To the Editor: In a recent *Nature Genetics* letter entitled "Estrogen receptor alpha (*ESR1*) gene amplification is frequent in breast cancer," Holst *et al.* report that more than 20% of breast cancers harbor genomic amplification of the *ESR1* gene[1]. The authors also suggest that *ESR1* amplification may help to identify a subgroup of estrogen-positive breast cancers likely to have a good response to anti-estrogen therapy. As the authors acknowledge, such an observation constitutes a rather unexpected finding in light of a number and variety of studies that have focused on the structure, expression and function of the estrogen receptor gene in breast cancer cells since its discovery more than 20 years ago.

Given the clinical importance of this finding, this report prompted us to investigate the status of the estrogen receptor gene in a series of 381 breast cancers studied by BAC-array CGH (aCGH). This series included 360 tumors (184 invasive ductal, 88 ductal *in situ*, 27 lobular, 24 micropapillary and 37 medullary carcinomas) and 21 breast cancer cell lines. The aCGH contained 3,342 sequence-validated BACs covering the human genome at the mean density of one BAC per megabase[2–4]. In particular, it included the RP11-450E24 BAC, which was used by Holst *et al.* to monitor *ESR1* gene amplification, and BAC CTD-2019C10, which contains the *ERBB2* gene. Cy5 (tumor DNA) to Cy3 (control DNA) ratios at each BAC locus were determined and analyzed using previously published spatial normalization, VAMP (visualization and analysis of CGH array, transcriptome and other molecular profiles) and GLAD (gain and loss analysis of DNA) analysis procedures[5–7] (**Supplementary Methods** online).

We investigated these two loci with reference to the twofold copy number increase (2× threshold) used by Holst *et al.* to define amplification. On our aCGH platform, the 2× threshold was calculated on the basis of the $\log_2$ ratios for single-copy gain of chromosome X loci in normal female/male hybridizations (the median $\log_2$ ratio of 130 chromosome X clones is +0.49, approximated to 0.5 in **Supplementary Fig. 1a**,**b** online). The single-copy loss was determined by chromosome 1p $\log_2$ ratios in a series of 34 oligodendrogliomas with 1p/19q deletions[3] (the median $\log_2$ ratio of 340 chromosome 1p deleted loci is –0.48). To estimate the relationship between FISH and aCGH data, we also took advantage of 49 cases that could be studied by both approaches at the *ERBB2* locus. Results obtained with both techniques were strongly correlated ($r = 0.83$), with an aCGH $\log_2$ ratio of 0.5 corresponding to an absolute FISH copy number of 4.8, hence close to the twofold copy number increase defined above if taking into account the hyperdiploid status of most breast cancers.

It seemed that the distribution of ratios for the *ERBB2* and *ESR1* loci were markedly different. All tumors defined as HER2-positive by immunohistochemistry[8] showed aCGH ratios higher than the 2× threshold (**Supplementary Fig. 1a**). In contrast, concerning the *ESR1* locus, only three cases (3/341 interpretable cases; 0.9%) crossed the 2× threshold (**Supplementary Fig. 1b**), one of these being clearly estrogen receptor (ER)-negative by immunohistochemistry[9]. Moreover, we did not observe any statistically significant difference in *ESR1* aCGH ratios between ER-positive and ER-negative tumors.

To rule out the possibility that low-level genomic amplification of *ESR1* may have escaped detection on aCGH because of technical reasons, we carried out quantitative PCR (qPCR) and FISH for validation. To investigate copy number at the *ESR1*, *ESR2* and *TGFBR3* loci, we used qPCR on a subset of 168 cases, including 2 out of the 3 cases with aCGH ratios higher than the 2× threshold (**Supplementary Table 1** online lists primer sequences). The aCGH and qPCR results were strongly correlated at corresponding loci ($r = 0.6$). Four cases, including the two cases previously detected by aCGH, had qPCR ratios higher than the 2× threshold (**Supplementary Fig. 1c**). In the other two cases, this increased qPCR ratio was rather due to a relative copy number loss at control loci, as suggested by decreased aCGH ratios at corresponding and flanking BACs. We did FISH analysis on two of the breast carcinomas with *ESR1* aCGH ratios over the 2× threshold and for which material was available. As positive controls for amplification, sarcomas with 6q amplicons encompassing the *ESR1* locus that were initially characterized on a dedicated aCGH[10] and further analyzed on the genome-wide aCGH used herein were also investigated. As for *ERBB2*, we observed a very strong correlation between aCGH ratios and FISH copy numbers at the *ESR1* locus ($r = 0.9$; **Supplementary Fig. 1d**,**e**), showing that the very few breast cancers with notably increased aCGH ratios indeed harbored an increased copy number by FISH. The aCGH ratios of 1.86 and 1.69 for the two breast cancers corresponded to FISH ratios (number of spots at the *ESR1* locus as compared to the control locus) of 1.65 and 1.56, respectively. This correlation curve pointed out that our aCGH platform was even more sensitive to detect copy number increase at the *ESR1* locus than at the *ERBB2* locus.

The study of this large series of breast cancers with aCGH, qPCR and FISH indicated that less than 1% of breast cancers harbored a notable increase of *ESR1* copy number and that, notwithstanding subtle variations between techniques, these increases never reached a level similar to that of the *ERBB2* amplification, even in ER-positive cases. These results sharply contrast with those reported by Holst *et al.*, although it has to be mentioned that cribriform and mucinous carcinomas, which showed the highest frequency of *ESR1* amplification in the series studied by Holst *et al.*, were not included in our analysis.

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Note: Supplementary information is available on the Nature Genetics website.

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To the Editor: Holst *et al.* have recently reported that 15.3% and 20.6% of breast cancers harbor *ESR1* copy number gains and gene amplification, respectively[1]. *ESR1* gains and amplification correlated with ERα expression; however, 2.4% of the gains and 0.6% of the amplified cases failed to show expression of the gene product. Although retrospective and uncontrolled, these data suggested that *ESR1* amplification could help identify a subgroup of individuals with breast cancer who would benefit most from endocrine therapy[1]. Holst *et al.*[1] described that *ESR1* gene amplifications were first identified in their unpublished analysis of breast cancers using 10K Affymetrix SNP arrays. They hypothesized that *ESR1* gene amplifications were not found in most previous comparative genomic hybridization (CGH) and microar-