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REVIEW

Estrogen receptor alpha gene amplification in breast cancer: 25 years of debate

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Abstract

Twenty-five years ago, Nembrot and colleagues reported amplification of the estrogen receptor alpha gene (*ESR1*) in breast cancer, initiating a broad and still ongoing scientific debate on the prevalence and clinical significance of this genetic aberration, which affects one of the most important genes in breast cancer. Since then, a multitude of studies on this topic has been published, covering a wide range of divergent results and arguments. The reported prevalence of this alteration in breast cancer ranges from 0% to 75%, suggesting that *ESR1* copy number analysis is hampered by technical and interpreter issues. To date, two major issues related to *ESR1* amplification remain to be conclusively addressed: (1) The extent to which abundant amounts of messenger RNA can mimic amplification in standard fluorescence *in situ* hybridization assays in the analysis of strongly expressed genes like *ESR1*, and (2) the clinical relevance of *ESR1* amplification: Such relevance is strongly disputed, with data showing predictive value for response as well as for resistance of the cancer to anti-estrogen therapies, or for subsequent development of cancers in the case of precursor lesions that display amplification of *ESR1*. This review provides a comprehensive summary of the various views on *ESR1* amplification, and highlights explanations for the contradictions and conflicting data that could inform future *ESR1* research.

Key words: Estrogen receptor alpha gene; Breast cancer; Tamoxifen; Gene amplification; Methodology

Core tip: The estrogen receptor alpha gene gene (*ESR1*) is one of the most important genes in breast cancer, but the prevalence of *ESR1* amplification is matter of ongoing debate. A number of studies suggest that technical issues and lack of standards contribute to the discrepant findings. Future studies should focus on the potential clinical relevance of this phenomenon.

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INTRODUCTION

In 1990, when Nembrot *et al*[1] reported on amplification of the estrogen receptor alpha encoding gene *ESR1* in breast cancer, it was not possible to foresee that, two and a half decades later, conflicting data on the prevalence and possible clinical significance of this alteration would lead to an ongoing debate[2,3]. The relevance of the controversy results from the importance of the gene for the treatment of breast cancer. *ESR1* encodes the estrogen receptor alpha (ERα), which is a cellular receptor for the steroid hormone estrogen, a key molecule that regulates the growth and differentiation of the mammary gland[4-8]. ERα is activated by estrogen and drives cell proliferation in breast cancer[9-11]. About two thirds of breast cancers express ERα at the time of diagnosis, making the ERα-protein the most frequently applied clinical biomarker and molecular therapy target for this tumor type[9,12-15].

Gene amplification is a critical mechanism for oncogenic activation of a gene[16,17], and is believed to be a marker for oncogene addiction[18,19]. The success of Herceptin® in treating breast and gastric cancers in which the *ERBB2* gene [that encodes the human epidermal growth factor 2 (HER2)] is amplified has impressively demonstrated the clinical value of gene amplification[20,21]. The report of frequent *ESR1* amplification as a candidate marker for optimal response of proliferating breast disease to anti-estrogenic Tamoxifen monotherapy, thus attracted considerable attention in the scientific community[22-29].

However, the accounts of *ESR1* amplification were challenged from the outset. Watts *et al*[30] used the same method as Nembrot *et al*[1], but reported an unexpected lower incidence of copy number increase in 1991. This already suggested that differences in laboratory protocols, interpretation of results, and tissue sampling may represent major challenges in the analysis of *ESR1* amplification. To date, articles that present a wide range of diverging data and arguments[22,31], continue to be published, and debate or address the topic of *ESR1* amplification. An intense dialog flared up following the report that frequent *ESR1* amplification was detected in a large series of breast cancers and that regarding clinical data suggested a particular benefit of Tamoxifen treatment for these patients[24-26,28,29,32-38]. This controversy was especially evident in response to the suggestion that pre-mRNA artifacts could explain the conflicting results reported in a 2012 fluorescence *in situ* hybridization (FISH) study on *ESR1* amplification[3,22,23,39,40], which appeared to be a self-fulfilling prophecy concerning mRNA artifacts that were discussed as far back as 2008[36]. Conclusions ranged from no *ESR1* amplification in breast cancer[38] and reports of *ESR1* amplification being "fictional"[39], to reports of frequent prevalence, with predictive significance for response or resistance to anti-estrogen therapy[24-26,40,41]. In addition, whether amplification of *ESR1* is an early or late event, and whether it can be implemented as a potential marker for prophylactic anti-estrogen treatment[3,42], is also unresolved.

PREVALENCE OF *ESR1* AMPLIFICATION

Definitions and references

Various studies have published *ESR1* amplification frequencies that range from 0% to 75% (Figure 1, Appendices A-D), depending on methods used, sample cohorts, and threshold definitions[31]. The amplification is typically described as occurring in a mosaic pattern, indicating heterogeneous and low level increases in copy number. Nuclei often show only few additional gene copies in tight clusters of the homogeneously staining region (HSR) type[40] (Figure 2).

This pattern of amplification is of particular relevance for understanding the scientific debate on *ESR1* amplification in breast cancer. To gain clarity on the prevalence of gene amplification requires first that the term be defined. In general, "gene amplification" is defined as an increase in the gene copy number in a cell, independent of the ratio of gene copy number to centromere copy number[43,44]. However, as testing for human epidermal growth factor receptor 2 (HER2, *ERBB2*) became more frequent, the term "gene amplification" was reserved for amplifications with an average gene to centromere ratio of $\geqslant$ 2.0 or $\geqslant$ 2.2 (or > 6 copies per nucleus), simply because the threshold for predicting the response to therapy was determined at this level[45-47]. As a consequence, low level gene amplification - with a ratio less than 2.0 but greater than 1.0 - was neglected. However, for studies in which low copy number increases associated with gene amplification are investigated, the exclusion of amplifications with these low ratios[48] can have major consequences with respect to the prevalence of the genetic alteration, and can decrease the frequencies determined to considerably lower numbers in study cohorts, as shown in Figure 1 and Appendices A-D.

