ESR1 gene amplification in breast cancer: a common phenomenon?

To the Editor: Using fluorescence *in situ* hybridization on tissue microarrays (FISH-TMA), Holst *et al.*[1] recently reported amplification of *ESR1*, the gene encoding estrogen receptor alpha, in 21% (358 of 1,739) of breast cancers. This prompted us to analyze *ESR1* copy number using either FISH-TMA or array CGH (aCGH) in a combined series of 725 breast cancers (see **Supplementary Methods** online for details of series and methodology).

We analyzed a total of 334 cases by FISH-TMA using the same FISH probe (end-sequence verified) for *ESR1* (RP11-450E24) as reported by Holst *et al.*[1] We carried out automated scoring of FISH signals using Metacyte (Metasystems) and considered cases to be amplified when the *ESR1* to centromere 6 ratio was ≥2 (ref. 2). We found *ESR1* to be amplified in four cases (1%, **Supplementary Fig. 1** and **Supplementary Table 1** online). Digital FISH images for *ESR1* and centromere 6 on the breast cancer TMAs are publicly available (http://www.gpecimage.ubc.ca/).

Holst *et al.*[1] validated *ESR1* gene amplification using a quantitative PCR (qPCR) assay comparing DNA copy numbers of *ESR1* and *ESR2* in four cases with and without *ESR1* amplification (as determined by FISH). We applied the same qPCR assay to 125 breast tumors that were included on our TMA. We observed increased DNA copy number of *ESR1* as compared to *ESR2* in 20 of 125 breast tumors (16%): two cases were considered amplified by FISH and 18 cases had a normal *ESR1* to centromere 6 ratio. The use of *ESR2* as the reference gene introduced an additional bias to the well-known limitations of qPCR in scoring copy number gains; this locus was lost in 12% of cases using aCGH (data not shown). We therefore used as controls two additional genes rarely altered in breast cancer: *EIF5B* (2q11.1) and *PVR* (19q13.2). Using this more rigorous qPCR assay, we found that three samples showed amplification of *ESR1* (and a further two when normalizing separately with each control gene) and only one of these was considered amplified by FISH.

Table 1 Analysis of copy number abnormalities at the *ESR1* locus using three different aCGH platforms

aCGH platform	No. of probes at *ESR1* locus	Breast tumors			ER status	
		N	Gains (%)	Amplification (%)	ER+ (%)	ER– (%)
Oligo aCGH[2]	4	171	13 (8)	2 (1)	113 (66)	58 (34)
BAC aCGH[4]	1	143	5 (3)	0 (0)	94 (66)	49 (34)
Agilent 244K aCGH	33	77	7 (9)	2 (3)	75 (97)	2 (3)

We studied a further 391 breast cancers with aCGH, the methodology first used by Holst *et al.* to identify *ESR1* amplifications. Three different platforms were used: a custom 30K oligonucleotide array[3,4] ($n = 171$), an OncoBAC array[5] ($n = 143$) and the Agilent 244K array[6] ($n = 77$). As shown in **Table 1**, both copy number gain (18 of 391, 5%) and amplification (4 of 391, 1%) at the *ESR1* locus were rare events. In contrast, we observed the expected frequency of commonly amplified regions, such as *ERBB2* at 17q12 and *CCND1* at 11q13 (refs. 3,5).

The reported *ESR1* amplicon was approximately 600 kb in size[1], and each of the aCGH platforms used here has the capability to detect an amplicon of this small size (see **Supplementary Methods** for details). The oligonucleotide 30K array contained four probes at the *ESR1* locus (**Supplementary Fig. 2a** online), and when we used our segmentation and calling algorithms, we found that the overall frequency of *ESR1* copy number gains was low (**Supplementary Fig. 2b** and **Supplementary Table 2** online), which contrasts with the high frequency of *ERBB2* copy number gains observed in the same tumors (**Supplementary Fig. 2c**). We verified this result by application of an algorithm specifically designed to detect low-level focal amplifications. The OncoBAC array contained a single BAC clone that spanned the *ESR1* locus (**Supplementary Fig. 2a**). The published OncoBAC array data was reanalyzed for this investigation (**Supplementary Methods**) and also showed a low percentage of copy number gains (**Supplementary Tables 3** and **4** and **Supplementary Fig. 3** online). The third aCGH platform contained 33 probes spanning from 152.2 to 152.5 Mb on 6q21, fully encompassing *ESR1*. Using this array, we observed a similar low frequency of amplification in the breast cancers (**Supplementary Table 5** and **Supplementary Fig. 4** online).

The results reported here (*ESR1* amplification in 1% of breast cancers) are clearly different from those published in this journal (*ESR1* amplified in 21% of breast cancers) by Holst *et al.*[1]. Several explanations for this disparity could be possible. The most trivial, given that Holst *et al.*[1] reported that *ESR1* amplification was exclusive of estrogen receptor (ER)-positive cases, would be that our series had a substantially larger proportion of ER-negative cases. However, that is not the case, as 69% of the 725 cases studied here were ER positive. Furthermore, the use of the CGH arrays described above rules out difficulty in identifying the amplicon because of its small size as a possible source of discrepancy. It is possible that natural copy number variation (CNV) in the reference DNA could mask our ability to observe amplification at the *ESR1* locus in the aCGH experiments. However, Redon *et al.*[7] reported no copy number variation at the locus where the clone used by Holst *et al.* maps. Moreover, we investigated this further in the oligonucleotide 30K array data by examining the signal in the refer-

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ence channel at the *ESR1* locus and found no evidence of CNV. Thus, CNV is unlikely to be the explanation for the discrepancy.

