CLINICAL TRIAL

CEF is superior to CMF for tumours with *TOP2A* aberrations: a Subpopulation Treatment Effect Pattern Plot (STEPP) analysis on Danish Breast Cancer Cooperative Group Study 89D

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Abstract The aim of this study was to examine *TOP2A* gene copy number changes as a means to identify groups of breast cancer patients with superior benefit from treatment with anthracyclines. Tumour tissue was retrospectively collected and successfully analysed for *TOP2A* in 773 of 980 Danish patients randomly assigned to receive intravenous CMF (cyclophosphamide, methotrexate and fluorouracil) or CEF (cyclophosphamide, epirubicin and fluorouracil) in DBCG trial 89D. Subgroup analyses on this material published by Knoop et al. (J Clin Oncol 23:7483–7490, 2005) and updated by Nielsen et al. (Acta Oncol 47:725–734, 2008) demonstrated that superiority of CEF over CMF is limited to patients with *TOP2A* aberrations, defined as patients whose tumours have *TOP2A* ratio below 0.8 or above 2.0. The Subpopulation Treatment Effect Pattern Plot (STEPP) technique was applied to these data to explore the pattern of treatment effect relative to *TOP2A* and to compare that pattern to the ranges previously used to define 'aberrations'. The pattern of treatment effect illustrated by the STEPP analysis confirmed that the superiority of CEF over CMF is indeed limited to patients whose tumours have high or low *TOP2A* ratios. The hypothesis of no treatment effect–covariate interaction was rejected ($P = 0.02$). Furthermore, results indicated that the interval of *TOP2A* ratios hitherto denoted as 'normal' could be narrower than previously assumed. A more optimal separation of *TOP2A* subgroups could be obtained by altering cut-points currently used to define *TOP2A* amplified and *TOP2A* deleted tumours by narrowing the *TOP2A* normal interval, and consequently enlarging the population with *TOP2A* aberrated tumours.

Keywords Breast cancer · *TOP2A* · Treatment · Anthracycline · STEPP

Introduction

The *TOP2A* gene encodes topoisomerase IIα (topo IIα), an enzyme catalysing the breakage and reunion of double-stranded DNA leading to relaxation of DNA supercoils; this enzyme is the primary target of anthracyclines [1].

A beneficial effect from substituting methotrexate with anthracyclines (epirubicin or doxorubicin) has been demonstrated in six symmetrically designed trials comparing CEF [2–4] or CAF [5–7] with CMF after radical surgery for early breast cancer, and this has been confirmed in an EBCTCG meta-analysis [8]. Superiority has also been shown for epirubicin followed by CMF compared to CMF [9], while four cycles of AC failed to demonstrate superiority to eight cycles of CMF [10].

Translational research has suggested that the modest benefit obtained on average originates mostly from a large benefit obtained by the small fraction of patients whose tumours have *TOP2A* aberrations [11]. In fact, while *TOP2A* aberrations are associated with worse prognosis in non-anthracycline treated cohorts [12, 13], recent reports show little influence of *TOP2A* on outcome for

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anthracycline treated cohorts [14, 15], providing indirect evidence that anthracyclines improve results for the TOP2A aberrant subgroup.

Copy number changes of the TOP2A gene may be classified as amplification or deletion using the fluorescence in situ hybridisation (FISH) ratio between signals of TOP2A and centromere 17 gene probes. However, there is no particular biological cut-point to adhere to and no consistent definition of cut-points exists, complicating comparisons among different studies. In the absence of a biologically based and well-defined cut-point, 1.5 was suggested in a pivotal study [16]. Later, a more conservative cut-point of 2.0 was developed for HER2 and it was argued that the same cut-point should be applied for TOP2A [17]. In retrospective studies evaluating the importance of TOP2A copy number changes for adjuvant anthracycline responsiveness in randomised trials, both 1.5 [18, 19] and 2.0 [11, 12] were applied as cut-points to define TOP2A amplification.

