Impact of Intertumoral Heterogeneity on Predicting Chemotherapy Response of BRCA1-Deficient Mammary Tumors

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Abstract

The lack of markers to predict chemotherapy responses in patients poses a major handicap in cancer treatment. We searched for gene expression patterns that correlate with docetaxel or cisplatin response in a mouse model for breast cancer associated with BRCA1 deficiency. Array-based expression profiling did not identify a single marker gene predicting docetaxel response, despite an increase in Abcb1 (P-glycoprotein) expression that was sufficient to explain resistance in several poor responders. Intertumoral heterogeneity explained the inability to identify a predictive gene expression signature for docetaxel. To address this problem, we used a novel algorithm designed to detect differential gene expression in a subgroup of the poor responders that could identify tumors with increased Abcb1 transcript levels. In contrast, standard analytical tools, such as significance analysis of microarrays, detected a marker only if it correlated with response in a substantial fraction of tumors. For example, low expression of the Xist gene correlated with cisplatin hypersensitivity in most tumors, and it also predicted long recurrence-free survival of HER2-negative, stage III breast cancer patients treated with intensive platinum-based chemotherapy. Our findings may prove useful for selecting patients with high-risk breast cancer who could benefit from platinum-based therapy. Cancer Res; 1–12. ©2012 AACR.

Introduction

Most forms of cytotoxic cancer chemotherapy also hit normal tissues. This is acceptable when the tumor responds, but frustrating when the tumor is intrinsically resistant and the patient only suffers from the side effects of an unsuccessful treatment. A major goal of molecular oncology is, therefore, to identify biomarkers that predict the response of tumors before treatment is started. Such predictive markers have been found for some targeted therapies in which the target and its interaction with drugs are well defined (1–7). For classical cytotoxic chemotherapy with DNA damaging drugs or antimitotics, however, predictive biomarkers have been harder to find.

In an attempt to find new biomarkers many investigators have turned to the analysis of genome-wide gene expression profiles. These profiles have been successful for predicting prognosis, that is, whether patients will require adjuvant chemotherapy after tumor removal (8). Prognostic and predictive biomarkers are fundamentally different, however (9). To detect predictive markers, considerable effort and money has been invested in the analysis of human breast cancer samples (10). In particular, the neoadjuvant setting seemed attractive to correlate gene expression profiles with therapy outcome. No clear response profile was obtained, however (11, 12). Other studies have gathered a number of unrelated signatures (9). These profiles either still await validation in an independent study; or the sensitivity and specificity was inadequate for clinical decision making; and some were based on flawed data (13–15). Moreover, cell line–based approaches to identify biomarkers suffer from the complication that the multidrug resistance transcriptome has been substantially altered during the long-term culture of these cell lines in vitro (16).

As progress in defining useful biomarkers using human tumor material has been limited, we have turned to a mouse model. In recent years, chemotherapy responses have been investigated in a new generation of genetically engineered mouse models (GEMM; ref. 17). These models employ conditional, tissue-specific activation of oncogenes and/or deletion of tumor suppressor genes in a stochastic fashion. The
Results

*Bra1<sup>−/−</sup>*/*p53<sup>−/−</sup> (KB1P) mammary tumors show individual and reproducible differences in docetaxel or cisplatin sensitivity

We have previously shown that individual KB1P mammary tumors differ substantially in their response to docetaxel (19). The response to cisplatin varied as well: although all tumors were sensitive to cisplatin, the time until relapse differed between tumors (19). To exploit these intertumoral differences, we analyzed docetaxel or cisplatin responses of 43 individual tumors (Supplementary Fig. S1).

The correlation of drug sensitivities with characteristics of a particular tumor is only possible if the responses are reproducible. We therefore explored the heterogeneity within a single tumor by orthotopic transplantation of multiple tumor fragments (Fig. 1). For this purpose, 3 animals carrying orthotopically transplanted mouse model were set out to find predictive markers of cisplatin or docetaxel response.