The key difference between our study and that of Holst *et al.*[1] is the methodology for scoring FISH-TMA (manual vs. automated) and the criteria used to call amplifications. Holst *et al.*[1] scored as amplified not only cases with an *ESR1* to centromere 6 ratio ≥2 but also "tumors with tight signal clusters…independent of their *ESR1*/centromere 6 ratio." They report using previously the same definition of amplification for *CCND1*, *ERBB2*, *MDM2* and *MYC*[8]. However, review of this publication reveals use of a single amplification criterion: signal ratio ≥2. As the authors state that "most amplified cases showed a clustered arrangement of additional *ESR1* copies"[1], we interpret this to mean that most of the *ESR1*-amplified cases were scored using subjective criteria. In contrast, the automated system we used to score FISH signals employs specific measurement algorithms to detect and quantify such clustered signals. We have previously reported a high correlation between manual and automated scoring of FISH signals and have implemented the use of this system for the scoring of gene amplification events[2]. The system is FDA approved for the automated scoring of *ERBB2* gene amplification (Metasystems). Using this objective set up, we found that only one case had a tight cluster of signals.

In summary, our data compiled from several institutions and obtained using two different techniques does not validate the findings of Holst *et al.*, and we conclude that *ESR1* amplification in breast cancer is a rare event of unknown clinical significance.

***Lindsay A Brown*[1,2], *Jeremy Hoog*[3], *Suet-Feung Chin*[4,5], *Yu Tao*[3], *Abd Alnaser Zayed*[1,2], *Koei Chin*[6], *Andrew E Teschendorff*[4,5], *John F Quackenbush*[7], *John C Marioni*[8], *Samuel Leung*[9,10], *Charles M Perou*[11], *Torsten O Neilsen*[9,10], *Matthew Ellis*[3], *Joe W Gray*[6], *Philip S Bernard*[7], *David G Huntsman*[1,2,9,10,12] & *Carlos Caldas*[4,5,12]**

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Measurement of DNA copy number ratios can be done by different methods, including FISH and CGH. Concordance between FISH and CGH results is generally high in breast carcinomas[2,3]. Public aCGH data are available for 266 individuals with breast cancer. Only Nessling *et al.*[4] had used the same BAC clones as Holst *et al.*[1]. Other groups used BAC or cDNA clones that include or overlap the *ESR1* gene (**Supplementary Table 1** and **Supplementary Note** online) and different cut-offs for calling gains and losses. In three studies, 12 of 266 (4.5%) breast tumor samples showed a gain, and of these 7 (2.6%) showed a higher-level amplification (ratio >2) for the *ESR1* gene (**Table 1**)[2,4,5].

We have analyzed DNA copy number ratios in 68 primary breast tumors (27 estrogen receptor (ER)-positive tumors and 39 ER-negative tumors; ER status was not available for two tumors) using a 3.2K aCGH (unpublished data and **Supplementary Note** online). We used the same BAC clones spanning the 600-kb *ESR1* amplicon as Holst *et al.*[1] We observed a gain in 5 of the 68 (7.4%) tumors, as determined by the 'fused lasso' method[6] (FDR < 0.01), for BAC clones RP3-44C4, RP11-450E24 and RP1-130E4 (**Supplementary Table 1**). The highest ratio for any of these BAC clones in these breast cancer samples was a 1.24-fold increase in copy number ratio. We also analyzed two unpublished 40K cDNA aCGH matrices (with two cDNA probes for *ESR1*) of 37 and 50 breast tumors (unpublished data and **Supplementary Note**), where together we found that 4 of the 87 (4.6%) samples showed gain of *ESR1*. Also available to us were CGH data from the Illumina 109K SNP array[7] containing 10 probes residing within the *ESR1* gene. We found that 5 of the 112 (4.4%) analyzed tumors from stage I and II breast cancer cases showed a gain of the *ESR1* gene as determined by the 'ACE score'[8] with a FDR of <0.00001. As we found a comparable frequency of gain and amplification for the *ESR1* locus in each of the datasets, with various different cohorts, array platforms, probe densities, and analysis algorithms, it is highly unlikely that we are underestimating the *ESR1* amplification rate in breast cancer.

To test whether we could correctly call twofold gain spanning just 600 kb for each of the different array platforms, we carried out an experiment swapping aCGH data of equivalent-sized random X-chromosome segments from a 48,XXXX versus 46,XX hybridization into normal female versus

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Table 1 Available aCGH data for *ESR1* locus 6q25.1 and *ERBB2* (*HER2*) locus 17q12

				ESR1 copy number		Total			ESR1		
No. probes on array CGH	Reference	Total cases	Available cases	Ampl. (%)	Gain (%)	ER+	ER–	n.a.	ER+	ER–	n.a.
287 BAC[a]	S.F. Chin, 2007	148	148	1 (1)	2 (1)	100	45	3	3	0	0
422 BAC[a]	Nessling, 2005	31	29	3 (10)	1 (3)			31			4
3,200 BAC[b]	H.M.H., unpublished data	68	68	0 (0)	5 (7)	39	27	2	1	4	0
40,000 cDNA[b]	D.-Y.N., unpublished data	37	37	0 (0)	2 (5)	17	20	0	0	2	0
	A.B., 2006	89	89	3 (3)	2 (2)	72	14	3	4	1	0
	A.B., unpublished data	50	50	0 (0)	2 (4)	26	22	2	2	0	0
Illumina 109,000 SNP[c]	Nordgard, 2008	112	112	5 (4)	0 (0)	64	40	8	2	2	1
	Total	535	533	12 (2.3)	14 (2.6)	318	168	49	12	9	5

