufacturer[a]	Antibody concentration	Dilution
FGD5	HPA019191	Sigma-Aldrich	0.05 mg/ml	1:40 [b]
FGD5	HPA019191	Sigma-Aldrich	0.05 mg/ml	1:500 [c]
Rabbit IgG polyclonal[d]	Ab27478	Abcam	0.2 mg/ml	1:160
Tubulin	T9026	Sigma-Aldrich	4 mg/mL	1:000
IRDye® 800CW	P/N 925-32210	LI-COR Biosciences	1 mg/mL	1:15000
IRDye® 680RD	P/N 925-68071	LI-COR Biosciences	1 mg/mL	1:15000

Abbreviations: FGD5= Faciogenital dysplasia 5. [a]Complete name and address of manufacturers: Sigma-Aldrich, St.Louis, USA; LI-COR Biosciences, Lincoln, Nebraska USA.
[b]Used for immunohistochemistry of primary tumours and lymph node metastases.
[c] Used for immunoblotting. [d]Used for isotype control.

Table 3. Characteristics of the study population according to FGD5 staining

	Cytoplasmic staining, staining index (SI)			Nuclear staining, 1% cut-off			Nuclear staining, 10% cut-off			
	SI <2	SI ≥ 2	OR (95% CI)	<1%	≥1%	OR (95% CI)	<10%	≥10%	OR (95% CI)	Total
Number of cases	227 (27)	602 (73)	-	297 (36)	532 (64)	-	616 (74)	213 (26)	-	829 (100)
Mean age at diagnosis (SD)	70.3 (11.2)	72.6 (10.0)	-	71.2 (9.9)	72.5 (10.7)	-	71.1 (10.5)	74.8 (9.6)	-	72 (10.4)
Mean follow-up, years (SD)	9.0 (8.7)	9.0 (7.7)	-	9.9 (8.2)	8.5 (7.8)	-	9.3 (8.3)	8.1 (7.1)	-	9.0 (8.0)
Breast cancer death	96 (42)	232 (39)	-	117 (39)	211 (40)	-	247 (40)	81 (38)	-	328 (40)
Death other causes	103 (45)	270 (45)	-	133 (45)	240 (45)	-	280 (45)	93 (44)	-	373 (45)
Grade, n (%)[1]										
I	28 (12)	67 (11)	1 (ref)	29 (10)	66 (12)	1 (ref)	61 (10)	34 (16)	1 (ref)	95 (11)
II	107 (47)	333 (55)	1.41 (0.85-2.36)	162 (55)	278 (52)	0.78 (0.48-1.28)	323 (52)	117 (55)	0.66 (0.40-1.09)	440 (53)
III	92 (41)	202 (34)	0.93 (0.52-1.67)	106 (36)	188 (35)	1.11 (0.63-1.96)	232 (38)	62 (29)	0.73 (0.40-1.31)	294 (35)
Chi^2 test	P=0.10			P=0.51			P=0.01			
Lymph node metastasis[2]										
Yes	92 (41)	201 (33)	0.96 (0.60-1.55)	98 (33)	195 (37)	1.02 (0.66-1.58)	215 (35)	78 (37)	1.05 (0.65-1.70)	293 (35)
No	77 (34)	245 (41)	1 (ref)	111 (37)	211 (40)	1 (ref)	240 (39)	82 (39)	1 (ref)	322 (39)
Unknown lymph node status	58 (26)	156 (26)	-	88 (30)	126 (24)	-	161 (26)	53 (25)	-	214 (26)
Chi^2 test	P=0.03			P=0.79			P=0.74			
Tumour size[2]										
≤2 cm	78 (34)	294 (49)	1 (ref)	149 (50)	223 (42)	1 (ref)	291 (47)	81 (38)	1 (ref)	372 (45)
>2 ≤5 cm	30 (13)	70 (12)	0.68 (0.37-1.24)	24 (8)	76 (14)	2.06 (1.10-3.87)	48 (8)	52 (24)	4.32 (2.39-7.82)	100 (12)
>5 cm	1 (0)	8 (1)	0.82 (0.09-7.66)	0 (0)	9 (2)	-	5 (1)	4 (2)	-	9 (1)
Uncertain, but >2 cm	51 (23)	89 (15)	0.52 (0.28-0.96)	45 (15)	95 (18)	1.54 (0.85-2.80)	104 (17)	36 (17)	1.47 (0.75-2.89)	140 (17)
Uncertain	67 (30)	141 (23)	-	79 (27)	129 (24)	-	168 (27)	40 (19)	-	208 (25)
Chi^2 test	P=0.002			P=0.002			P<0.001			
Stage at diagnosis[1]										
1	99 (44)	306 (51)	1 (ref)	159 (54)	246 (46)	1 (ref)	304 (50)	101 (47)	1 (ref)	405 (49)
2	101 (45)	227 (38)	0.75 (0.53-1.04)	111 (38)	217 (41)	1.33 (0.98-1.82)	238 (39)	90 (42)	1.32 (0.93-1.87)	328 (40)
3	13 (6)	37 (6)	0.95 (0.47-1.89)	12 (4)	38 (7)	2.09 (1.04-4.19)	38 (6)	12 (6)	0.95 (0.46-1.94)	50 (6)
4	13 (6)	28 (5)	0.67 (0.33-1.37)	11 (4)	30 (6)	1.94 (0.93-4.06)	31 (5)	10 (5)	1.14 (0.52-2.48)	41 (5)
Chi^2 test	P=0.37			P=0.02			P=0.65			
Molecular subtype[1]										
Luminal A	104 (46)	292 (49)	1 (ref)	121 (41)	275 (52)	1 (ref)	266 (43)	130 (61)	1 (ref)	396 (48)
Luminal B (HER2-)	56 (25)	169 (28)	1.28 (0.84-1.95)	84 (28)	141 (27)	0.67 (0.46-0.98)	169 (27)	56 (26)	0.74 (0.49-1.11)	225 (27)
Luminal B (HER2+)	19 (8)	44 (7)	1.04 (0.55-1.94)	32 (11)	31 (6)	0.37 (0.20-0.65)	58 (9)	5 (2)	0.18 (0.07-0.49)	63 (8)
HER2 type	22 (10)	34 (6)	0.80 (0.41-1.57)	22 (7)	34 (6)	0.51 (0.26-0.99)	45 (7)	11 (5)	0.58 (0.26-1.26)	56 (7)
5 negative phenotype	15 (7)	15 (3)	0.38 (0.17-0.81)	13 (4)	17 (3)	0.53 (0.25-1.16)	27 (4)	3 (1)	0.24 (0.07-0.81)	30 (4)