Low-level gene copy number alterations such as *ESR1* amplifications often present as a continuum of one-to-several additional *ESR1* copies, and minor changes of the threshold cut off value can have a major impact on study outcome. For example, using a cut off of > 2.0 instead of $\geqslant$ 2.0 for amplification calling, or a ratio 2.2 instead of 1.8, can change the amplification frequency by almost 50%[22,49,50]. In a recent study done with use of next-generation sequencing (NGS), the threshold of $\geqslant$ 6 average copies [as recommended for *ERBB2* (HER2) testing[46]] in tissue samples with tumor purity of > 20% resulted in only 0.8% *ESR1* amplification across samples[51].

The low level and heterogeneous character of *ESR1* amplification suggests that "classical *ERBB2* (HER2)" thresholds may not be optimal for *ESR1* analysis[26,40]: This is even more true when non-morphologic methods are applied for analyzing isolated DNA, wherein the choice of normalization references has a critical impact on analysis outcome. Indeed, several investigators employing quantitative polymerase chain reaction (qPCR) have demonstrated that the prevalence of *ESR1* amplification depends massively on the choice of the reference genes or sequences[32,34,40], and have suggested that variable

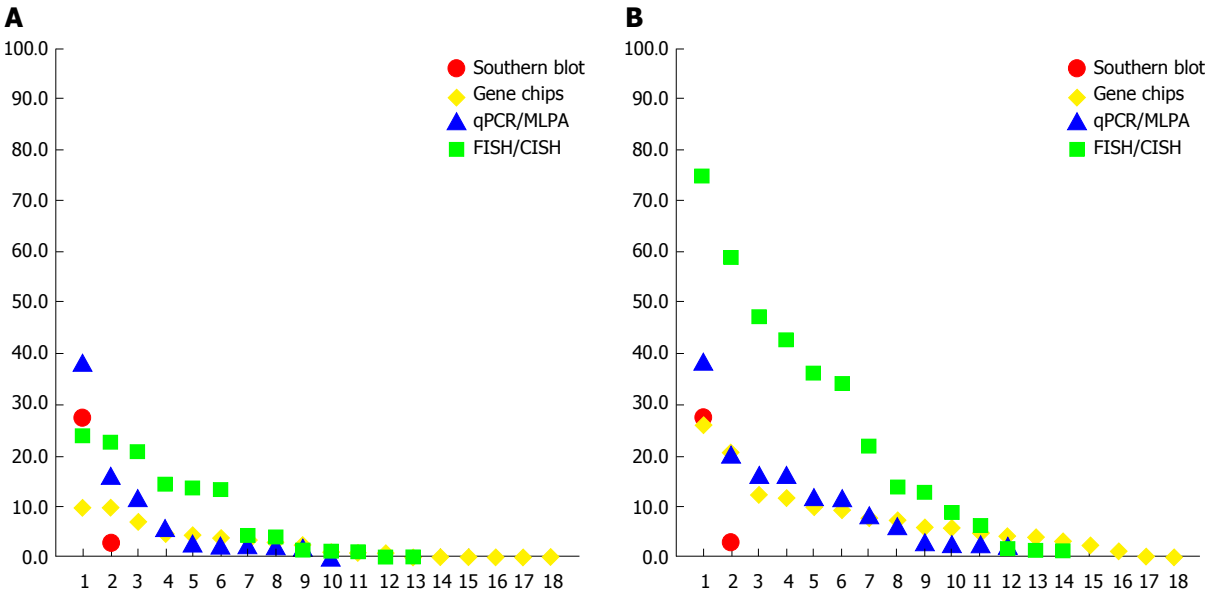

Figure 1 Prevalence of estrogen receptor alpha gene amplification in the literature. The fraction of altered cases (y-axis) is indicated across published studies (x-axis) and is shown separately for different detection methods. The studies are sorted in descending order of *ESR1* amplification frequency, as detected. A: Prevalence of *ESR1* amplification defined according to the diagnostic criteria for *ERBB2* (HER2) amplification; B: Prevalence of *ESR1* copy number increase including amplification and gain. For study citations see Appendices A-D. *ESR1*: Estrogen receptor alpha gene; MLPA: Multiplex ligation-dependent probe amplification; FISH: Fluorescence *in situ* hybridization; CISH: Chromogenic *in situ* hybridization.

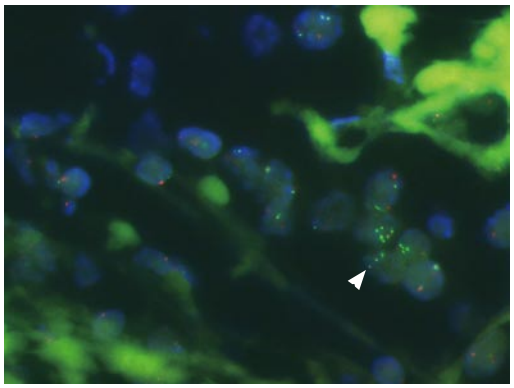

Figure 2 Heterogeneous estrogen receptor alpha gene amplification detected by fluorescence *in situ* hybridization analysis. Green and red spots represent estrogen receptor alpha gene (*ESR1*) gene probe and centromere 6 probe, respectively. White arrowhead points to tumor cell nuclei (blue) with increased numbers of *ESR1* fluorescence *in situ* hybridization signals next to tumor cell nuclei without increased numbers of signals. From Moelans *et al*[40].

deletion frequencies of reference genes are responsible for this phenomenon[32,34].