Materials and Methods

Mice and drug treatments

KB1P mammary tumors were generated, genotyped, orthotopically transplanted, and treated as described (18, 19). Additional details including the generation of KB1P mammary tumors can be found in Supplementary Materials. All experimental procedures on animals were approved by the Animal Ethics Committee of the Netherlands Cancer Institute.

Genome-wide expression profiling

RNA extraction, amplification, and microarray hybridization using dual channel MEEBO arrays (Illumina BV) were carried out as described (19, 22, 23). For the gene expression analysis using single channel 45K MouseWG-6 v2.0 BeadChips (Illumina), total RNA was processed according to the manufacturer’s instructions (http://www.illumina.com/products/mousewg_6_expression_beadchip_kits_v2.ilmn). More details on the processing and analysis of the microarray data are presented in Supplementary Materials.

Quantitative RNA analyses using reverse transcription–multiplex ligation–dependent probe amplification or TaqMan low-density arrays

These procedures were carried out as reported previously (20, 21, 24). Additional information is presented in Supplementary Materials.

ArrayCGH

ArrayCGH data was available from a recent study (25). Segmentation of the CGH profiles was done with the CGHseg package (26). The CGHcall R package (v 2.12.0) Bioinformatics. 2007 Apr 1:23(7):892-4 pmid: 17267432 was used to call aberrations in CGH profiles.

FISH

Three samples per individual tumor were investigated in a blinded fashion using tissue microarrays of the trial cohort. At least 100 nuclei per sample were assessed. More information on the protocol is presented in Supplementary Materials. If the number of cells with no *XIST* RNA clouds was more than 60%, the sample was classified 0 for “*XIST* RNA cloud.” In the presence of one X chromosome detected by the RNF12 DNA probe, *XIST* RNA was usually absent (loss of Xi). In the presence of 2 X chromosomes, loss of Xi and a XaXa configuration was defined as a more than 50% reduction in the number of expected *XIST* RNA clouds based on the RNF12 DNA FISH.

Patients

In a previous study, stage III HER2-negative breast cancer patients were randomly selected from a large randomized controlled trial (RCT) carried out in the Netherlands between 1993 and 1999 (27) and analyzed for aCGH classification (25). Further details on these patients are presented in Supplementary Materials. All trials described in this article were approved by the Institutional Review Board of the Netherlands Cancer Institute.

Supervised gene expression profiling does not yield a general signature that correlates with docetaxel response

In our model, we used the tumor volume as the basis for a response classifier. We found that after completion of the
initial docetaxel treatment on day 14, 22 tumors did not shrink below their original size when treatment was started (100%) and eventually continued growing (“poor response”). In contrast, 21 tumors regressed to a volume below 50% of the original size (“good response”) and took on average 28 days (SD 11d) after the last docetaxel treatment to grow back to 100% (Fig. 2A and Supplementary Table S1). With such an obvious separation, we expected to identify genes that are differentially expressed between these 2 groups. To detect these, RNA of all 43 untreated tumors was analyzed using 39K mouse exonic evidence-based oligonucleotide (MEEBO) gene expression microarrays and 45K Illumina Sentrix mouse V6 single-channel bead arrays. Unsupervised hierarchical cluster analysis did not separate good from poor responders (Supplementary Fig. S2). For the supervised analysis, we used significance analysis of microarrays (SAM; ref. 28), which is frequently applied to detect differential gene expression. SAM uses nonparametric statistics to compute for each gene a delta that measures the strength of the relationship between gene expression and docetaxel sensitivity. Permutations of repeated measurements are employed to estimate the false discovery rate (FDR). Using this approach, we expected to find several differentially expressed genes between good and poor docetaxel responders with a delta >0.7 (FDR <5%). Remarkably, this analysis did not detect a single gene that correlated with drug sensitivity with a meaningful delta (Fig. 2B).