Basal phenotype	11 (5)	48 (8)	2.16 (1.01-4.61)	25 (8)	34 (6)	0.45 (0.24-0.85)	51 (8)	8 (4)	0.31 (0.13-0.72)	59 (7)
Chi² test	P=0.01			P=0.02			P<0.001			
Ki67[3]										
Ki67 <15%	133 (59)	327 (54)	1 (ref)	155 (52)	305 (57)	1 (ref)	325 (53)	135 (63)	1 (ref)	460 (55)
Ki67 ≥15%	94 (41)	275 (46)	1.61 (1.11-2.34)	142 (48)	227 (43)	0.79 (0.57-1.11)	291 (47)	78 (37)	0.80 (0.55-1.16)	369 (45)
Chi² test	P=0.27			P=0.15			P=0.007			
Mitoses/10HPF[4]										
≤2	74 (33)	192 (32)	1 (ref)	96 (32)	170 (32)	1 (ref)	188 (31)	78 (37)	1 (ref)	266 (32)
>2 to ≤6	42 (19)	138 (23)	1.27 (0.81-2.00)	64 (22)	116 (22)	1.19 (0.79-1.80)	136 (22)	44 (21)	0.90 (0.57-1.41)	180 (22)
>6 to ≤13	55 (24)	126 (21)	0.88 (0.56-1.38)	65 (22)	116 (22)	1.32 (0.85-2.03)	129 (21)	52 (24)	1.33 (0.84-2.12)	181 (22)
>13	56 (25)	146 (24)	1.05 (0.64-1.70)	72 (24)	130 (24)	1.54 (0.98-2.42)	163 (26)	39 (18)	0.96 (0.57-1.63)	202 (24)
Chi² test	P=0.50			P=0.999			P=0.067			

Numbers are number of observations (%) unless otherwise specified. Abbreviations: SD= standard deviation, HER2= human epidermal growth factor receptor 2, HPF= high power fields.

[1] In the logistic regression analysis, mutual adjustments were made for age, grade, stage, and molecular subtype, as applicable.

[2] In the logistic regression analysis, mutual adjustments were made for age, grade, lymph node status, tumour size and molecular subtype, as applicable.

[3] In the logistic regression analysis, adjustments were made for age, grade, and stage. [4] In the logistic regression analysis, adjustments were made for age, stage, and molecular subtype.

