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INVITED COMMENTARY

Re-searching anthracycline therapy

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> "I have yet to see any problem, however complicated, which, when you looked at it in the right way, did not become still more complicated."
> Paul Alderson (1926–2001)

More than a decade of research into identification of individual predictive biomarkers for anthracyclines has been disappointing. Despite extensive attempts to identify clinically robust predictive biomarkers, the issue of the specific biological subgroup of early breast cancer patients to derive anthracycline benefit remains elusive. In particularly, the use of HER2 and the anthracycline drug target, topoisomerase II alpha (topo IIα), as sole markers has yielded inconsistent results. Regulation of topo IIα, DNA repair pathways and host features complicate the anthracycline story. See Fig. 1. We may need to abandon the quest for a sole predictive marker and re-search anthracycline activity with multifactorial predictive tools.

Anthracyclines are DNA-damaging agents; however, they cannot bind directly to DNA. They bind and inhibit the topo IIα enzyme. Topoisomerase enzymes relieve the torsional stresses created by separation of the supercoiled DNA double-helix during transcription and replication. Disruption of topo IIα leads to double-strand DNA breaks, with subsequent cell death if the cell is unable to repair the damaged DNA. Theoretically, anthracyclines would be particularly active in the setting of overexpression of the topo IIα protein, coupled with impaired DNA repair. However, prediction of anthracycline benefit by topo IIα is inconsistently reported. Indeed, prediction of anthracycline activity has been reported in a conflicting mix of reports for several markers, including HER2, topo IIα and duplication of the chromosome 17 centromere.

Regarding HER2, the Canadian MA.5 trial showed a significant predictive role for positive HER2 status [1]. In contrast, a substudy of the UK BR9601 trial showed improved outcomes from anthracyclines in the setting of normal HER1-3 status [2]. Many other studies showed a trend in favour of HER2, but were individually underpowered to robustly test treatment interactions [3–7]. A meta-analysis based on abstracted data from eight previously published studies concluded that anthracycline benefit was confined to HER2-positive disease [8]. However, diversity in patients, treatment and biological subtypes in the meta-analyses limit robust conclusions. A clear mechanistic link between HER2 and anthracycline activity remains elusive.

Results also conflict for topo IIα, with support for [4, 5, 9–13] and against a predictive role [2, 14–16]. The Canadian MA.5 trial reported significant predictive value of pooled *TOP2A* aberrations, but not of *TOP2A* deletion or amplification independently [10]. Combined analysis of the UK NEAT/BR9601 trials showed no predictive role for *TOP2A* [14]. A recently presented meta-analysis assessed *TOP2A* in four phase III adjuvant studies comparing anthracyclines with CMF [17]. The interim analysis showed a clinically modest and statistically borderline predictive value for *TOP2A* amplification. The only prospective study to address this question, the TOP trial, was a single arm, single-agent neoadjuvant epirubicin analysis [9]. Whilst not yet formally published or peer reviewed, this trial is important as it is also the only trial to assess