				ERBB2 copy number	
No. probes on array CGH	Reference	Total cases	Available cases	Ampl. (%)	Gain (%)
287 BAC[a]	S.F. Chin, 2007	148	148	10 (7)	19 (13)
422 BAC[a]	Nessling, 2005	31	31	7 (23)	6 (19)
3,200 BAC[b]	H.M.H., unpublished data	68	68	9 (13)	2 (3)
40,000 cDNA[b]	Bergamaschi, 2006	89	89	19 (21)	5 (6)
	D.-Y.N., unpublished data	37	37	13 (35)	3 (8)
	A.B., unpublished data	50	50	7 (14)	4 (8)
Illumina 109,000 SNP[c]	Nordgard, 2008	112	112	23 (21)	0 (0)
	Total	535	535	88 (16.4)	39 (7.3)

[a]Cut-off levels for gain were used as in the original papers. Chin, 2007: ratio >1.35, gain; ratio >2, amplification. Bergamaschi 2006: ratio >1.32, gain, ratio >1.59, amplification. [b]Determined by fused lasso algorithm[7] (FDR < 0.01). [c]Determined by ACE score[8] (FDR < 0.00001). n.a., not available; ER, estrogen receptor; ER+, estrogen receptor positive based on immunohistochemistry; IHC, immunohistochemistry; CGH, comparative genomic hybridization.

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normal female data, thereby modeling two-fold gain (**Supplementary Methods** online). We found that 49 of 100 randomly swapped segments were correctly called 'gain' for the cDNA array, 71 for the BAC array, and 60 for the SNP array. However, given that the authors actually claimed that the 600-kb *ESR1* area was subject to amplification, and not just simply gain, the same analysis was run on 6X and 8X inserted into the 2X set. We found that 85% of segments in the 6X/2X and 99.8% in the 8X/2X were called as 'amplified' for the SNP-CGH set, indicating that the frequency of 5% for *ESR1* copy number gain in breast cancer, as identified by us in several datasets, is correct and not flawed by technical problems in detecting *ESR1* copy number gain.

We find it unlikely that the difference between the results found by FISH and aCGH is explainable by the small size of the 6q25.1 amplicon. For example, well-known small amplicons in breast cancer, including the one containing the *ERBB2* (also known as *HER2*) gene (measuring approximately 280 kb), can be detected by aCGH. From published and our own aCGH studies, 88 of 535 (16.4%) breast cancer samples showed amplification for *ERBB2* and gain in an additional 39 (7.3%) tumors (**Table 1**), comparable to frequencies reported using FISH on tissue microarrays[9]. For the SNP-CGH dataset containing 112 breast cancer samples that were profiled on a 109K SNP array[6], FISH for the *ERBB2* gene was done and all 23 *ERBB2*-amplified samples were also detected using the Illumina platform.

In summary, we have found in our own unpublished and in published aCGH data only 12 of 533 (2.3%) samples with amplification for the *ESR1* gene and only an additional 14 (2.6%) breast tumors with gain (**Table 1**). We think that it is highly unlikely that the frequency of *ESR1* gene amplification is as high as 21%; the frequency is more likely to be less than 5%. It remains to be explored whether the high frequency reported by Holst *et al.*[1] is due to selection of breast cancer cases or to technical issues related to the assessment of *ESR1* gene copy number status.

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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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We investigated the prevalence of *ESR1* gene amplification in a cohort of 245 invasive breast carcinomas (80.1% ERα-positive) arranged in a tissue microarray (TMA) containing representative replicate tumor cores. The characteristics of this cohort were reported elsewhere[2] (**Supplementary Methods** online). Sections of the TMA were subjected to chromogenic *in situ* hybridization (CISH) with in-house generated probes for the *ESR1* gene composed of two BACs (RP11-655I21 and RP11-517B22, see **Supplementary Methods**). Out of 245 cases, 148 (85.1% ERα-positive) rendered optimal results and only 2 cases (1.35%) harbored *ESR1* gene amplification in the form of large signal clusters. None of the cases showed >5 individual copies of the gene. *ESR1* gene status in the two amplified cases and eight additional CISH not-amplified cases was confirmed by FISH. The prevalence of *ESR1* gene amplification we observed was, therefore, much lower than that reported by Holst *et al.*[1]