This negative result might be due to the lack of sensitivity of the gene expression platforms used for genes that are relevant for drug resistance. This is exemplified by the work of Orina and colleagues (29) on drug transporters of the ATP-binding cassette (ABC) family. Using the NCI-60 panel of cell lines, they showed that TaqMan low-density arrays (TLDA) are more precise and more sensitive in measuring the expression of these transporter genes than oligonucleotide arrays (29).
Within this ABC family, a number of genes has been associated with docetaxel resistance, including ABCB1/P-glycoprotein (P-gp; ref. 30), ABCC2 (31), and ABCC10 (32). We therefore examined whether the more quantitative TLDA analysis of the 49 genes that encode mouse ABC proteins would reveal differences between poor and good docetaxel responders. As shown in Fig. 2C and Supplementary Table S2, none was found at a significance level of $P < 0.01$ (Mann–Whitney $U$ test).
We note in passing that on both platforms used to analyze gene expression (Supplementary Fig. S2), 2 poor responders (T26 con and T41 con) form a separate branch which correlates with the sarcomatoid morphology (carcinosarcoma) of these tumors (Supplementary Table S1). Most likely, these 2 tumors have undergone an epithelial–mesenchymal transition, as in the K14creBra1^{f/f}p53^{flo/flo} model the Cre-mediated deletion of the Bra1 and p53 genes selectively occurs in epithelial cells driven by the K14 promoter. Whether such a morphologic change correlates with drug resistance is under investigation.

**Increased gene expression of the Abcb1a and Abcb1b genes is frequently found in acquired docetaxel resistance**

Because our analysis of gene expression did not turn up a single gene that correlated with intrinsic docetaxel resistance, we tested tumors with acquired docetaxel resistance. Genes responsible for the acquired resistance might also cause intrinsic docetaxel resistance. We therefore compared RNA from samples of the same tumor before treatment and after they had become resistant to docetaxel. Unsupervised hierarchical cluster analysis did not separate sensitive from resistant tumors, but the docetaxel-resistance cluster analysis did not separate sensitive from resistant tumors. Hence, tumors have undergone an epithelial–mesenchymal transition, as in the K14creBra1^{f/f}p53^{flo/flo} model the Cre-mediated deletion of the Bra1 and p53 genes selectively occurs in epithelial cells driven by the K14 promoter. Whether such a morphologic change correlates with drug resistance is under investigation.

**Increased expression of the Abcb1a and Abcb1b genes can explain poor docetaxel response of 5 of 22 nonresponders**

In addition to conventional SAM analyses, we also tested an algorithm designed to specifically detect differential gene expression that only occurs in a subgroup of tumors within the nonresponding group (de Ronde and colleagues; submitted for publication). This algorithm places a threshold on the gene expression corresponding to the highest expression level in the docetaxel responder group. For the docetaxel poor responders that exceed this threshold, the sum of the differences of the expression is then calculated. Using this algorithm we found that Abcb1b was among the top outliers and formed a cluster with several other genes (Fig. 3 and Supplementary Table S4). This suggested that Abcb1b is not only involved in acquired docetaxel resistance but may also contribute to upfront docetaxel resistance of some tumors. To further investigate whether an increased expression of the Abcb1a/b genes can explain the poor intrinsic docetaxel response of some of the 22 poor responder tumors (Fig. 2A), we quantified the RNA levels in the untreated tumors by RT-MLPA (Fig. 4A). In 5 of 22 tumors, we found a 7- to 9-fold increase in Abcb1 RNA above the average level of the good responders. Abcb1a RNA was elevated as well in these 5 tumors (Fig. 4A). We have previously shown that a modest upregulation of Abcb1 by a factor of 7 to 9 is sufficient to cause drug resistance in these tumors (21). Indeed, we found that the 5 tumors with increased Abcb1 gene expression also did not respond to the P-gp substrate doxorubicin (Fig. 4B and Supplementary Fig. S1), whereas the poor docetaxel responders without increased Abcb1 RNA usually shrunk below 50% with doxorubicin (Fig. 4C and Supplementary Fig. S1). As expected, there was no correlation of Abcb1 transcript levels with cisplatin relapse-free survival (Fig. 4B and C), as cisplatin is not a substrate of P-gp.