In fact, use of some assays with reference genes that have a lower frequency of deletion in breast cancer (approximately 18% *vs* approximately 30%) - according to The Cancer Genome Atlas (TCGA)[52] - also led to the detection of lower frequencies of *ESR1* amplification (*ASXL2*, *EIF5B* and *PVR vs ESR2*). Note, however, that when reference genes such as *PIEZO2* (*FAM38B*) are used, which have higher deletion frequencies (approximately 30%), the frequency of *ESR1* amplification remains low[34], suggesting that factors other than reference gene alterations may also contribute to the outcome of qPCR studies. This is also exemplified in cases when two qPCR assays lead to different *ESR1* amplification results, even though the different reference genes (*ESR2* and *SOD2*) used had similar deletion frequencies (approximately 30%) according to TCGA.

These assays highlight a huge difference (approximately half a dimension) in the dynamic range for the same samples, pointing to the impact of technical issues, in addition to the status of the reference gene, on study outcome[53] (Supplementary Tables S1-S3 and Supplementary Graphs S1-S4).

Heterogeneity and counting

Tumor heterogeneity and plasticity have been increasingly recognized over the last few years as common properties of cancer[54-64] that make molecular diagnosis difficult[22,29,40,57,58,64-66]. Heterogeneity is an obvious issue in the analyses of isolated DNA, where mixtures of cells with normal copy numbers and low level amplification may easily result in an "average" copy number that ranges below the definition threshold for the amplification status or even within the background noise of measurement[40]. In the case of heterogeneous cancers analyzed with use of *in situ* methods (such as FISH), the choice of the tumor area(s) for analysis is of the utmost importance[40]. Accordingly, approaches that classify the tumor gene status based on a minimum of successful hybridized tumor cells[67] might fail to detect aberrations that are only present only in a minor portion of the cancer bulk.

Signals and copies

Besides the purity and analyzability of tumor cells, the interpretation of FISH signals also contributes to analysis outcome. For example, automated FISH scoring

systems are limited in their ability to count individual signals in tight clusters, and instead measure the ratio of fluorescence intensities[68]. But because fluorescence intensities and signal numbers can vary due to various technical issues that are independent of copy number, automated analysis may be considerably less sensitive than the human eye. This may be particularly true in the case of the low-level clusters that are typical of *ESR1*[31,32], because identification of individual gene copies is highly dependent on the interpretation of signal patterns. A major challenge in determining low level gene copy numbers that are also heterogeneously distributed is the identification of FISH signals that represent a single gene copy. For example, distinguishing dense clusters of multiple gene copies (which often appear as one large signal) from true single gene copies is difficult; and additionally it is virtually impossible to detect tandem gene duplications (which occur commonly in breast cancer genomes[69]) with use of established FISH scoring criteria that recommend that "doublets" (*i.e.,* two tightly adjacent signals) be interpreted as a single gene copy.

Such a FISH signal-counting recommendation is often applied, based on the interpretation guidelines for *ERBB2* (HER2) FISH testing[70]. However, the recommendation to count two adjacent signals as one is based on studies that determine numerical chromosome aberrations, not focal changes in gene copy number[71,72]. This approach is warranted for chromosome enumeration that uses probes that do not hybridize within the centromere region: Following the s-phase of the cell cycle, chromosomes consist of two chromatids, each of which contains one gene copy. Accordingly, FISH analysis displays two signals nearby for these two gene copies[73] (Figure 3), even though chromosome number is not pathologically increased in these cases and such signals should be counted as one.

In contrast to chromosome enumeration, the increased number of gene copies on one chromatid or chromosome is relevant for determining gene copy number: In this case, signal doublets should be considered to represent two gene copies. Studies that combine FISH and gene chip or Southern blot technologies show that FISH signal doublets represent two gene copies on one chromosome, and as such constitute gene amplification[74-77] (Figure 3 and Supplementary Figures S1-S7).

Gene chip challenges

Gene chip technology and NGS are powerful techniques for detecting genomic alterations with high accuracy and objectivity, compared to morphologic methods that evaluate results based on the interpretation of individual observers. Nevertheless, use of these methods is associated with serious pitfalls. In 2008, a published summary of gene chip analyses listed a huge range of amplification frequencies reported in different studies and different tumor populations, ranging from 7% to 35%, even for *ERBB2* (HER2)[33]. Over the years, various limitations have led to a reconsideration of these methods. Most importantly, it has to be taken into account that the isolated DNA was analyzed as an average of many different cells. But also methodological limitations due to technology-related background of measurement and the quality of the gene chip hybridization (call rates) have to be considered[22,29,36,40,78]. The large amount of data involved in these studies also makes these methods strongly dependent on the specific computational approaches and algorithms that are used[78-80].

The general impact of the normalization reference and the computational method used to analyze raw data played a key role in the rediscovery of *ESR1* amplification, based on the analysis of 22 breast cancers with use of Affymetrix 10K SNP gene chips[24,81]. This is illustrated in Supplementary Figures S1-S7 and the Supplementary Optical Dataset S1, as well as in a video documentation (Supplementary Video Clips S1 + S2).