The present study was undertaken to analyse further the ranges previously used to define TOP2A aberrations and to explore other cut-points to improve classification of groups of patients whose disease responds better to treatment with anthracyclines. To that end we will apply the Subpopulation Treatment Effect Pattern Plot (STEPP) technique to the data from Knoop et al. [11].

Materials

The data investigated arise from a clinical trial and a corresponding sub-study, both of which have been previously described in detail [2, 11].

Between 1990 and 1998, the Danish Breast Cancer Cooperative Group recruited 980 Danish high risk patients with primary breast cancer for the trial DBCG 89D. The patients were randomised to nine cycles of adjuvant chemotherapy with either i.v. CMF (cyclophosphamide, methotrexate and 5-fluorouracil) or i.v. CEF (cyclophosphamide, epirubicin and 5-fluorouracil) repeated every three weeks.

Archival paraffin embedded tissue blocks were obtained from 806 Danish patients enrolled in the trial, corresponding to 82% of the patients enrolled, between September 2001 and August 2002 and analysed centrally for HER2 and TOP2A status. Copy number aberrations of TOP2A were identified using FISH. The TOP2A ratio for each sample was defined as the ratio of TOP2A gene to centromere 17 signals.

Methods

STEPP is an *exploratory* statistical method developed to illustrate the pattern of treatment effect with respect to a covariate. The technique, introduced in 2000 by Bonetti and Gelber [20], is based on analysing a hypothesised treatment effect in a number of subgroups, which are made to overlap, in order to both uphold the number of subgroups and their size, and thereby the precision of the estimates from each group [20, 21]. The resulting correlation between the groups is then taken into account when an overall test for interaction between covariate and treatment effect is carried out.

Two different strategies for dividing the data into overlapping subgroups have been proposed. With the 'sliding window' strategy, a certain number of subgroups is generated, so that the number of patients in each group and the number of patients exchanged between groups remains roughly constant.

The 'tail oriented' strategy produces one subgroup that encompasses all the patients, while the remaining subgroups decrease in size at a nearly constant rate as the covariate value tends to the extremes. By generating the subgroups using the 'tail oriented' strategy, the focus is directed at the tail of the covariate distribution.

The two strategies are illustrated in Fig. 1.

Having generated subgroups according to the chosen strategy, it is then necessary to define some measure of treatment effect and compute its value in each subgroup. Such a measure can, for example, be the difference in 10-year disease-free survival between two treatment arms.

A STEPP plot then illustrates how the treatment effect changes with the covariate by depicting the estimated treatment effects against the median covariate values in the corresponding subgroups.

The technique makes a point of defining overlapping subgroups so that the estimated treatment effects are correlated; this correlation should be taken into consideration when interpreting patterns illustrated in STEPP plots. Having estimated the (joint asymptotic) distribution of the subpopulation-specific treatment effects, it is possible to obtain the P value of a test for the absence of treatment effect–covariate interaction and a confidence band, taking the correlation between groups into account [21].

Results

Figure 2 shows the distribution of TOP2A ratios for the 773 patients who received CEF or CMF, and for whom TOP2A assessment was achieved. The cut-points usually applied are indicated with vertical lines; tumours were prospectively classified as being TOP2A deleted if the ratio was below 0.8, and TOP2A amplified if the ratio was ≥2.0, with ratios between 0.8 and 2.0 denoted as TOP2A normal. The corresponding group sizes are also denoted in Fig. 2.