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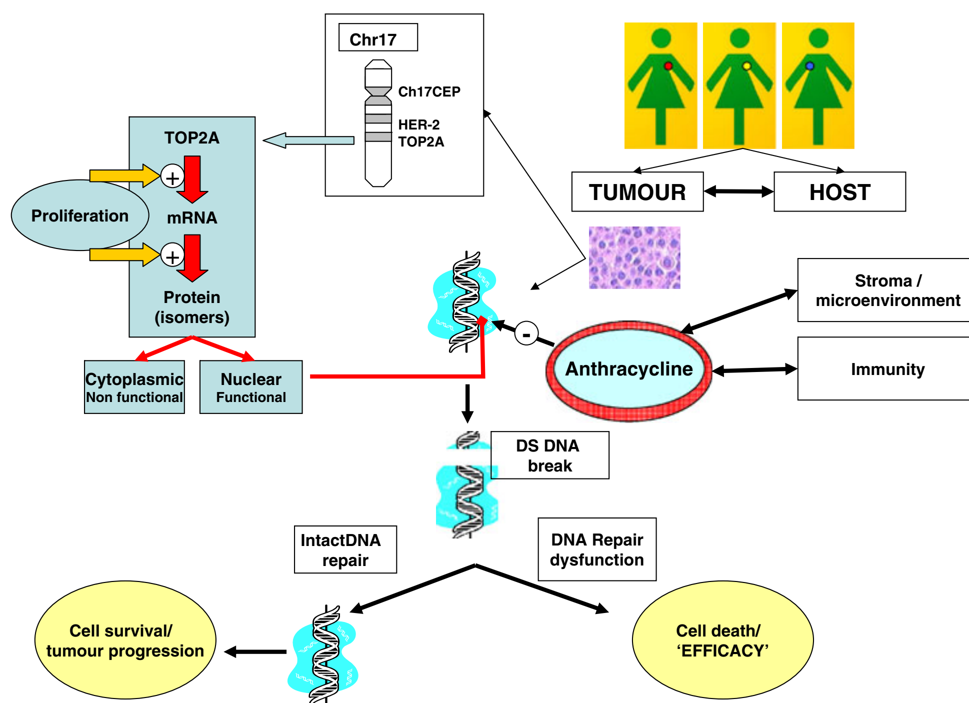

Fig. 1 Considerations in anthracycline efficacy: *TOP2A* status is one of many factors which may impact on the activity of anthracyclines

topo IIα at levels of gene, mRNA and protein. *TOP2A* amplification, but not mRNA or protein, correlated with pathological complete response.

Recent studies associate amplification of chromosome 17 centromere enumeration probe (Ch17CEP) with incremental anthracycline benefit. Ch17CEP detects a pericentromere polymorphic region with no known biochemical function. A prospectively planned combined analysis of the UK NEAT/BR9601 studies found benefit of epirubicin over CMF alone confined to patients with Ch17CEP duplication, independent of HER2 and *TOP2A* status [14]. A role for Ch17CPE, but not HER2 or *TOP2A*, was also strongly supported by a recent meta-analysis of four phase III adjuvant trials, which was well powered to detect treatment interactions [18]. In contrast, the TOP trial did not reveal such an association [9]. Like HER2, Ch17CPE duplication lacks a clear mechanistic link with anthracycline activity.

Thus, results for HER2, topo IIα and Ch17CEP are conflicting. Explanations include underpowered individual studies, diverse patient populations, variable anthracycline treatment, variable accompanying treatment, imprecise detection methods and inconsistent detection thresholds. Beyond these factors, however, these discrepancies may be highlighting inherent heterogeneity in biology and treatment sensitivity in early breast cancer. A key question remains: Can a single biomarker reliably predict incremental benefit from anthracycline based therapy?

In this edition, Gunnarsdottir et al. [19] report a retrospective Subpopulation Treatment Effect Pattern Plot (STEPP) analysis confirming superiority of epirubicin-based therapy in patients with *TOP2A* aberrations from the Danish Breast Cancer Cooperative Group (DBCG) Study 89D. The original study, conducted between 1990 and 1998, compared nine cycles of 3-weekly CEF (cyclophosphamide 600 mg/m^2, epirubicin 60 mg/m^2 and 5-fluorouracil 600 mg/m^2) and intravenous CMF (cyclophosphamide 600 mg/m^2, methotrexate 40 mg/m^2 and 5-fluorouracil 600 mg/m^2) in 980 pre- and postmenopausal early breast cancer patients. It showed benefit from CEF in the overall trial population (Hazard ratio (HR) for overall survival 0.79; 95% CI 0.66–0.94) [20]. Primary tumour tissue was collected retrospectively for centralised assessment for HER2 positivity and *TOP2A* amplification and deletion in 803 and 773 patients, respectively. *TOP2A* amplification and deletion were defined as a fluorescent in situ hybridization (FISH) ratio of *TOP2A* to Ch17CEP ≥ 2.0 and < 0.8, respectively. Two published sub-analyses have since reported that additional benefit from CEF was restricted to patients with *TOP2A* amplifications, and perhaps deletions [5, 21].