Table 4. *FGD5* copy number status and FGD5 immunohistochemical expression in primary tumours

		FGD5 copy number status							
		FGD5/CEP3 ratio			Mean *FGD5*			*FGD5* amplification status	
		≤2 FGD5 copies/cell	Ratio <2[a]	Ratio ≥2[b]	≤2	>2 to <4	≥4	Ratio <2 and mean *FGD5*<4	Ratio ≥2 and/or mean *FGD5*≥4
FGD5 immunohistochemistry	Cytoplasmic staining index, n (%)								
	<2	22 (18)	62 (23)	5 (21)	31 (19)	54 (24)	4 (13)	84 (22)	5 (13)
	≥2	97 (82)	213 (77)	19 (79)	135 (81)	167 (76)	27 (87)	295 (78)	34 (87)
	Chi^2 test		P=0.66			P=0.19		P=0.18	
	Nuclear staining, n (%)								
	<1%	38 (32)	90 (33)	4 (17)	63 (38)	63 (29)	6 (19)	126 (33)	6 (15)
	≥1%	81 (68)	185 (67)	20 (83)	103 (62)	158 (71)	25 (81)	253 (67)	33 (85)
	Chi^2 test		P=0.27			P=0.04		P=0.02	
	Nuclear staining, n (%)								
	<10%	79 (66)	193 (70)	15 (63)	115 (69)	150 (68)	22 (71)	261 (69)	26 (67)
	≥10%	40 (34)	82 (30)	9 (38)	51 (31)	71 (32)	9 (29)	118 (31)	13 (33)
	Chi^2 test		P=0.61			P=0.92		P=0.78	

[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 5. FGD5 cytoplasmic and nuclear staining in primary tumours and lymph node metastases

		Primary tumour, n (%)		
Lymph node metastasis	Cytoplasmic staining index	Negative (<2)	Positive (≥2)	Total
	Negative (<2)	25 (32)	21 (14)	46
	Positive (≥2)	52 (68)	133 (86)	185
	Total	77	154	231
	Chi^2 test	P=0.001		
	Nuclear staining 1% cut-off	Negative (<1%)	Positive (≥1%)	Total
	Negative (<1%)	11 (15)	7 (4)	18
	Positive (≥1%)	61 (85)	152 (96)	213
	Total	72	159	231
	Chi^2 test	P=0.004		
	Nuclear staining 10% cut-off	Negative (<10%)	Positive (≥10%)	Total
	Negative (<10%)	103 (62)	23 (35)	126
	Positive (≥10%)	63 (38)	42 (65)	105
	Total	166	65	231
	Chi^2 test	P<0.001		

Table 6. Absolute and relative risks of death from breast cancer according to cytoplasmic and nuclear immunohistochemical FGD5 staining

	Cytoplasmic staining index		Nuclear staining, 1% cut-off		Nuclear staining, 10% cut-off	
	Negative (<2)	Positive (≥2)	Negative (<1%)	Positive (≥1%)	Negative (<10%)	Positive (≥10%)
Cum. risk (%) until 5 years after diagnosis (95% CI)	27.6 (22.2-33.9)	23.9 (20.7-27.6)	23.0 (18.6-28.2)	26.0 (22.5-30.0)	25.0 (21.7-28.6)	24.8 (19.5-31.3)
P-value Gray's test	p=0.21		p=0.25		p=0.98	
Cum. risk (%) until 15 years after diagnosis (95% CI)	41.8 (35.4-48.7)	37.5 (33.7-41.6)	37.7 (32.3-43.6)	39.3 (35.2-43.7)	38.8 (35.0-42.9)	38.1 (31.7-45.2)
P-value Gray's test	p=0.25		p=0.44		p=0.95	
HR[a] (95% CI)	1 (ref)	0.88 (0.69-1.11)	1 (ref)	1.14 (0.91-1.43)	1 (ref)	1.02 (0.80-1.32)
HR[a] adjusted for age (95% CI)	1 (ref)	0.88 (0.70-1.12)	1 (ref)	1.12 (0.89-1.41)	1 (ref)	0.96 (0.74-1.24)
HR[a] adjusted for age, grade, stage (95% CI)	1 (ref)	0.85 (0.66-1.08)	1 (ref)	1.10 (0.79-1.26)	1 (ref)	1.07 (0.82-1.39)

Abbreviations: Cum.= cumulative, CI= confidence interval, HR= hazard ratio, ref.= reference

[a]Hazard ratios from Cox regression analyses for the entire observation period