TCGA provides the largest and most advanced gene chip copy number database for isolated DNA from tumor samples[82,83]. A meta-analysis of TCGA dataset offers new insights into *ESR1* copy number alterations beyond the published FISH studies. For example, two tumor entities with the most frequently reported *ESR1* amplification (by FISH analysis), *i.e.,* breast and endometrial cancers[24,84], are top ranked also by Genomic Identification of Significant Targets in Cancer (GISTIC) analysis (2016-06-01 stddata_2015-04-02 regular peel off)[52]. For GISTIC, TCGA defines gene amplification as a linear copy number increase that exceeds the genome-wide median (adjusted to diploid) by more than 0.1 copies[83,85]. Overall, focal (smaller than half a chromosome arm) and high level (increase of more than one copy) *ESR1* amplifications are determined by GISTIC in 16.2%, 6.8% and 2.2% of breast cancers (n = 1080), respectively. Furthermore GISTIC analysis demonstrates that *ESR1* undergoes focal amplifications significantly (threshold q = 0.25) above the genome-wide average rate in breast cancer (residual q after GISTIC peel-off = 0.096)[52,86,87]. TCGA also confirms the existence of very small amplifications, with *ESR1* and *CCDC170* being the only genes in the GISTIC peak[52] (Figure 4): Although some amplifications are due to structural alterations that involve only untranslated parts of *ESR1*, others are limited specifically to the entire gene and its flanking regions (Figure 4)[52,88], providing additional strong support for a clonal selection process that targets the *ESR1* locus[86]. Amplifications that were limited to *ESR1* - but included parts of *CCDC170* - were also found in another SNP gene chip study[81] (supporting Figures 1 and 5) and by NGS, in a breast tumor that was sensitive to estradiol treatment[89] (Figure 5). Obviously, novel and publicly available databases such as TCGA collection[52] were not available at the time when the debate on *ESR1* amplification started, but the latest upgrade is still not considered in all publications to date[90].

Fact or phantom

FISH allows the analysis of gene copy number variations at a single cell level, and its morphological localization.

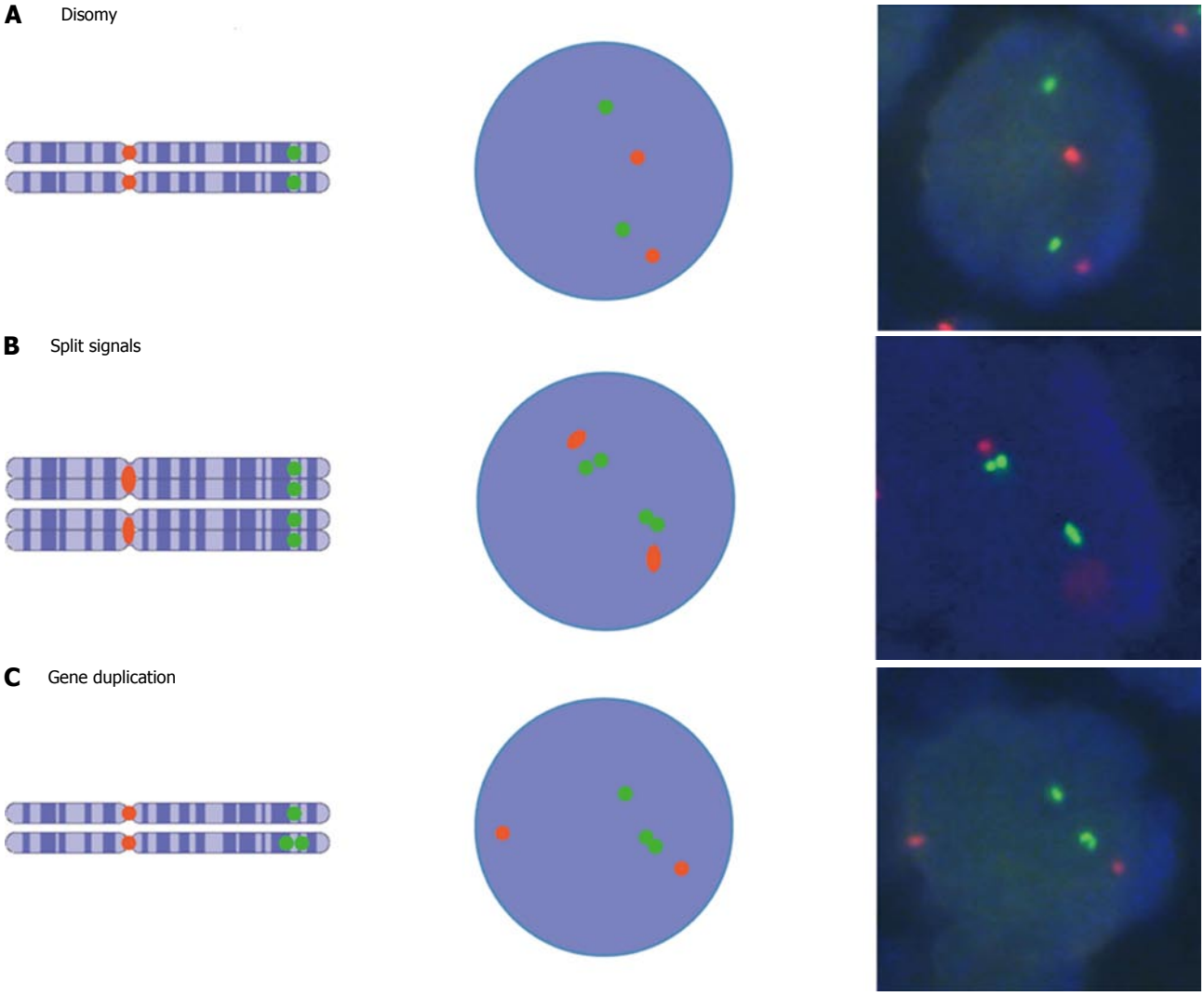

Figure 3 Estrogen receptor alpha gene single and split signal patterns and their supposed appearance as detected with fluorescence *in situ* hybridization assays. Green and red spots represent estrogen receptor alpha gene gene probe and centromere 6 probe, respectively. A: Normal disomy with two chromosomes and two gene copies; B: Normal disomy after s-phase with four chromatids and four gene copies; C: Disomy harboring mono allelic gene duplication with two chromosomes and three gene copies[38]. Photos from Moelans *et al*[40].

