# *CHL1* hypermethylation as a potential biomarker of poor prognosis in breast cancer

## SUPPLEMENTARY FIGURES AND TABLES

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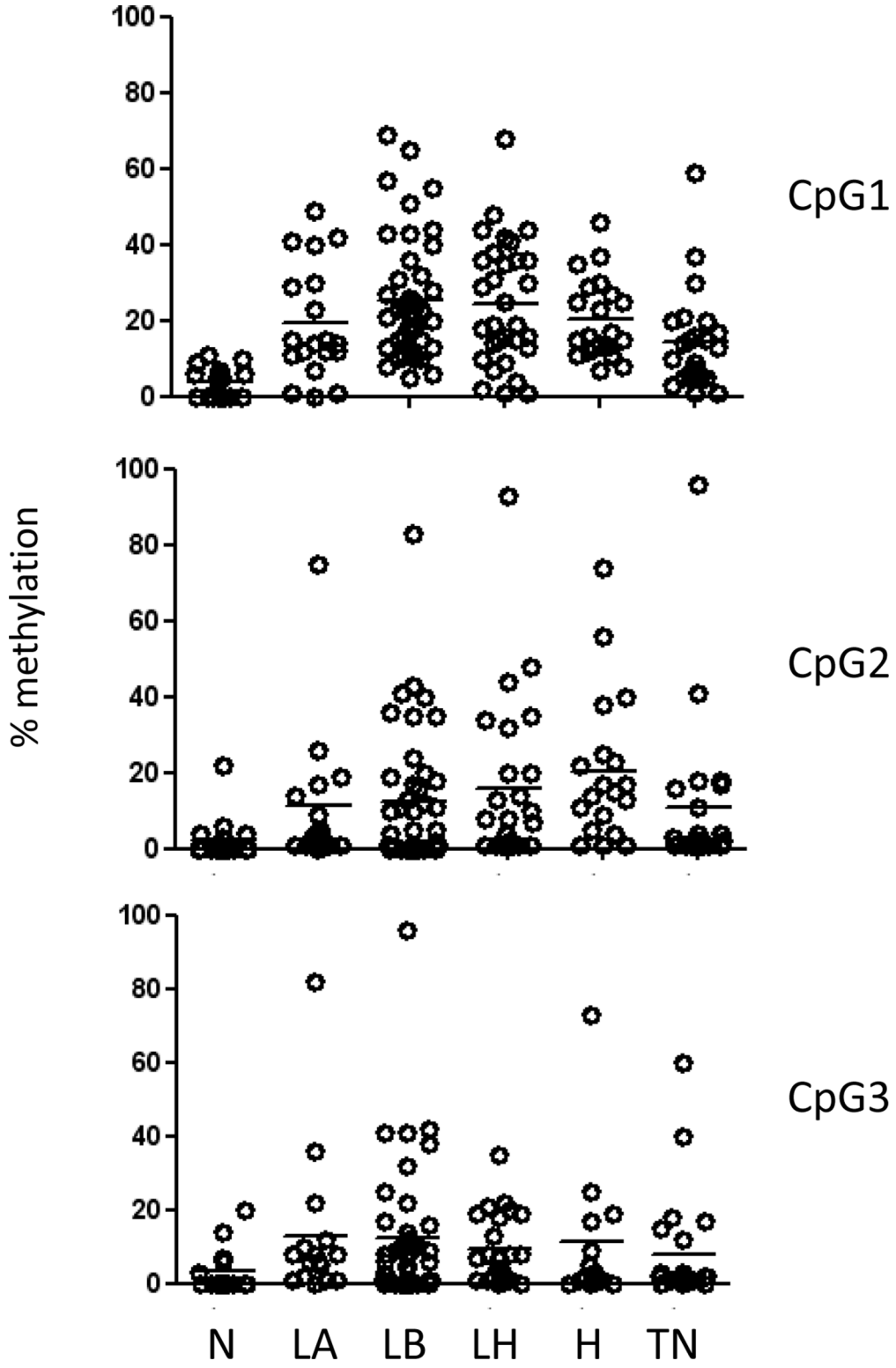
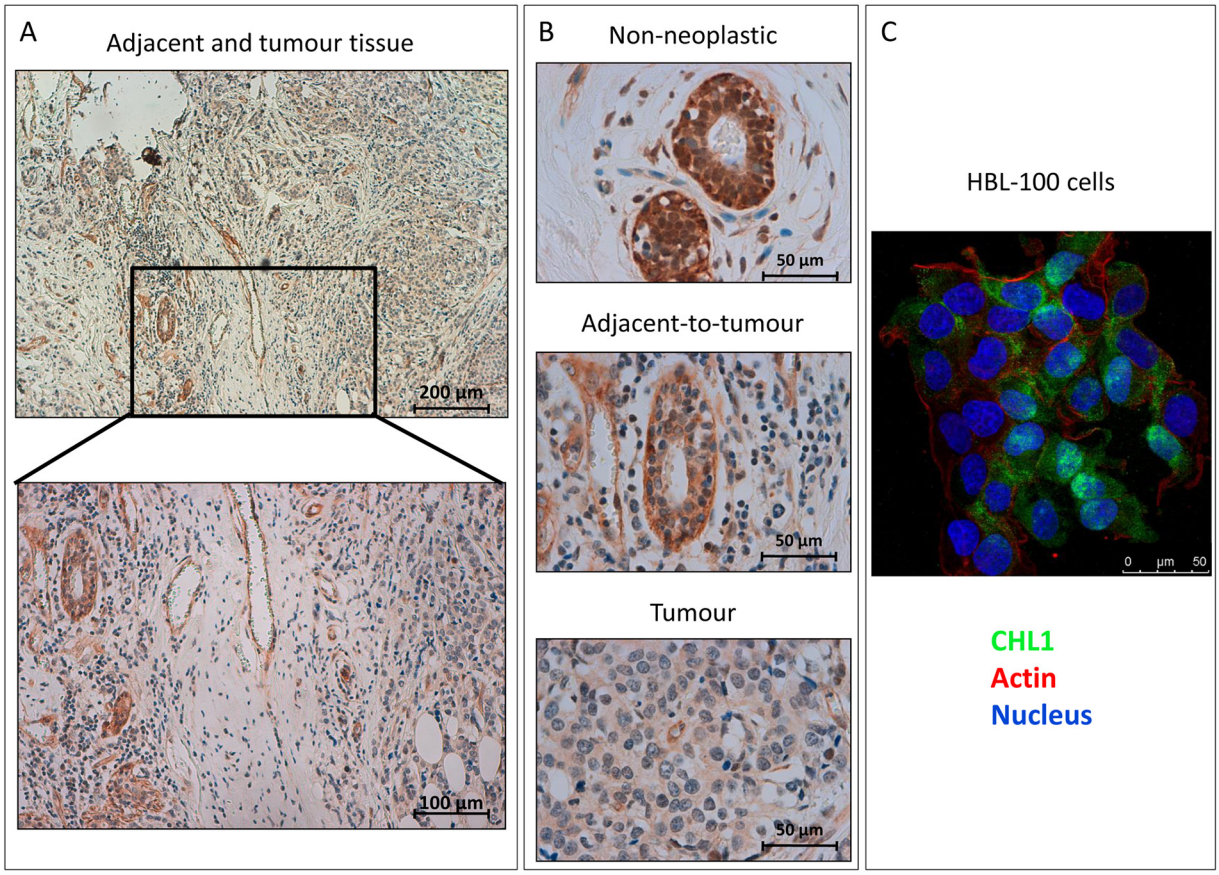
GGTTTTCGGTCGGTTCGGGGGAGAAGGCGTTCGAGGGGAGGCGTCGGATAGATCGCGTTTCGGAGGCGGC
GTAG