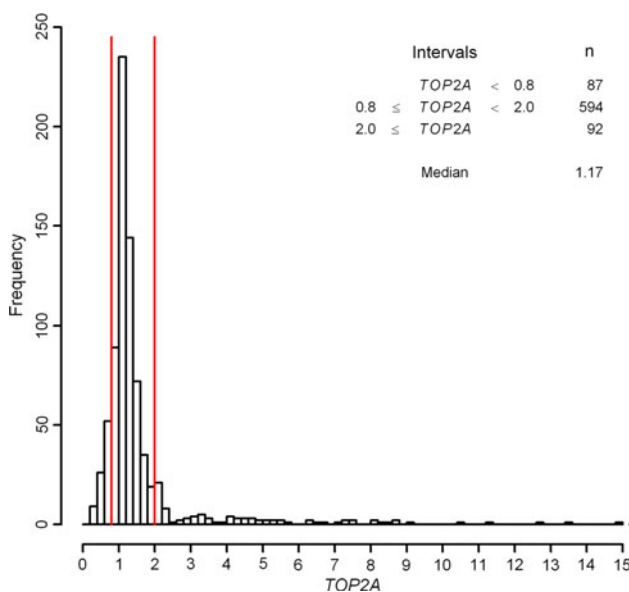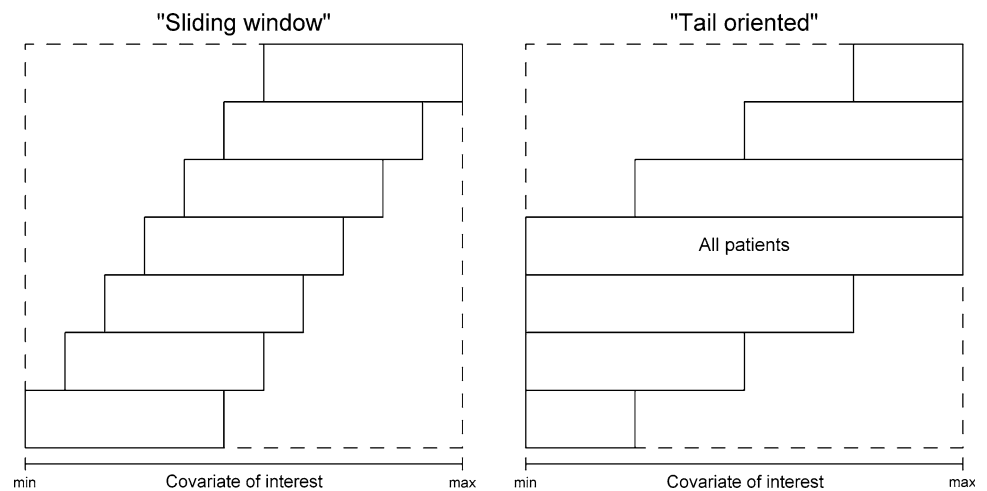**Fig. 1** The principle of two strategies for generating subgroups (*Source*: Bonetti and Gelber [20])

Fig. 2 Histogram of *TOP2A*

adjusted relative risk was 0.61 ($N = 86$, 95% CI 0.35–1.07). In contrast, little difference was seen in disease-free survival on treatment with CEF versus CMF for patients with *TOP2A* normal tumours; the adjusted relative risk for that group was 0.94 ($N = 589$, 95% CI 0.73–1.20). The observed interaction between *TOP2A* status and benefit from treatment with CEF over CMF was significant ($P = 0.02$).

In the present study, the 773 patients were included in a STEPP analysis, where treatment effect was defined as the difference in 10-year disease-free survival on treatment with CEF versus CMF with disease-free survival referring to the time from randomisation to recurrence, other malignancy, death or completion of 10 years of regular follow-up, whichever came first.

The results are illustrated in Fig. 3. The median *TOP2A* ratio within each subgroup is shown on the horizontal axis, and the number of patients in the corresponding subgroup is shown in parentheses (note: values are only provided for some subgroups). The vertical axis shows the difference in 10-year disease-free survival, with positive values favouring CEF and negative values favouring CMF. The treatment effect in each subgroup is plotted against the median *TOP2A* ratio in that group, and the points are connected to show the overall pattern of treatment effect with respect to *TOP2A*. Furthermore, 95% pointwise confidence intervals are shown as bars on the plot and 95% confidence bands, where the correlation between subgroups is taken into account, are indicated. Finally, overall departure from the null hypothesis of no treatment effect–covariate interaction is indicated by a *P* value.

In the sliding window analysis, subgroups were generated so that their size was ∼200 patients and the adjacent subgroups had around 180 patients in common. The resulting number of subgroups was 21, corresponding to the number of points on the plot.