This makes FISH especially well-suited for taking tissue heterogeneity into account. But signal interpretation with use of FISH is dependent on observer subjectivity and is prone to influence by signal artifacts; FISH probes may also detect pre-mRNA transcripts of *ESR1*, and such hybridization could mimic DNA hybridization signals[39,40]. Such mRNA artifacts may lead to *ESR1* amplification calling by FISH that is false positive in cases with amplification signals that are exclusively limited to the nucleotide sequence of *ESR1*[24]. These artifacts may also explain the lower rates of amplification found by other methods[39]. The significance of this phenomenon has, however, not yet been fully evaluated, and some reports indicate that it does not have a major impact: These include cases with *ESR1* amplifications whose extensions have been mapped (by FISH) to the gene locus only[24]. For example, FISH amplification signals are detectable even after RNase treatment of the tissue section prior to the FISH analysis, indicating that DNA is targeted[40] (Figure 6).

Also, in at least 50% of tested cancers, *ESR1* amplification identified by FISH can be confirmed by multiplex ligation-dependent probe amplification PCR[40] (Figure 6), and additionally, a qPCR could show that tumors with *ESR1* amplification (determined by FISH) average significantly higher *ESR1* copy numbers than do tumors without *ESR1* amplification (also detected by FISH) (Supplementary Tables S1-S3, Supplementary Graphs S1-S4)[53].

It is important to understand that failed validation of FISH-determined *ESR1* amplifications by qPCR or gene chip analysis may be due to tumor purity and heterogeneity. Quantitative analysis of DNA isolated from tissue samples is always prone to underestimation of gene copy numbers - especially when tumor samples are not microdissected. This is because, due to the presence of non-cancerous cells present in a tumor sample, cancer cell purity typically ranges between 20%-80% in breast cancer, and is often overestimated in histological analyses[91]. The "contaminating" non-