| | |
|---|---|
| Forward primer CpG1 | Forward primer CpG2 and CpG3 |
| Sequencing primer CpG1 | Sequencing primer CpG2 and CpG3 |
| Reverse primer CpG1 | Reverse primer CpG2 and CpG3 |

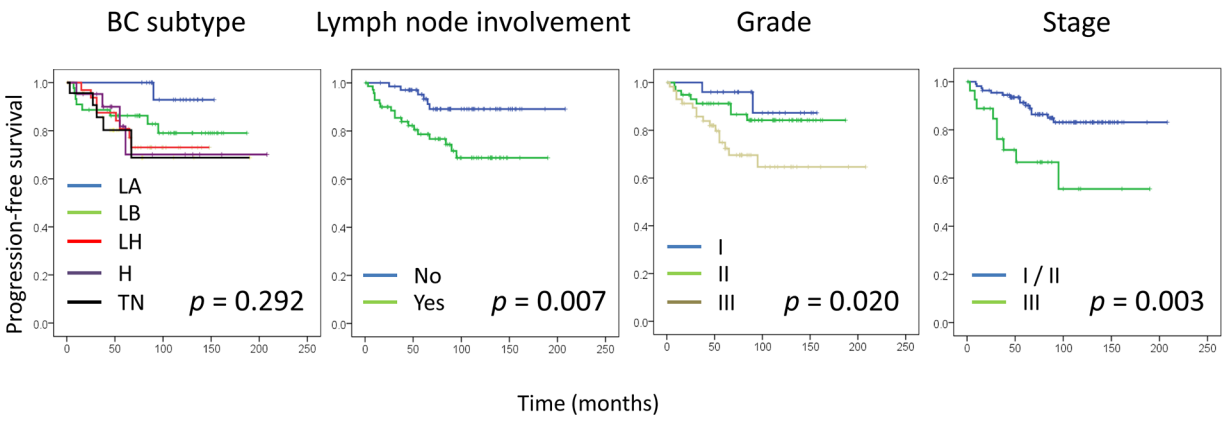
**Supplementary Figure 1: The *CHL1* gene.** Bisulphite-converted sequence of the *CHL1* promoter (lower case) and the first exon (upper case). The three CpG sites examined in this study are highlighted.