To rule out the possibility that amplification of *ESR1* was not detected because of CISH analysis thresholds, we used TaqMan q-PCR. Assays were designed to determine *ESR1*, *ESR2*, *FAM38B* and *ASXL2* (**Supplementary Table 1** online) copy numbers in a separate set of 35 primary invasive breast tumors, 32 of which (91.4%) were ERα-positive. We selected the reference genes *FAM38B* and *ASXL2*, instead of *ESR2*, whose locus is often deleted in breast cancers[3,4], as they have been shown to not be affected by copy number polymorphisms (http://projects.tcag.ca/variation/). Furthermore, gains or losses affecting these genes are exceedingly rare in breast cancers, as defined by analysis of a large breast cancer CGH database[3]. Derivation of the ratios between *ESR1* and reference genes (*ESR2*, *FAM38B* and *ASXL2*) made evident the importance of selecting appropriate reference genes for q-PCR copy number analysis (that is, those not deleted or gained in cancer)[3]: when we normalized *ESR1* to *ESR2*, as in Holst *et al.*, we detected 2/35 (5.7%) gains (ratio between 1.5 and 2.0) and 2/35 (5.7%) amplifications (ratio >2). However, when normalized to the reference genes *FAM38B* and *ASXL2*, 0/35 tumors showed a gain of *ESR1* and 1/35 (2.8%) showed an amplification; this amplified tumor had both a high enzyme immunoassay score (500 fmol/mg versus a mean of 82 fmol/mg) and a high immunohistochemical score (248.6 versus a mean of 138) for ERα. Out of the three amplified cases as defined by q-PCR, only one identified by *ESR1*/*ESR2* ratios showed discordant results by CISH and FISH. These discrepancies may reflect the fact that the *ESR2* locus varies in copy number in breast cancer[3]. CISH analysis of an additional 11 cases deemed not amplified by q-PCR confirmed *ESR1* copy number status in 10 cases. Further verification by FISH confirmed the results of both methods in five out of six cases. Therefore, the prevalence of *ESR1* gene amplifications in this cohort was also markedly lower than that described by Holst *et al.*[1].

HSRs on chromosome 6q have been reported in breast cancers; however, their prevalence seems to be much lower than 20%. Several studies using CGH and aCGH have demonstrated the presence of recurrent 6q21–q22 amplifications (ref. 5 and references therein); however, recurrent amplifications of 6q25 have been reported to be significantly rarer[6]. We therefore investigated the prevalence of *ESR1* gene amplifications using tiling-path aCGH in two series of invasive breast cancers (see **Supplementary Methods**). aCGH analysis of these series of 70 invasive breast cancers (45 of which were ERα-positive, M.D. and J.S.R.-F., unpublished results) revealed amplification of the *ESR1* gene in three cases (4.3%), all of which were ERα positive (**Supplementary Fig. 1a** online). This is in agreement with the aCGH data from Fridlyand *et al.*[7], who analyzed the aCGH profiles of 50 sporadic invasive ductal carcinomas and tumors from four *BRCA1* mutation carriers: by using objective criteria based on variable thresholds[7], they found that 2 of the 54 cases (3.7%) showed high-level *ESR1* gene amplification as identified by the BAC clone GS-59B4, which spans the *ESR1* gene locus; both cases were ERα positive. In addition, our analysis revealed gains of the *ESR1* gene in five cases (two ERα negative and three ERα positive, **Supplementary Fig. 1b**) and deletions in nine cases (two ERα negative and seven ERα positive), of which eight were in the form of large-scale deletions involving most of 6q (6q12–qter, **Supplementary Fig. 1c**). We did not observe any significant correlations between *ESR1* gain or amplification and ERα expression as defined by immunohistochemical analysis in our series. Furthermore, *ESR1* mRNA expression levels did not correlate with *ESR1* gene copy number in a series of 54 breast cancer cell lines[8].

Although we have also identified *ESR1* gene amplification in separate cohorts of invasive breast cancers (total of 253 cases), we found this phenomenon to be substantially less prevalent than described by Holst *et al.*[1] In our study, *ESR1* gains and amplifications did not show a significant correlation with ERα protein expression. Our findings do not support *ESR1* amplification being "instrumental in defining a subtype of primary breast cancers…optimally suited for hormonal therapy"[1]. Further independent analyses of large series of breast cancers are warranted to determine the definite prevalence of *ESR1* amplifications and whether *ESR1* is the target of 6q HSRs.

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Holst *et al.* reply: In our recent study, we reported 20.6% amplification and 14% gain of the *ESR1* gene, encoding estrogen receptor alpha (ER)[1]. We also described that *ESR1* amplification is typically low and contains only the *ESR1* gene. Four Correspondences by Brown *et al.*, Vincent-Salomon *et al.*, Reis-Filho *et al.* and Horlings *et al.* presented in this issue challenge these findings and suggest that *ESR1* amplification might occur markedly less frequently[2–5]. We believe that this controversy highlights the technical challenges of DNA measurement in clinical tissue samples.

Using array CGH, Brown *et al.*, Vincent-Salomon *et al.*, Reis-Filho *et al.* and Horlings

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Holst *et al.* reply: In our recent study, we reported 20.6% amplification and 14% gain of the *ESR1* gene, encoding estrogen receptor alpha (ER)[1]. We also described that *ESR1* amplification is typically low and contains only the *ESR1* gene. Four Correspondences by Brown *et al.*, Vincent-Salomon *et al.*, Reis-Filho *et al.* and Horlings *et al.* presented in this issue challenge these findings and suggest that *ESR1* amplification might occur markedly less frequently[2–5]. We believe that this controversy highlights the technical challenges of DNA measurement in clinical tissue samples.