This study used a STEPP analysis to further explore the concordance between *TOP2A* gene copy number and anthracycline benefit in 773 patients. Treatment effect, in terms of difference in 10-year disease-free survival between CEF and CMF, was analysed by gene copy number, with gene copy number assessed as a continuous variable. The authors show differential anthracycline benefit according to *TOP2A* status and furthermore, show variation in efficacy within the currently defined 'normal' *TOP2A* range (0.8 < *TOP2A*: Ch17CEP < 2.0). From this univariate analysis, the authors suggest that more optimal

TOP2A assessment may be achieved by adopting and standardizing a reduced 'normal' ratio from 1.1 to 1.3.

STEPP analysis is a valuable methodology for assessing interaction between treatment effect and a defined covariate, particularly covariates with low prevalence. As the authors note, this is a univariate analysis which lacks multivariate analysis and external validation. Despite this, the study does allow observations, which may in turn spark hypothesis for future work. An observation of interest for the authors was variation in anthracycline efficacy within the current 'normal' *TOP2A* range. The authors hypothesise that a standardised narrower range of normal *TOP2A* may improve statistical power in assessment of *TOP2A* and anthracyclines. Certainly, a narrower range may improve statistical power; however, the high concentration of patients around the proposed cutoffs is fraught with difficulties in terms of reproducibility, with potential misclassification of many patients.

This study confirms results of two prior retrospective analyses of DBCG 89d, supporting a predictive role for *TOP2A* for anthracyclines. However, this study, even with proposed new thresholds, remains one positive study amongst conflicting studies. In the case of *TOP2A*, the specifics of the 'normal' range are unlikely to be the main hurdle. Inconsistent results more likely reflect inaccurate level of topo IIα analysis, and factors upstream and downstream of the drug target which impact on efficacy. Whilst TOP2A is likely to influence anthracycline efficacy, it is only one of many factors involved.

To date, standardised guidelines for detection and reporting of topo IIα status do not exist. Analyses vary in terms of the level of detection: gene, mRNA or protein. Gene-based analyses are limited by poor reproducibility, poor specificity and variable cutoffs. A concerning rate of interlaboratory *TOP2A* FISH discordance was reported in the preliminary *TOP2A* meta-analysis, with 31% discordance between local and central laboratories [17]. Reported FISH *TOP2A* copy number to centromere 17 ratio thresholds range from 0.67 to 0.8 for deletion and 1.5 to 2.0 for amplification [4, 10, 13–15]. Furthermore, a recent study used representational oligonucleotide microarray analysis (ROMA), which has a higher resolution technique for detection of genomic aberration, reported low incidence of *TOP2A* amplification, suggesting FISH overestimation of amplification [22].

Gene-based analyses are challenging to interpret as amplification and deletion are often grouped together and described as a *TOP2A aberration*. Rationale for pooling together these biologically different lesions is lacking. Whilst *TOP2A* amplification has a strong biological rationale for correlating with efficacy, it is counterintuitive that deletion of a drug target would be associated with improved efficacy. Regarding clinical application, an individual will not have an aberration, they will have either amplification or deletion.