**P-gp–deficient mammary tumors are docetaxel hypersensitive**

To improve our ability to detect P-gp–independent mechanisms of docetaxel resistance, we introduced the Abcb1a/b null alleles into the K14creBra1^{f/f}p53^{flo/flo} model. The lack of functional P-gp did not affect mammary tumor latency or morphology of the female K14creBra1^{f/f}p53^{flo/flo}Abcb1a/b^{+/−} mice (data not shown). P-gp–deficient mice carrying spontaneous mammary tumors cannot be treated with the docetaxel maximum-tolerated dose (MTD) because P-gp contributes to the normal disposition of docetaxel in the mouse. We therefore grafted Bra1^{+/−}p53^{+/−}Abcb1a/b^{+/−} tumors (KB1PM) orthotopically into syngeneic wild-type mice (Fig. 5A). In sharp contrast to Abcb1a/b wt tumors (KB1P), tumors unable to make P-gp were hypersensitive to the docetaxel MTD: only 1 of 11 individual KB1PM tumors responded poorly to docetaxel and the mouse had to be sacrificed 40 days after the start of treatment (Fig. 5B). The median recurrence-free survival (RFS)
time increased significantly \( (P < 0.0004) \) from 14 (T7-T43) to 51 days (KB1PM-1 and KB1PM-3 to -11) and for 1 tumor (KB1PM-2), no relapse occurred within 250 days, suggesting that this tumor was even eradicated (Fig. 5B and C). With the exception of KB1PM-5 none of the P-gp-deficient tumors acquired docetaxel resistance and, eventually, the mice had to be killed because of cumulative docetaxel toxicity. The median survival of animals carrying orthotopically transplanted P-gp-deficient tumors increased significantly \( (P < 0.0001) \) to 164 days (±69 SD, \( n = 11 \)) compared with 45 days (±28 SD, \( n = 37 \)) of animals with P-gp-proficient tumors (Fig. 5D). These data showed that P-gp is a major contributor to docetaxel resistance of KB1P mammary tumors \textit{in vivo}.

**Low expression of the \textit{Xist} gene correlates with high cisplatin sensitivity of KB1P tumors and predicts benefit of platinum-based chemotherapy in patients with high-risk primary breast cancer**

Because we found variation in the response to drug, not only for docetaxel but also for cisplatin (Fig. 6A), we wondered whether standard gene expression analyses would also fail to identify predictive markers for this treatment. All KB1P tumors were cisplatin sensitive, but 23 tumors relapsed already within 39 days, whereas 12 tumors only grew back to 100% after 49 days. When we stratified the gene expression profiles of the untreated tumors based on their cisplatin sensitivity (above or below the mean time to relapse), we found a low expression of the \textit{Xist} gene to correlate significantly with cisplatin hypersensitivity on 2 independent gene expression platforms (Fig. 6B).

The physiologic role of the noncoding RNA \textit{Xist} is to coat one X chromosome of female cells in \textit{cis} and subsequently trigger chromatin remodeling to form the heterochromatic Barr body [condensed inactivated X chromosome (Xi)]. \textit{XIST} is transcribed exclusively from the Xi to achieve equal X-linked gene dosage between the sexes. The analysis of genes correlating with a low \textit{Xist} expression in our tumor model revealed a reduced expression of 3 other X-linked genes: \textit{Utx}, \textit{Jari1c}, and \textit{Ej253x3} (Supplementary Fig. S5). As all of these genes are known to escape X inactivation (34), they are independent markers for the loss of the Xi.