In the tail-oriented analysis, eight subgroups were generated in each tail, with 17 subgroups in total. The smallest

The relationship between *TOP2A* status and benefit of treatment with CEF over CMF was investigated by Knoop et al. [11] in a retrospective study elaborating on DBCG trial 89D, and the findings are corroborated by an update, published by Nielsen et al. [13].

Ejlertsen et al. [2] reported an adjusted relative risk with respect to disease-free survival on treatment with CEF compared with CMF of 0.84 (95% CI 0.71–0.99). Nielsen et al. [13] conducted subgroup analyses on a subset of the same data consisting of the 767 Danish patients eligible for multivariate analysis. They found that looking at the three *TOP2A* groups separately, a significant improvement in treatment effect was found in disease-free survival on treatment with CEF versus CMF for patients with *TOP2A* amplified tumours; the adjusted relative risk was 0.39 ($N = 92$, 95% CI 0.22–0.70), and a similar non-significant improvement for patients with *TOP2A* deleted tumours; the
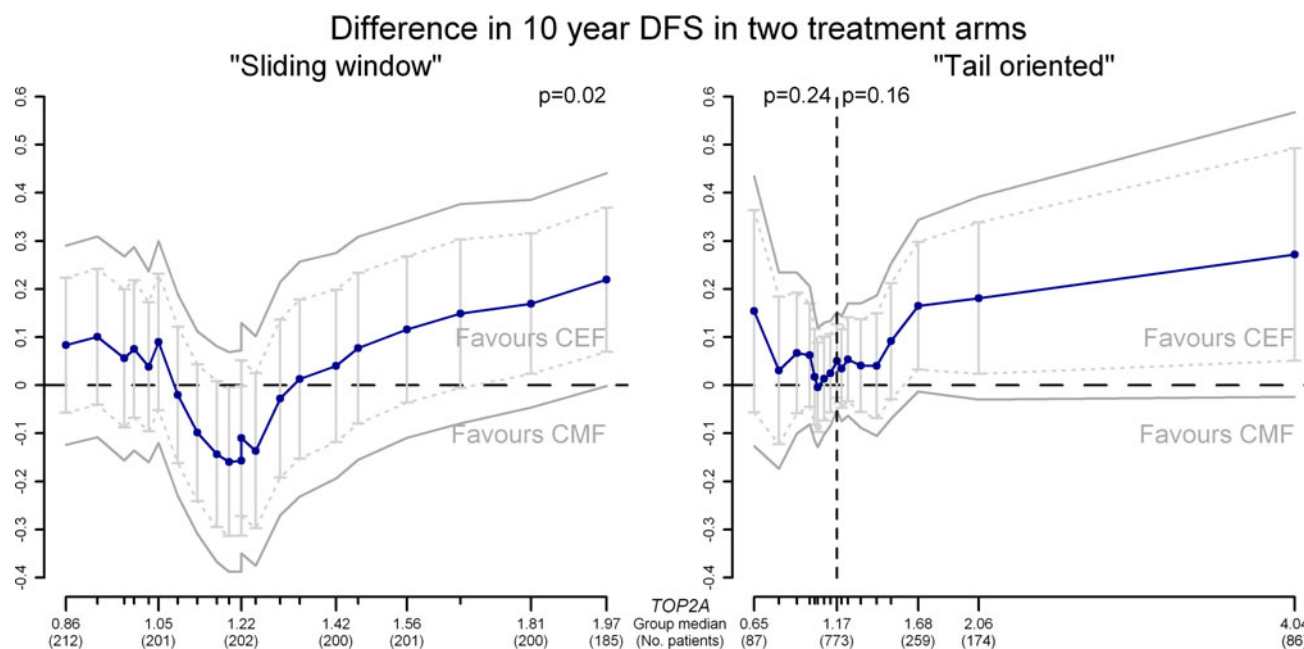
Fig. 3 Example of STEPP results, using both the "Sliding window" and "Tail oriented" strategies for subgroup division

subgroups at the extremes had <90 patients and the largest subgroup in the middle contained all 773 patients.