The topo IIα protein, not the gene, is the anthracycline target. Assessing *TOP2A* as a marker of drug target assumes concordance between gene and protein expression. However, in contrast to HER2, topo IIα shows variable correlation between gene and protein. Protein expression is strongly influenced by the cellular proliferative rate. There is cell-cycle phase dependence of mRNA transcription [23, 24]. Proliferative signals can upregulate topo IIα protein levels independently of gene status, as evidenced by a study showing no correlation between *TOP2A* gene and topo IIα protein, but a strong correlation between topo IIα protein and proliferation [24]. Similarly, in a retrospective analysis of 31 patients with triple negative primary breast cancer, 0% had *TOP2A* gene amplification but 79% had high expression of topo IIα protein [25]. This contrasted with the non-triple negative patients in whom 10% had *TOP2A* gene amplification and 52% had high protein expression. Clinical evidence that *TOP2A* alone is inadequate may come from triple negative breast cancer, in which *TOP2A* amplification occurs rarely if at all [9, 26]. Whilst evidence is conflicting, some studies in triple negative disease suggest incremental anthracycline benefit [16, 27, 28].

Another topo IIα complication presents during translation. Variable mRNA splicing creates diverse topo IIα protein isomers with different activity and subcellular localization [29]. Cytoplasmic isoforms appear functionally inactive, whilst nuclear isoforms appear active and may be sensitive [30]. Detection of *TOP2A* cannot assess these downstream effects. Meaningful clinical information may be best derived from quantification and intracellular localisation of topo IIα protein. A sub-analysis is underway in the TOP study, using an immunofluorescence-based automated quantitative analysis, to quantify and localise the topo IIα protein.

To our minds, the most interesting observation of this analysis by Gunnarsdottir et al. [19] is the bimodal distribution of benefit of anthracycline-based therapy over non-anthracycline-based therapy. The extremes of the sliding window plot show incremental benefit of anthracyclines in individuals with either *TOP2A* amplification or deletion. It is reasonable to assume that amplification, especially in hyperproliferating tumours, will correlate with anthracycline benefit. However, increased sensitivity from gene deletion does not make obvious sense. We wonder whether *TOP2A* is not a marker for the topo IIα protein, but a surrogate for chromosomal and genomic instability. In fact, *TOP2A* amplification and deletion, *HER2* amplification and Ch17CEP duplication may all be surrogate markers for impaired DNA repair, with resultant incremental benefit from DNA-damaging anthracyclines. They may predict

sensitivity to DNA-damaging chemotherapy, not to anthracyclines specifically. Tools to assess DNA damage repair may evolve as meaningful clinical predictive markers [31, 32].

Regarding potential future tools, other DBCG 89d sub-studies have reported a predictive role for tissue inhibitor of metalloproteinases (TIMP)-1. TIMP-1 plays a key regulatory role in the breast stromal microenvironment, inflammation and immunity [33, 34]. In CEF-treated patients, TIMP-1 negative tumours had significantly improved outcome [33], and the combination of TIMP-1 negativity and *TOP2A* alteration was highly significant for improved outcome [34].

Anthracycline-specific predictive multigene signatures have been proposed based on in vitro assessment [35]. One of the only clinically validated predictive signatures comes from the TOP trial. A *TOP2A* signature, which is the averaged sum of genes frequently reported co-amplified with *TOP2A,* showed a significant correlation with pCR in HER2-positive tumours [9]. It was validated in ER negative, HER2-positive patients from the EORTC 10994/BIG01-00 trial by predicting response to FEC, but not to a taxane. Subsequently, this *TOP2A* expression signature was combined with host stroma and immunity expression signatures in an anthracycline-based chemosensitivity score, with a very high negative predictive value [36].

An intriguing evolving field is the study of anthracycline predictive stromal signatures. Results for anthracycline prediction by a 50-gene insilico-derived stroma-related signature (metagene) are reported [37]. High metagene expression was predictive for resistance to FEC in the EORTC 10994/BIG00-01 trial. This suggests a compelling interaction between chemotherapy and stroma in determining outcome.

Thus, the last decade has witnessed conflicting results regarding topo IIα status in prediction of anthracycline benefit. Evidence to date cannot support clinical application of *TOP2A* for women with early breast cancer. Importantly, this decade has also fostered research into the many components and complications of the breast cancer–host-treatment interaction. Incorporation of these features into mulitparameter predictive tools may lead the way forward.

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