**Supplementary Figure 2: Methylation status of *CHL1* promoter in BC subtypes.** (A) Levels of methylation of three CpG sites were measured by pyrosequencing in our series of 142 BC cases, classified into five major subtypes: LA, luminal A (n=20); LB, luminal B/HER2-negative (n=44); LH, luminal B/HER2-positive (n=33); H, HER2-positive (n=21); TN, triple-negative (n=24); and N, non-neoplastic mammary tissues from reduction mammoplasties (n=19). The horizontal line indicates the median of each group.




**Supplementary Figure 3: CHL1 protein expression pattern in BC.** Representative pictures of mammary tissues immunostained with CHL1 antibody. **A.** The upper panel shows an area at low magnification (100x) with the tumour (right side) and the adjacent-to tumour (left side) tissues. The bottom panel shows detail at a higher magnification (200x): non-neoplastic ductal cells strongly express CHL1, while unstructured tumoral cells do not show CHL1 expression. **B.** High magnification (630x) pictures of a non-neoplastic, adjacent-to-tumour and tumoral tissue, showing the cytoplasmic pattern of CHL1 expression. All of the images were acquired using a Leica DMD 108 digital microscope (Leica, Wetzlar, Germany). **C.** CHL1 protein expression was explored by immunofluorescence in cultured immortalised but non-neoplastic mammary cells (HBL-100 cells). Green, red and blue stained CHL1, actin filaments and the nuclei, respectively. Images were captured at 400x magnification with a Leica TCS SP5 laser scanning microscope (Leica, Wetzlar, Germany).




**Supplementary Figure 4: Clinical value of important factors in BC prognosis.** Associations between progression-free survival and BC subtype (LA: Luminal A-like; LB: Luminal B-like/HER2-negative; LH: Luminal B-like/HER2-positive; H: HER2-positive (non-luminal); TN: triple-negative), lymph node involvement, histological grade and stage were analysed in our series of 142 BC patients.

**Supplementary Table 1: Pathological and clinical characteristics of our BC patient series**

| Feature | Frequency (%) |
|---|---|
| **BC subtype** | |
| LA | 20/142 (14.1) |
| LB | 44/142 (31.0) |
| LH | 33/142 (23.2) |
| H | 21/142 (14.8) |
| TN | 24/142 (16.9) |
| **Grade** | |
| I | 25/142 (17.6) |
| II | 59/142 (41.5) |
| III | 58/142 (40.8) |
| **Lymph node involvement** | |
| No | 68/139 (48.9) |
| Yes | 71/139 (51.1) |
| **Stage** | |
| I | 49/138 (35.5) |
| IIA | 34/138 (24.6) |
| IIB | 27/138 (19.6) |
| IIIA | 19/138 (13.8) |
| IIIC | 9/138 (6.5) |
| **Age** (years) | Mean: 60<br>Range: 30-95 |
| **Tumour size** (cm) | Mean: 2.2<br>Range: 0.3-10 |
| **Progression-free survival** (months) | Mean: 82.9<br>Range: 1-208 |
| No | 115/141 (81.6) |
| Yes | 26/141 (18.4) |
| **Overall survival** (months) | Mean: 86.9<br>Range: 1-208 |
| Exitus | 27/140 (19.3) |
| **Chemotherapy** | |
| No | 49/138 (35.5) |
| Yes | 89/138 (64.5) |
| **Hormone therapy** | |
| No | 43/136 (31.6) |
| Yes | 93/136 (68.4) |

For the BC subtype: LA, Luminal A-like; LB, Luminal B-like/HER2-negative; LH, Luminal B-like/HER2-positive; H, HER2-positive (non-luminal); TN, triple-negative.

**Supplementary Table 2: Primers used for pyrosequencing of the *CHL1* gene in 3 CpG sites**

| | CpG1 | CpG2 and CpG3 |
|---|---|---|
| **Forward primer** | TTTTTAAATGAAGGAAAGT AAGAAGATAAT | GTAATGGGAGAAAAGTAGATTGG |
| **Reverse primer** | [Btn]CCAATCTACTTTTCTC CCATTACT | [Btn]ACAAAAAACCAAACCAAAA ACTTTAATC |
| **Sequencing primer** | GTATATGGTATTATATTTT TTTAAG | ATTTGTGTGTGTAATATGAA |

They were designed using PyroMark Assay Design software from Qiagen (Hilden, Germany).