Using array CGH, Brown *et al.*, Vincent-Salomon *et al.*, Reis-Filho *et al.* and Horlings

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et al.[2–5] found elevated *ESR1* copy numbers in 2–13% of breast cancers. In our own unpublished CGH array study, we found a comparable frequency (1/22; 4.5%). As contaminating normal DNA (for example, from stroma) is a challenge for detection of low-level amplicons, it is not surprising that Reis-Filho *et al.*[3], who carried out microdissection, found the highest rates of increased *ESR1* copies (11.4%). We do not consider 11.4% to be fundamentally different from the 35% found by the more sensitive FISH approach. This especially applies in the light of the highly variable CGH array data on *ERBB2* amplification (7–35%) summarized by Horlings *et al.*[4]. *ERBB2* amplification typically occurs at much higher levels (20–50 copies) than *ESR1* amplification. In our set of 358 *ESR1*-amplified tumors, only 15% had more than 10 gene copies, whereas most amplified cancers (40.9%) had clusters of 5–6 copies only. *ERBB2* amplification should therefore be identified more easily by array CGH than *ESR1* amplification.

Contamination of tumor DNA with normal DNA is also a major drawback in qPCR. Significant differences are therefore expected for studies analyzing a low-level amplicon. In the studies published in the current issue[2–5], the rate of increased *ESR1* copy number ranged between 2.3% and 19.2%. Again, we feel that the highest rate reported by qPCR comes relatively close to our FISH rate of 35%. Reis-Filho *et al.*[3] emphasized that the choice of the reference gene has marked impact on the study outcome, and they demonstrated that using alternative reference genes (*FAM38B* and *ASXL2*) reduced the frequency of *ESR1* copy number gains from 11.4% to 2.8%. To test the described impact of the reference gene, we analyzed ten randomly selected breast cancers with known *ESR1* amplification and ten breast cancers with normal *ESR1* copy number by qPCR after microdissection using *ASXL2* as a reference. A significant difference in the *ESR1*/*ASXL2* ratio between *ESR1*-amplified and non-amplified cancers was also found by this 'low-sensitivity approach' (**Fig. 1**). In our opinion, the generally low level of amplification explains the failure of several *ESR1*-amplified tumors (by FISH) to reach the threshold for amplification (*ESR1*/*ASXL2* ratio >2.0).

Most of the controversy on *ESR1* reported in this issue comes from the FISH/CISH analyses. In our initial report, we described 20.6% amplification and 14% gain by FISH. We are aware that we have previously not sufficiently described issues related to the *ESR1* FISH analysis. In our opinion, the small amplicon size in combination with the low level of amplification changes requirements for probe manufacturing and slide reading. Although larger FISH probes combining multiple BACs are superior to smaller probes for most FISH analyses, larger probes lead to more confluent signals that can barely be separated in case of *ESR1*. In our laboratory, most *ESR1*-amplified tumors have small gene clusters that could be considered as one signal if '*ERBB2* criteria' were applied. The distance between the signals is often smaller than the diameter of one FISH signal (**Supplementary Fig. 1** online). Such clusters are difficult to count although the tumor appears amplified at first sight during visual inspection. We therefore feel that estimating the *ESR1* gene copy number may—given the currently available reagents—enable a more reliable identification of amplified cancers than classical counting.

Apparently, this FISH analysis approach bears the risk of scoring artifacts. However, the marked association of amplifications and 'gains' with protein expression based on a double-blinded evaluation of FISH and IHC convinced us that we have not substantially overestimated the prevalence of additional *ESR1* gene copies. We are not aware of a possibility to reliably and reproducibly detect RNA by BAC FISH probes, the only theoretical alternative explanation for such a notable association between *ESR1* amplification and protein expression. In a follow-up study on 678 breast cancers, we have meanwhile confirmed the relationship between *ESR1* FISH and IHC (**Supplementary Table 1** online). We also found a high reproducibility of manual FISH scoring between two medical students (**Supplementary Table 2** online).

We can only speculate that some of the issues described above may have contributed to the lower rates of *ESR1* amplification published in this issue. It may be possible that Reis-Filho *et al.* missed a fraction of low-level amplifications in their TMA analysis using chromogenic *in situ* hybridization (CISH) as a result of the large 360-kb probe[3]. CISH usually produces larger signals than FISH, which might have further complicated the detection of small *ESR1* gene clusters. In another paper, van de Vijver *et al.* compared FISH and CISH in a series of 35 breast cancers with low-level *ERBB2* amplification and emphasized a markedly reduced sensitivity of CISH[6]. Using a large FISH probe (380 kb) and manual scoring, Ejlertsen *et al.* reported 14% *ESR1* amplifications (ratio ≥2.0) in 94 ER-positive breast cancers (B. Ejlertson, Danish Breast Cancer Cooperative Group, personal communication). Brown *et al.* used the

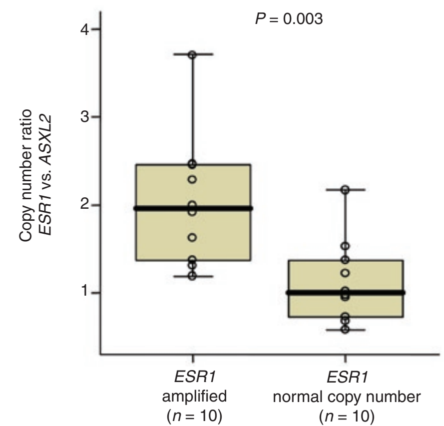

Figure 1 Box plot showing the DNA copy number ratio between *ESR1* and *ASXL2* as measured by qPCR in ten cases of FISH amplified and FISH normal breast cancers. Circles indicate the individual qPCR ratio measured for each tumor.

same small FISH probe as we did but employed an automated scoring system approved for *ERBB2* analysis[2]. Given the small size of the *ESR1* gene clusters, it may be possible that an algorithm trained for detection of *ERBB2*-like amplicons may be less suited for *ESR1* analysis. The highest *ESR1* amplification rate reported so far is by Nembrodt *et al.*, reporting *ESR1* amplification by DNA blot in 6/14 ER-positive tumors[7].