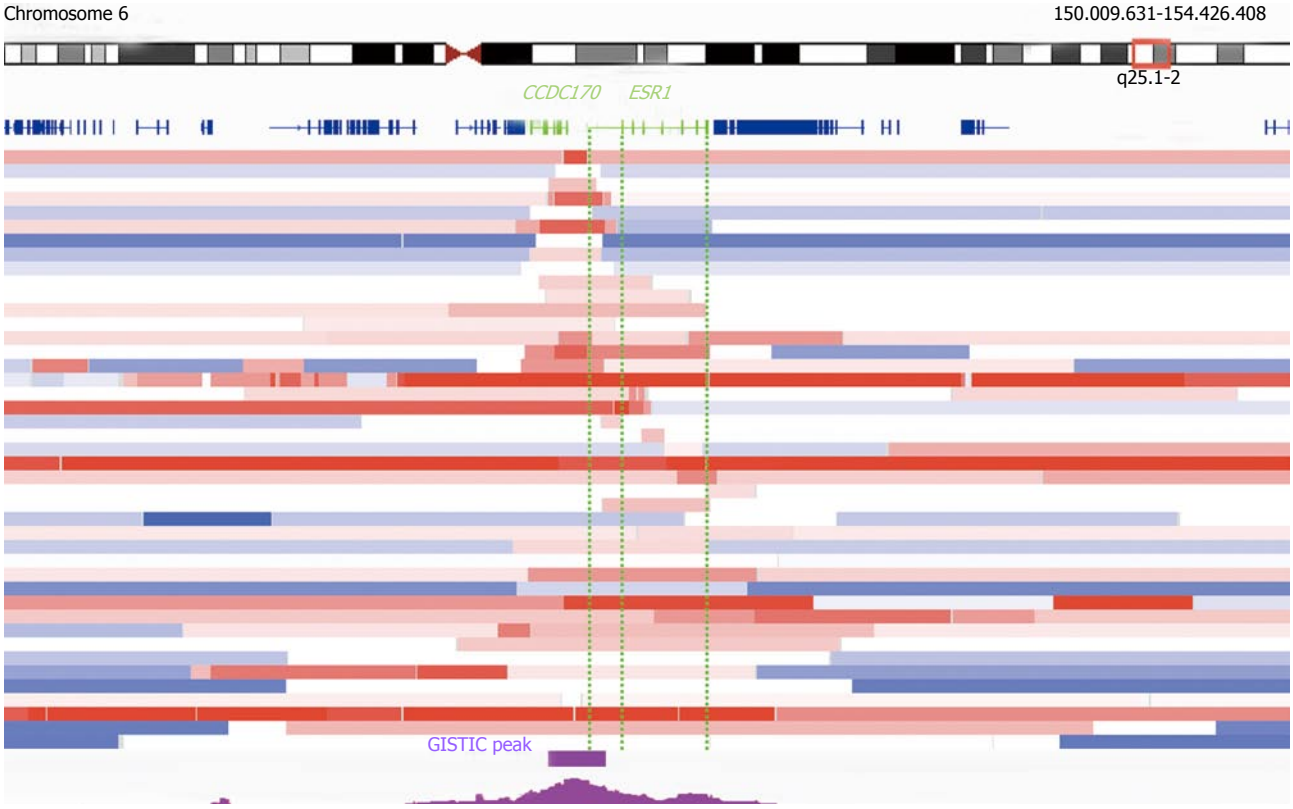

Figure 4 Architecture of the most focal estrogen receptor alpha gene amplifications in The Cancer Genome Atlas. Segmented log2 gene copy number ratios in 43 TCGA breast cancers are represented as horizontal bars (red: increased, white: neutral/normal, blue: decreased/deleted). Focal amplifications within and smaller than the region that is 2 Mb up and downstream of estrogen receptor alpha gene (*ESR1*) (150.009.631-154.426.408 bp) and that harbor any amplified *ESR1* sequences in relation to their flanking regions are shown. The 19 cases at the top of the figure show amplifications that overlap any *CCDC170* sequences and only parts of *ESR1*. In the lower 24 cases, the amplicon includes either the full *ESR1* sequence, or parts of *ESR1* without overlapping *CCDC170*. Positions of genes are indicated in dark blue; *ESR1* as well as *CCDC170* are highlighted in green. Dotted green lines indicate regions of *ESR1* with (right) or without (left) translated exons. The position of the GISTIC-log10 q-value peak is indicated in magenta. The position of the significant GISTIC *q*-value (< 0.25) region is indicated as a separate magenta colored bar (95%CI)[52,83,86]. TCGA: The Cancer Genome Atlas; GISTIC: Genomic Identification of Significant Targets in Cancer.

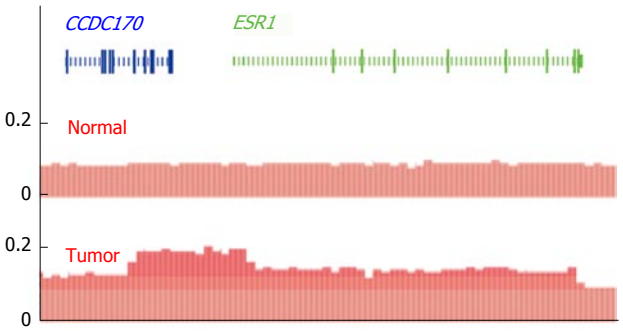

Figure 5 Estrogen receptor alpha gene amplification in a breast cancer tumor responding to estradiol treatment. DNA copy number profiles (red) in relation to the genomic position of *CCDC170* (blue) and estrogen receptor alpha gene (*ESR1*) (green) in normal and a breast cancer tumor tissue that harbors amplification of the *ESR1* gene. The amplified DNA sequence extends from *CCDC170* throughout the promoter region and the coding sequence of *ESR1*. The mapping of amplification was performed using read counts obtained during whole genome sequencing. Read counts above normal and max reads including *ESR1* are indicated in increased darker red shading. Data and graphic illustration according to Li *et al*[89].

cancerous tissue inevitably "dilutes" the tumor DNA, and leads to underestimation of changes in gene copy number[22,29,40,52,91]. Clearly this dilution effect is even greater when low-level and heterogeneous alterations (*e.g.*, the *ESR1* amplification) are analyzed[52,62,88].

CLINICAL SIGNIFICANCE OF *ESR1* AMPLIFICATION

Gains for expression

A causal relationship between *ESR1* copy numbers and increased expression of ERα protein, which drives cell proliferation[9-11], provides the molecular underpinning of the potential clinical relevance of *ESR1* amplification. Expression of the ERα-protein itself has long been used for decades as a biomarker for initiating anti-estrogen treatment in breast cancer[9]. However, regardless of the prevalence of gene amplification across a tumor type, the increase in copy number for a given gene is a well-known mechanism for increasing its expression[16,17,44,92,93]; accordingly, gene amplification is assumed to be a marker for a tumor's addiction to the expression of the amplified gene[18,19].

Several reports, including those that use DNA-specific methods for *ESR1* copy number determination, have documented a significant correlation between *ESR1* gene amplification and ERα protein expression[24,25,28,50,67,83-97] (Table 1).

Nevertheless, it is important to keep in mind that other

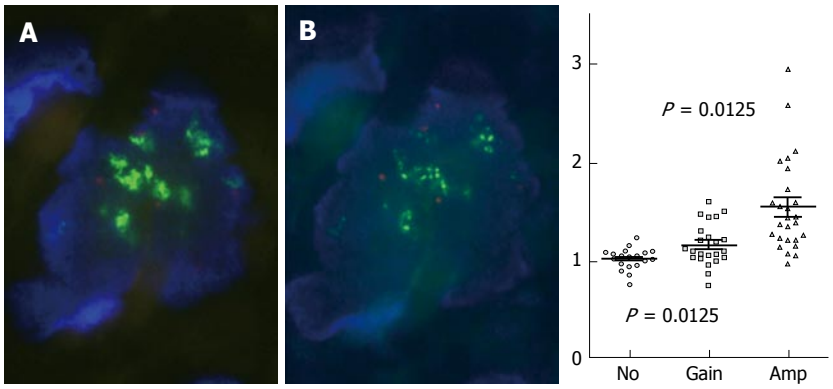

Figure 6 Effect of RNase treatment on fluorescence *in situ* hybridization signal patterns. RNase pretreatment resulted in a higher fraction of tumor cells that showed point-shaped fluorescence *in situ* hybridization (FISH) signals, when fuzzy clouds of estrogen receptor alpha gene (*ESR1*) signals detected by standard FISH were eliminated (A). *ESR1* copy number ratios determined by multiplex ligation-dependent probe amplification (MLPA) (y-axis) of "not increased" *ESR1* copy number (no), *ESR1* copy number gain (gain) and *ESR1* amplification (amp) determined by FISH (x-axis) according to *ERBB2* (HER2) testing criteria (B). Results suggest an association of increased DNA copies of *ESR1* determined by MLPA with increased *ESR1* signals detected by FISH (B). MLPA ratios of groups "no" and "amp" as well as of "no" and "gain" are significantly different (see dot plot). Modified from Moelans *et al*[40].