In general, the shape of the sliding window pattern shown in Fig. 3 corroborates previous findings that patients with *TOP2A* normal tumours on average demonstrate similar outcomes for CEF and CMF, while patients whose tumours have low or high *TOP2A* ratios demonstrate greater benefit of treatment with CEF compared with CMF. Furthermore, substantial fluctuation is noticeable in the CEF versus CMF comparison for *TOP2A* ratios in the range between 0.8 and 2.0, with cohorts at the extremes of this range benefiting more from CEF and ratios in the middle favouring CMF. This observation may indicate that the lack of superiority of CEF over CMF might be limited to an interval narrower than the *TOP2A* ratio range from 0.8 to 2.0 used in the publications by Knoop et al. [11] and Nielsen et al. [13].

The tail-oriented pattern in Fig. 3 illustrates that as *TOP2A* values increase or decrease above or below the average value for all patients, the benefit of CEF over CMF increases.

Sensitivity analysis and small sample properties

A sensitivity analysis (results not shown) varying the choice of subgroup parameters indicated that while the *P* values for the test for interaction differ, patterns similar to those seen in Fig. 3 were observed.

The small sample performance of STEPP was also explored. In particular, we carried out a simulation study to establish the coverage of the confidence bands and the false positive rate of the overall test for no interaction between treatment effect and covariate value in a simulated setting.

Survival times were simulated in three different settings, all with no interaction between *TOP2A* and treatment effect, by randomly generating observations from the exponential distribution and choosing parameters appropriately according to the original data set. The generated survival times were then combined with *TOP2A* ratios from the original dataset, thus producing, in each of the three settings, 300 data sets of the correct dimension.

Each data set was then analysed by means of the STEPP technique, using the 'sliding window' approach to generate subgroups.

The recovery of the Type I error for the test of no covariate-treatment effect interaction was verified by noting the number of data sets in each setting where the test showed significance. The level of recovery was satisfactory in all settings; a significant covariate–treatment interaction was found between 15 and 21 of the 300 data sets, corresponding to a false positive rate of between 5.0 and 7.0%. Similarly, the coverage of the 95% confidence band was assessed by counting the number of cases in each setting where the known 'true' value of the treatment effect was not encompassed by the confidence band for at least one subgroup. The confidence band excluded the true value in 8–9% of cases, suggesting that the confidence bands are slightly narrower than they should be. This is likely to be due to the use of approximate asymptotic results in the construction of the band. Note that while here the small sample properties of the methods appear to be satisfactory, this might not always be the case. In such cases, an alternative inferential procedure has recently become available [22].

Discussion

Subgroup studies using ad hoc cut-off points to categorise patients into groups according to the *TOP2A* ratio of the tumour have established *TOP2A* as an indicator for responsiveness to treatment with anthracyclines in breast cancer patients [11]. In contrast to earlier studies, a recent subset analysis of the NEAT/BR9601 study gave conflicting results [23]. In univariate analysis, amplification of *HER2* and deletion of *TOP2A* were associated with a poorer prognosis while copy number of chromosome 17 centromere (CC17) was not. In a multivariate analysis, CC17, however, was associated with incremental benefit from epirubicin, while *HER2* and *TOP2A* were not [23]. Different cut-offs were used for *HER2*, *TOP2A* and CC17. Biological or historical evidence that substantiates particular cut-off points between groups is lacking, and it is therefore of interest to discover whether a more optimal separation of patient subgroups could be obtained by applying different cut-points. A unified definition of cut-points would furthermore facilitate comparison between different studies on the subject.

The STEPP analysis suggests the presence of subgroups of patients who, contrary to the overall conclusion of Ejlertsen et al. [2], do not benefit from treatment with CEF. The sliding window strategy for subgroup division was given the highest importance in the STEPP analysis, since the tail-oriented strategy focuses on the extremes, where the superiority of CEF seems irrefutable, at least for patients with *TOP2A* amplified tumours.

Although the DBCG 89D study was not prospectively designed for that purpose, the availability of tumour tissue samples relating to patients enrolled in the study rendered it ideal as the basis for a subsequent biomarker sub-study investigating the relationship between *TOP2A* and the benefit of treatment with anthracyclines. Furthermore, treatment allocation in DBCG 89D was randomised, thus minimising the risk of obtaining biased results.