In our opinion, the varying data emphasize that detection of *ESR1* amplification is not trivial and will probably require modifications of the established procedures. Substantial discrepancies exist not only with respect to our previous communication but also among the four correspondences presented in this issue. Because of the high reproducibility of the FISH results in our laboratory and its strong association with ER protein expression, we still tend to believe that the true frequency of increased *ESR1* copy numbers is not too far from the numbers described in our initial article.

Frederik Holst[1], Phillip Stahl[1], Olaf Hellwinkel[1], Ana-Maria Dancau[1], Antje Krohn[1], Laura Wuth[1], Christian Heupel[1], Annette Lebeau[1], Luigi Terracciano[2], Khawla Al-Kuraya[3], Fritz Jänicke[4], Guido Sauter[1] & Ronald Simon[1]

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The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturegenetics/.

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HDAC2 deficiency and histone acetylation

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To the Editor: Histone hyperacetylation is a commonly recognized molecular marker of histone deacetylase (HDAC) inhibition and is considered to be a permissive requirement for the radiosensitizing ability of HDAC inhibitors[1]. We have previously reported on the radiosensitization of tumor cells by HDAC inhibitors[2,3], and so we were intrigued by the *Nature Genetics* report by Ropero *et al.* describing the apparent absence of histone hyperacetylation in HDAC2-deficient cancer cell lines following treatment with the HDAC inhibitor trichostatin A (TSA)[4], and by the possibility of using such cell lines in mechanistic studies on the role of HDAC inhibitors and histone acetylation status in radiotherapy.

The authors screened several cancer cell lines for the presence of mutations in the coding sequences of a number of 'epigenetic modifier genes' and identified a specific truncating mutation in *HDAC2* exon 1 in colorectal carcinoma cell lines RKO and Co115 but not HCT116. The inactivating mutation resulted in abrogation of HDAC2 protein expression and enzymatic activity, and a resultant loss of biochemical and biological effects of HDAC inhibitors was hypothesized. Following TSA treatment (250 nM) for 24 h, baseline acetylation of histones H3 and H4 was observed in the HDAC2-deficient cell lines, whereas hyperacetylation was present in HDAC2-proficient cell lines. The authors concluded that "trichostatin A was unable to induce the hyperacetylation of either histone (H3 or H4) in the HDAC2-deficient RKO and Co115 cell lines, but it was effective in the cells with wild-type HDAC2." Furthermore, the authors demonstrated that both HDAC2-deficient cell lines and xenografts were resistant to the cytotoxic action of TSA.

We recently reexamined a number of these introductory experiments, initially using an 'in-house' RKO cell line (termed RKO-L; cultured for some time in our laboratory) and later a variant obtained directly from the American Type Culture Collection (termed RKO-ATCC). Surprisingly, the HDAC2 expression of both RKO variants was identical to that of the HCT116 cells (results shown only for RKO-ATCC). The RKO cell clone used in the previous study[4] (termed RKO-ES; cell line provided by Ropero *et al.*) was confirmed negative for HDAC2 expression, as was also the Co115 cell line (**Fig. 1a**). We then sought to verify the identity of the three RKO cell lines (RKO-L, RKO-ATCC and RKO-ES), specifically to exclude inadvertent contamination or mislabeling that might have contributed to the differential HDAC2 expression. The three RKO variants were subjected to DNA fingerprinting analysis using 15 genetic markers that segregate independently (done at the National Institute of Forensic Medicine, Oslo, Norway). According to the results, the RKO-L and RKO-ATCC cell lines are confirmed to be identical, whereas the RKO-ES cell line harbors distinct discrepancies when compared to the RKO–L and RKO-ATCC variants but is probably of the same origin (**Supplementary Fig. 1** online).

Given the variation in HDAC2 expression, we analyzed the histone acetylation patterns of the four representative cell lines (RKO-ES, RKO-ATCC, HCT116 and Co115) following incubation with 100 nM TSA for