mechanisms in addition to amplification also regulate a gene's transcription, and its translation into protein. In fact, some studies[1,34,39,98,99] challenge the correlation between *ESR1* copy number and expression levels (Table 1). Nonetheless, it is clear that here, too, the majority of discrepant findings are likely due to technical and methodological reasons. These could include a low number of cases analyzed, false negative ERα expression test results, or lack of statistical power due to low frequencies of tumors with *ESR1* copy number alterations detected. However, *ESR1* amplification has been described in ERα-protein negative breast cancers with poor survival[99] and it is conceivable that general genetic instability drives ERα-independent 6q amplification in these tumors. Accordingly, such cases are unlikely to be associated with ERα-protein expression and could contribute to the findings that challenge the correlation between *ESR1* copy number and ERα expression levels.

Response or resistance

The controversy about the relevance of *ESR1* amplification in breast cancer derives from claims that this genetic aberration is a potential predictive marker for optimal response to endocrine therapy. Three studies used FISH for gene copy number determination, and reported that breast cancer patients who were treated with Tamoxifen, and who showed *ESR1* amplification in their tumors, had a better disease-specific survival than did patients without this alteration. These studies also included a retrospective analysis of the Tamoxifen-only arm of the prospective randomized ABCSG-06 trial (Figure 7)[24,25,41]. Additionally, a qPCR study found a worse outcome for patients whose tumors were ERα-negative and had *ESR1* amplification, while there was no association with survival in patients with ERα-positive cancers that received Tamoxifen treatment[99]. In contrast, another study suggested that *ESR1* amplification predicted resistance to Tamoxifen therapy[26], although the results were not reproduced in a follow-up study of the randomized Danish cohort of the BIG-98 trial[27].

These discrepant results can be explained by differences in the kinds and mechanisms of amplification. For example, gene amplification driven by general genetic instability may be a marker for aggressive tumors[100] with unfavorable prognosis, that are less likely to respond to any therapy. Such amplifications, with lack of ERα protein expression[99], would dominate the results of survival analysis in aggressive tumor subsets[26]. Other tumors might amplify a gene specifically driven by the tumors' addiction to the respective pathway[19]. Indeed, this mechanism has been suggested in two independent studies that observed focal *ESR1* amplifications of low-level copy number change in long-term estrogen-deprived (LTED) MCF7 breast cancer cell lines, with use of DNA-specific gene chips and qPCR for *ESR1* copy number determination. And yet another experimental study showed that breast-cancer-derived xenografts respond to estrogen treatment of tumor cells that harbor *ESR1* amplification, as determined by NGS[89,101].

Furthermore, in one clinical phase Ⅱ study for evaluating anti-estrogen treatment, a focal *ESR1* amplification appeared after therapy in one out of 49 tumors analyzed by NGS[102]. These functional studies provide strong evidence for the potential clinical relevance of *ESR1* amplification as a mechanism of ERα pathway regulation. One additional study used LTED MCF7 cells to show a change of *ESR1* gene status detectable by FISH; however, the FISH signals were RNase-sensitive and no *ESR1* copy number increase was detectable by *ESR1* qPCR, suggesting that the FISH results may have been due to probe hybridization to abundant RNA[103].

Gene amplifications in human cancers are markers of the tumor's dependence on the encoded protein, and point to a potential target of therapy[18,20,21,45-47,104,105]. However, the effects of therapy depend on effective target neutralization, and indicate that target levels must be relevant for effective inactivation by antagonistic drugs.

Table 1 Published studies testing interrelations of estrogen receptor alpha gene (*ESR1*) amplification with estrogen receptor alpha protein expression over cases, studies are separated for test results by correlation or association and no correlation or association found

	Ref.	Patients (*n*)	*ESR1* CNI (%)	ERα-negative CNI (%)	Method for CNI detection
Correlation or association found					
1	Nembrot *et al*[1]	22	27.3	0	Western blot
2	Holst *et al*[24]	1652	36.1	1.3	FISH
3	Tomita *et al*[25]	133	33.8	0	FISH
4	Moelans *et al*[28]	135	8.1	27.3	MLPA
5	Tsiambas *et al*[50]	60	21.6	-	FISH
6	Dunbier *et al*[95]	44	20.5	0	Gene chip
7	Laenkholm *et al*[96]	220	42.4	8.8	FISH
8	Singer *et al*[41]	394	47.5	1	FISH
9	Lin *et al*[67]	150	12.7	5.9	FISH
10	Pentheroudakis *et al*[97]	1010	58.8	12.5	FISH
11	Li *et al*[93]	219	-	-	Gene chip
12	Soysal *et al*[3]	58	15.5	0	FISH
No correlation or association not found					
1	Watts *et al*[30]	37	2.7	0	Western blot
2	Reis-Filho *et al*[34]	70	11.4	25	Gene chip
3	Vincent-Salomon *et al*[35]	341	0.9	66.70%	Gene chip
4	Moelans *et al*[125]	39	Approximately 20	-	MLPA
5	Ooi *et al*[39]	51	5.9	0	FISH/MLPA
6	Markiewicz *et al*[99]	281	11.7	66.7	qPCR
7	Chen *et al*[2]	301	8.6	46.2	FISH

Studies are separated with regard to correlation or association and no correlation or association. Frequency of *ESR1* copy number increase (CNI), size (*n*) of study cohort, and proportions of ERα negative (ERα-) tumors in cases with *ESR1* CNI, as available. MLPA: Multiplex ligation-dependent probe amplification; FISH: Fluorescence *in situ* hybridization; qPCR: Quantitative polymerase chain reaction.