Given the high frequency of reduced \textit{Xist} expression in cisplatin hypersensitive mouse tumors, we tested whether \textit{XIST} expression could serve as a biomarker to predict response to platinum-based chemotherapy in human breast cancer. For this purpose we took tumor samples of 60 stage III, HER2-negative breast cancer patients who had been randomized between 2 treatment arms: intensive platinum-based chemotherapy or a standard anthracycline-based regimen (conventional chemotherapy; ref. 27). The patient information is summarized in Supplementary Table S5. To quantify \textit{XIST} gene expression levels of FFPE material (>60% tumor cells), we used RT-MLPA including 2 independent probes hybridizing to the exon 2–3 or exon 4–5 boundary of \textit{XIST} cDNA (Supplementary Table S6 and Supplementary Fig. S6). Analysis of the RFS showed that patients with a low \textit{XIST} expression significantly benefited from the intensive platinum-based therapy compared with conventional chemotherapy: the 5-year RFS increased from 37% to 75% (Fig. 6C, adjusted HR: 0.30, 95% confidence interval (CI): 0.11–0.82 for the probe of exon 4–5, Supplementary Table S5B). In patients with \textit{XIST} gene expression above the cut-off, no significant survival benefit was observed of platinum-based chemotherapy (5-year RFS 33% both treatment arms, Fig. 6C; adjusted HR: 0.81, 95% CI: 0.23–2.89 for the exon 4–5 probe, Supplementary Table S5B). Analysis with the exon 2–3 probe confirmed those obtained with the exon 4–5 probe (Fig. 6C and Supplementary Table S5B).

To determine whether loss of the \textit{XIST} gene could explain the low \textit{XIST} gene expression detected with both RT-MLPA probes, we analyzed the DNA of 37 patients with arrayCGH using probes flanking the \textit{XIST} locus. Indeed, a \textit{XIST} gene was lost in 16 of 37 patients. Loss of \textit{XIST} DNA correlated significantly with low RNA expression for all 60 patients \( (P < 0.017 \) for the exon 2–3 probe, Fisher exact test). For 24 of the 60 samples, we also managed to carry out \textit{XIST} RNA FISH analyses on the available FFPE material (Fig. 7). A DNA probe targeting \textit{RNF12} was taken along as X chromosome marker (Fig. 7A). RNA FISH confirmed that patients with low \textit{XIST} gene expression had significantly fewer \textit{XIST} clouds (Fig. 7B and C). Moreover, the
Figure 4. Quantification of the expression of the mouse Abcb1 genes in untreated mouse mammary tumors. A, RT-MLPA analyses of Abcb1a or Abcb1b transcript levels of 43 individual KB1P tumors. Error bars indicate SD of 3 independent reactions. B, time until relapse of KB1P tumors treated with the MTD of cisplatin, docetaxel, or doxorubicin. The 5 tumors that showed increased Abcb1 gene expression are highlighted in red. In panel C, the remaining 15 poor docetaxel responders that were also treated with cisplatin and doxorubicin are indicated in red.
A combination of RNA and DNA FISH showed for all cases with aCGH-based XIST deletion that only the Xa was present (Fig. 7B). In several patients with low XIST gene expression, but no XIST gene deletion detectable by aCGH, we found 2 active X chromosomes and loss of Xi (Fig. 7B). Together, our data showed that loss of Xi is the main cause of low XIST gene expression ($P < 0.027$, Fig. 7C).

High prevalence of a predictive marker is required for its detection

Because Xist was readily identified as predictive marker for cisplatin sensitivity in our mouse model by SAM, it remains remarkable that our initial search to detect predictive markers for docetaxel sensitivity (Fig. 2) failed. When we analyzed only the tumors with an intrinsically high Abcb1 expression (T8/C3 con, T9/C3 con, T15/C3 con, T26/C3 con, and T41/C3 con) versus the 21 docetaxel-sensitive tumors as defined in Fig. 2A, Abcb1b was one of the most significantly increased genes on both the MEEBO and Illumina gene expression platforms (Supplementary Fig. S4C and D). Also the TLDA expression data showed a significant difference for Abcb1a and Abcb1b when only the 5 poor responders were compared with the docetaxel-sensitive tumors (Abcb1a: $P < 0.0064$; Abcb1b: $P < 0.0043$; Mann–Whitney U test). However, as increased expression of the Abcb1 genes is only found in a subgroup of the poor docetaxel responders, this significance is lost when samples with other docetaxel resistance mechanisms are added (Supplementary Fig. S7). In fact, addition of 5 samples without Abcb1 upregulation suffices to dilute the Abcb1 signal below significance.

In contrast to Abcb1b in the case of docetaxel treatment, the prevalence of low Xist expression was high in cisplatin hyper-sensitive tumors: 11 (MEEBO platform) or 10 (Illumina platform) of the 12 showed Xist gene expression below the median (Supplementary Fig. S7).