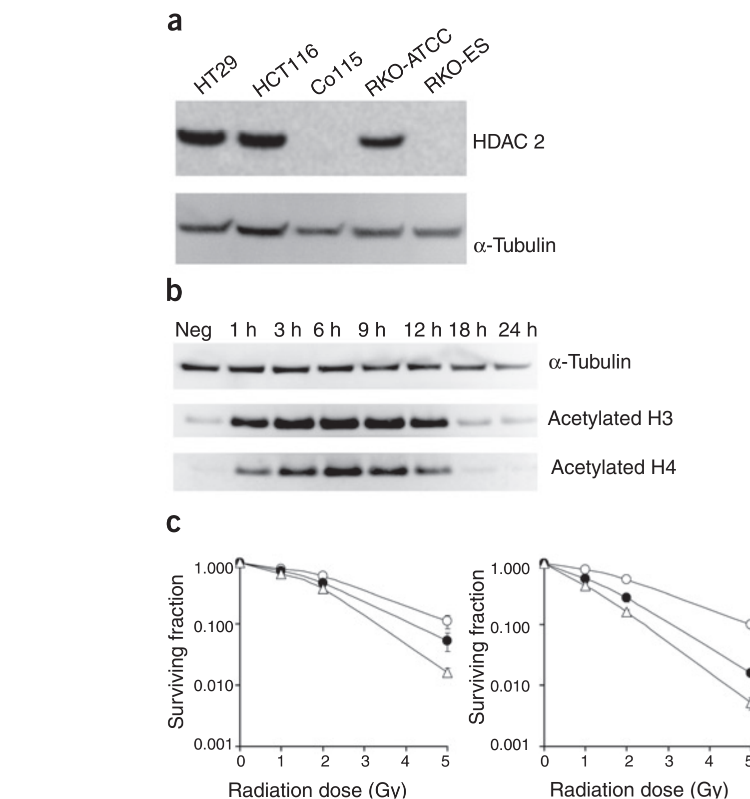

Figure 1 HDAC2 status, histone acetylation, and clonogenicity of colorectal carcinoma cell lines. (**a**) Absence or presence of HDAC2 expression in HT29, HCT116, Co115, RKO-ATCC and RKO-ES cells. The full-length immunoblot is presented in **Supplementary Figure 4** online. (**b**) Levels of acetylated H3 and H4 in RKO-ES cells following incubation with TSA (100 nM) for 0–24 h. The full-length immunoblot along with corresponding immunoblots of the RKO-ATCC, HCT116 and Co115 cells lines are presented in **Supplementary Figure 2**. (**c**) RKO-ATCC (left panel) and RKO-ES (right panel) cells were exposed to ionizing radiation without (open circle) or following pretreatment for 18 h with 30 nM (filled circle) or 100 nM (triangle) TSA, to determine relative colony formation (mean ± s.e.m., $n = 3$). Experimental procedures are described in **Supplementary Methods** online.

decay (NMD). The variant lies on the third most frequent haplotype (**Fig. 1**), which is either 'mildly' protective or neutral in AMD, thereby not confirming the degradation hypothesis. It is possible, however, that R38X has an effect other than NMD.

Although the study by Weber and colleagues suggests a very plausible functional mechanism explaining the association of the 10q26 locus with AMD, it does not eliminate all other possibilities. In all fairness, the same holds true for most other AMD-associated loci. For example, although nobody disputes the role of complement genes in AMD, the exact disease-associated variants and their functional consequences are not known for *CFH*, *CFB/C2* and/or *C3*. For the *ABCA4* locus, it took over 10 years for the functional research to catch up with the genetic studies to prove the association with AMD. The disease-causal consequence of *ABCA4* mutations, an elevated accumulation of the cytotoxic lipofuscin fluorophore A2E in the retinal pigment epithelium, is now directly linked to complement activation both *in vitro*[15] and *in vivo* in the *Abca4*$^{-/-}$ mouse model (R.A. Radu, G.H. Travis and D. Bok, Jules Stein Eye Institute, personal communication). The current study provides hope that the functionally relevant variants will soon be unequivocally identified in all AMD-associated loci to directly aid the development of therapeutic applications.

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Conflicting evidence on the frequency of *ESR1* amplification in breast cancer

Donna G Albertson

An earlier report of high-frequency *ESR1* amplification in breast cancer is now challenged by correspondence from four groups. This discussion of whether or not there is something 'FISHy' about *ESR1* amplification highlights the difficulty of validating such observations, leaving the frequency and clinical significance of *ESR1* amplification in breast cancer an open question.

Solid tumors may acquire gains and losses of copies of genomic regions encompassing oncogenes and tumor suppressors. Amplification, defined as a relatively high increase in copy number of a restricted region, may also occur[1]. Last year, Holst and colleagues[2] reported that amplification of *ESR1* as detected by FISH occurs in a significant proportion of breast cancers (20.6%), as well as in pre-malignant lesions and benign breast disease. They also found that *ESR1* amplification was an indicator of good prognosis for a subset of individuals with breast cancer. The observation of the high frequency of amplification was notable, as its presence in breast cancer had not been widely appreciated. Holst *et al.*[2] suggested that *ESR1* amplification was missed because the recurrent region of amplification is small (600 kb). On page 806 of this issue, Correspondences from four groups[3–6] contest the high frequency of *ESR1* amplification and together find that amplification occurs at a frequency of only 0–10% as detected by a variety of methods, including array comparative genomic hybridization (CGH), FISH and quantitative PCR. In a reply to this challenge, Holst and colleagues[7] stick to their guns and offer additional data to support their observations.

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Selection pressure drives amplification

Amplicons are unstable, in contrast to the statement to the contrary in the original report from Holst *et al.*[2]. When extra copies of the locus no longer provide an advantage, they are likely to be lost. Thus, amplicons harbor genes currently under selection pressure in a tumor, indicating that these genes are contributing to the functioning of the tumor and further that the genes are potential therapeutic targets. The frequency with which overexpression of particular oncogenes is accomplished by amplification varies with both the oncogene and tumor type[1]. In most cases, amplification is only one of several mechanisms promoting overexpression, and expression of oncogenes is usually only modestly correlated with copy number[8]. Upregulation of *ERBB2*, on the other hand, is unusual in that it is almost always associated with high level amplification[1]. This feature allows copy number of the locus, as determined by FISH, to be an effective diagnostic for *ERBB2*-positive breast tumors and identification of individuals with breast cancer likely to benefit from Herceptin[9]. Amplification of *ESR1* might similarly be of diagnostic utility.