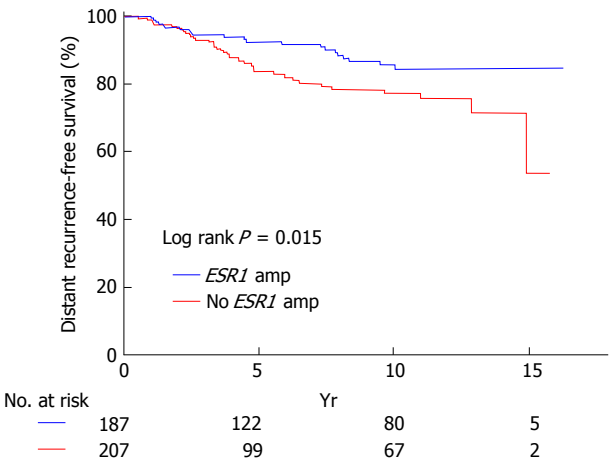

Figure 7 Kaplan-Meier plot for distant recurrence-free survival of 394 breast cancer patients treated with Tamoxifen. Patients with (blue) and without (red) estrogen receptor alpha gene amplification in primary tumor. From Singer *et al*[41].

In other words, the success of therapy might depend on the fold change in the amplified gene's copy number.

Accordingly, gene amplification is a well-known mechanism that underlies drug resistance[106-113]. Even in the case of HER2 (*ERBB2*), the mechanism of "receptor overcrowding" (and thus the level of receptor gene expression) was believed to be responsible for turning a marker for response into one for resistance depending on the level of gene expression[113].

And while the threshold for therapy response was determined at a doubled gene dose in the case of *ERBB2* (HER2), amplifications of other genes [*e.g.*, *EGFR*, *ERBB3* (HER3), and *PIK3CA* in lung cancer] might be relevant at lower levels[40,73,114-123]. This, as well as a tumor's heterogeneity regarding the amplification status of a gene, should be taken into account when considering gene amplification as a maker for therapeutic response or resistance.

Early or late

There is growing evidence that low-level gene copy number amplifications represent an adaptation by tissues to selective pressures[89,101], even in normal (non-transformed) cells[62]. It is obvious that such alterations in growth-regulating pathways can increase the risk for cancerous outgrowth[10,93]. The appearance of *ESR1* amplification in precancerous lesions, and their increased frequency during neoplastic transformation, suggest that such amplification is an early event that is potentially cancer-initiating and that drives cell proliferation[3,24,42,124,125]; what is not as clear is whether *ESR1* amplification alone is sufficient to transform cells. Nevertheless, detection of *ESR1* amplification in breast cancer precursor lesions might help to identify patients at high cancer risk, and it has thus been suggested that such patients could benefit from prophylactic anti-estrogen treatment[3].

FUTURE DIRECTIONS AND CHALLENGES

Rat runs and maps of malignancy

The debate on *ESR1* amplification in breast cancer is mainly based on methodological issues, including technical limitations, quality of application, and interpretation of results using the standard methods that are available today. The controversy about the frequency of

low level *ESR1* amplifications in particular, highlights the need for methodically advanced and sensitive approaches that will allow consistent findings.

The power of high throughput screening methods has enabled us to draw integrated maps of malignancy schematic landscapes from a bird's eye perspective. But cancer is not yet vanquished, and zooming in to details of these genetic landscapes could open new dimensions of insights into hidden and undiscovered molecular pathways of malignancy (rat runs) that might be missed from a bird's eye viewpoint.

A future perspective could comprise a combination of the existing cancer landscapes and detailed information derived from sensitive targeted approaches that will enable us to develop eagles eyes. Use of established morphological imaging methods such as FISH, as well as newly developed NGS-based approaches, could combine the objectivity of computational analysis algorithms with the resolution of single-cell analyses. These methods could integrate spatial and morphological, objective and high resolution measurement within tissues[126], and are in the process of being developed. Initial results have been published with use of single-nucleus sequencing[56,61,127], but challenges of using NGS data processing and whole genome amplification still remain to be tackled[79,80,128].

Gnosis and medicine's 5 sigma

The reproducibility of research on potential drug targets is low - successful only in about a fifth of studies published[129-131]. While the established FISH method seems to be a valuable approach for studying the clinical relevance of *ESR1* amplification or gene status in breast cancer[3,24-26,40,41,50,96] (Figure 7), there is no established consensus on how the interpretation of signal patterns or of gene status classification thresholds and definitions. As such, the nature of the *ESR1* gene status on the level of nucleic acids (DNA or RNA) might appear to be of secondary importance when considering a reproducible phenomenon that has an established standard diagnostic method and that is potentially applicable as a clinical marker[3]. In contrast, studies on the potential clinical significance and status definitions of detectable phenomena seem to be rather reasonable. In this context, the robustness and predictive power of a clinically applicable marker may be more important than its molecular properties.

Richard Horton recently commented that "much of the scientific literature, perhaps half, may simply be untrue", pointing to the recent *P*-values of "5 sigma" set in particle physics. And the idea, that, regarding scientific publications, "something has gone fundamentally wrong with one of our greatest human creations", highlights the need for open debates and paper critiques in science[132]. However, scientific debates will be rewarded when, besides the *P*-values and technical methodology, we do not lose sight of the goals of medical research[133].

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