The STEPP results indicate that an improved separation of subgroups might be obtained by altering the cut-points currently being applied to define the *TOP2A* normal cohort. In particular, the results suggest that the cut-point for defining *TOP2A* deletions could be higher than 0.8 and that the cut-point for defining *TOP2A* amplifications could be less than 2.0. If this were the case, the *TOP2A* normal cohort would be smaller than it is currently believed to be, and the groups of patients with *TOP2A* deleted or *TOP2A* amplified tumours would be correspondingly larger.

For example, setting the cut-point for defining *TOP2A* deletions at 1.1, and the cut-point for defining *TOP2A* amplifications at 1.3 could be considered. These newly identified *TOP2A* cut-points differ considerably from cut-points previously used as an indicator for responsiveness to treatment with anthracyclines. For instance, the identified cut-point for amplifications of 1.3 is considerably lower than the 2.0 threshold applied by Knoop et al. [11], Tanner et al. [24] and Nielsen et al. [13] and nearer the cut-point of 1.5 applied by Järvinen et al. [16] and Di Leo et al. [18]. The distribution of *TOP2A* ratios is denser around the suggested new cut-points compared to 0.8 and 2.0, and the higher concentration of observations around the cut-points leads to a numerically larger group of patients that could potentially be misclassified.

Using the original cut-points 0.8 and 2.0, only 87 patients are classified as having *TOP2A* deleted tumours and 92 as having *TOP2A* amplified tumours, while 594 patients are considered *TOP2A* normal. These imbalanced group sizes can diminish the statistical power of subgroup analyses aiming to detect a putative treatment benefit within each *TOP2A* group. Shifting the cut-points, as we have suggested, to 1.1 and 1.3, the groups are more similar in size: There are 308 patients with tumours that have a *TOP2A* ratio <1.1; 269 patients have tumours with *TOP2A* ratio ≥1.3; the remaining group has 196 patients. The individual *TOP2A* subgroups being larger and more equal increases the statistical power with which conclusions about differences in treatment benefit within these groups can be drawn. Indeed, this is also likely to apply in those studies where no predictive value of *TOP2A* has been detected, as lack of power due to the relatively low numbers of *TOP2A* aberrated tumours [23] will have affected the power to detect the *TOP2A*-treatment interaction.

In any case, further validation is required to define *TOP2A* aberration cut-points for use in the clinic.

Traditional subgroup analyses can be misleading, partly because repeated testing on the same data increases the risk of finding false positive results. In addition, studies are designed to have, as a whole, the power to detect a certain treatment effect; the power might not be sufficient to detect a treatment difference in subsets, thus possibly leading to false negative results. While this is also true for a STEPP analysis, the STEPP methodology has the advantage that the precision of estimates is increased due to the overlapping subgroups, while the number of analysed subgroups is still sufficiently large for the pattern of treatment effects to be illustrated in a clinically useful way. A further advantage is that the method allows the treatment effect to be defined using traditional statistics, such as fixed time survival estimates.

In this article, we have focused on STEPP analyses with the difference in 10-year disease-free survival as a measure of treatment effect. This is a univariate measure, but in fact the STEPP technique could also be applied in a multivariate setting, for example, defining treatment effect in terms of adjusted relative risk.

Interpreting such results would be more difficult, but a test for the absence of interaction between treatment effect and covariate could still be carried out, to amend the STEPP results. When the data arise from a randomised study, as in the present case, adjusted risk estimates are less imperative; however, in this case, a number of patients have been excluded from the original randomised population because of the availability of tissue samples.

The suggested new cut-off values should not be treated as definitive, and they could indeed be too close to each other: STEPP is an *exploratory* method designed to illustrate variations in treatment effects, and it is not designed to define specific cut-points for subgroups, which we identified without taking into account their statistical variability. In order to verify that a modification of the cut-points is indeed appropriate, we suggest that this possibility be examined on additional data material.

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