Amplification detection by different means

The study carried out by Holst *et al.*[2] used FISH, and in the original publication, the authors state that they based their analysis on the scoring system used by the US Food and Drug Administration–approved *ERBB2* amplification test kits (PathVysion). The package insert for this test directs the user to identify regions of bright hybridization signals, count closely spaced signals as one signal and determine the average *ERBB2* to centromere 17 copy number ratio from 20 nuclei. Now, in their reply, Holst *et al.*[7] emphasize the importance of scoring clusters of signals, rather than following the standard procedure. Moreover, Holst *et al.*[7] point out that *ESR1* is not highly amplified. They scored only ~3% of amplified cases with >10 *ESR1* copies, whereas the majority had 3–6 copies. By comparison, they found that *ERBB2*

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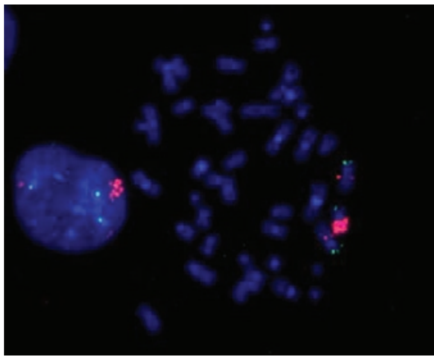

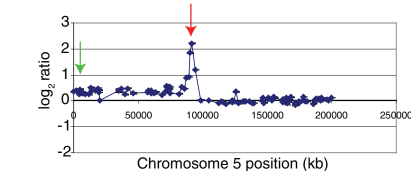

Figure 1 Compare FISH and CGH studies with caution. FISH and CGH are quantitatively concordant when scoring criteria are unambiguous and samples are homogeneous, as shown here for a ~fourfold amplification (12:3 copies, 5q, red: 5p, green). However, in heterogeneous samples, CGH will underestimate the highest copy number and FISH interpretation is uncertain with closely spaced spots.

amplicons typically have 20–50 copies. Thus, *ESR1* amplification will be more difficult to detect than *ERBB2* amplification, and detection of *ERBB2* amplification is not an indicator of the capability of other measurement techniques to detect *ESR1* amplification.

The Correspondences report failure to find high-frequency *ESR1* amplification using several methods[3–6]. Two of the groups used FISH and the more traditional scoring methods. All four groups used CGH, which unlike FISH measures the average copy number of the population of cells and so is sensitive to tumor heterogeneity and dilution of tumor cells by normal cells. The data reported by Vincent-Salomon *et al.*[6] and our own published observations[10] indicate that when detected by CGH, the elevated copy number of *ESR1* is low, consistent with the FISH data of Holst *et al.*[7] The question then is: how reliably could the methodologies used by the corresponding groups have detected a twofold copy number increase (the criterion used for scoring the locus amplified by FISH) of a 600-kb region (the length of the core *ESR1* amplicon)? Brown *et al.*[3] attempted to answer this question for their custom oligonucleotide CGH platform, as did Horlings *et al.*[4] for their three CGH platforms. These analyses showed that for all but one of the four platforms, the majority of such amplicons would have been found. Although admixed normal cells and tumor heterogeneity were not taken into account, the frequencies of amplification found by CGH, which ranged from 2% to 7.4%, are still well below the 20.6% published by Holst *et al.*[2]. Other confounding factors in comparisons of CGH and FISH include variable and/or overly stringent definitions of 'amplification' and possible misidentified clones on arrays.

Nevertheless, the clusters seem to be a recognizable feature of breast tumors associated with high estrogen receptor protein expression, as the capability to perform the *ESR1* FISH scoring method has been transferred to two newly trained medical students[7] with results similar to the original publication[2]. Three additional studies[11–13] have found *ESR1* amplification at comparable frequencies. In the most recent one[11], however, carried out with a cohort of postmenopausal women receiving tamoxifen therapy following radical surgery, *ESR1* amplification was associated with shorter time to recurrence, whereas Holst *et al.*[7] found *ESR1* amplification to be a good indicator of prognosis for a subset of women.

Considering clinical relevance

Can these conflicting data be rationalized? If the CGH measurements are taken to indicate infrequent true amplification—that is, high level copy number increase—then the FISH signal clusters observed by Holst *et al.*[2] might not reflect gene amplification, but rather hybridization to open, highly transcribed chromatin. In such a case, one would be comparing apples and oranges by FISH and CGH. (**Fig. 1**) Nevertheless, the clinical significance concerning prognosis remains unresolved. Thus, it seems that the jury is still out on the question of *ESR1* amplification and its clinical significance.

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Lung stem cells in the balance

Saverio Bellusci

Wnt ligands are secreted glycoproteins with critical roles in organogenesis, cancer initiation and progression, and maintenance of stem cell pluripotency. A new study strengthens considerably our understanding of the role of Wnt signaling in progenitor cells of the lung epithelium during development and injury.

Since the discovery and sequencing of *Drosophila wingless* more than 20 years ago, many more members of the Wnt family have been identified. The study on page 862 of this issue by Yuzhen Zhang and colleagues[1] provides new insight into the role of Wnt signaling in lung epithelial cells, both during development and after injury.

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From Gata6 to β-catenin

Canonical Wnt ligands act extracellularly to promote the stabilization of β-catenin in the cytoplasm. β-catenin is in turn translocated to the nucleus to form a complex with other transcription factors[2]. In the paper by Zhang *et al.*[1], the authors show that Gata6, a zinc-finger transcription factor expressed in the epithelium, regulates the expression of an epithelial Wnt receptor called Frizzled 2. The authors propose that the function of