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ESR1 gene amplification in breast cancer: a common phenomenon?

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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

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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.

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 reference 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.

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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)