Discussion

We have investigated whether predictive markers for chemotherapy benefit can be identified in a GEMM using genome-wide expression profiling. GEMMs should be ideal...
for this purpose, as they lack the profound genetic heterogeneity of tumors from human patients. The tumors originate from the targeted deletion of \textit{Brca1} and \textit{p53}, and all differences between tumors originate from random mutations in the period between the initiating deletions of \textit{Brca1} and \textit{p53} and the development of a mammary tumor. These additional mutations are responsible for the marked and stable differences in sensitivity to docetaxel and cisplatin that we find in individual tumors.

Even in this genetically homogeneous tumor system, we did not find a signature predicting docetaxel response, using genome-wide expression profiling. This negative result is instructive, however, because it has allowed us to delineate what is required to get useful predictive signatures. In our collection of 22 poor docetaxel responders, 5 tumors contained a substantial increase in \textit{Abcb1} RNA, known to be sufficient to cause drug resistance (21). Nevertheless, this increase in \textit{Abcb1} RNA was completely missed by 2 independent platforms measuring gene expression profiles. The \textit{Abcb1} transcript was readily detected in the 5 tumors with elevated transcript levels, as long as these tumors were analyzed as a group. However, when the results were pooled with those from only 5 tumors without elevated \textit{Abcb1} RNA, the positive result was completely lost. This shows why it is difficult to develop predictive markers, based on genome-wide expression arrays: only if the response to a drug is primarily determined by the expression level of a gene in most tumors, one can expect that gene to show up in the array-based gene expression analyses. We found such a gene in analyzing the response of the mouse tumors to cisplatin. The low \textit{Xist} expression associated with tumors hypersensitive to cisplatin was present in 10 of 12 tumors and therefore detectable in our array analysis. The detection sensitivity can only be increased by the use of special algorithms that can identify subgroups within the samples. We show that such an algorithm is able to identify \textit{Abcb1} as outlier within the poor docetaxel responders. However, even with a more sophisticated analysis, the problem remains that probes on the arrays are not sensitive enough to detect all relevant expression differences of genes causing therapy resistance. Gillet and colleagues found in a panel of cancer cell lines...
that the expression of the 380 "resistance-relevant" genes could only be reliably measured by quantitative PCR. For many genes the results obtained by microarrays were useless because of low sensitivity (29, 35).

Given all these hurdles in finding predictive markers for chemotherapy, it is gratifying that we identified a gene that correlated with cisplatin response. It is encouraging that the low expression of XIST predicting high sensitivity to cisplatin in drug-naive mouse tumors also predicted an increased RFS of high risk, primary breast cancer patients treated with intensive platinum-based chemotherapy. Although detected in a rather small group of 60 patients, the effect found is considerable. Intensive chemotherapy has largely been abandoned for the treatment of breast cancer because for many patients the therapeutic benefit is limited (36). Nevertheless, several studies suggest that there are subgroups of patients that do benefit from this therapy, but the predictive tests to identify them are lacking (37, 38). Hence, the analysis of XIST gene expression may be a useful tool to decide whether intensive platinum-based chemotherapy should be considered as alternative therapy for patients with HER2-negative, high-risk breast cancer. Not all patients with a low XIST expression that we investigated benefited from the platinum-based therapy. An optimized cut-off for the level of XIST expression, validated in prospective clinical trials, may increase the positive predictive value, as may a combination with other classifiers, such as BRCA1-like CGH profiles (25).

Why tumors with a low expression of XIST are platinum hypersensitive is under investigation. Low XIST gene expression may be a flag for genomic instability as we found loss of Xi as the main cause underlying low transcript levels. The loss of Xi is most likely the consequence of chromosome segregation errors, which may be enhanced in BRCA1-defective cells due to a compromised spindle checkpoint (39). It has recently been found that mis-segregation stress induces a DNA damage response (40), and it was observed that aneuploid cells are more sensitive to antiproliferative drugs (41). Mammary tumor...
cells with defects in DNA repair that are additionally stressed by improper execution of mitosis may therefore be hypersensitive to intensive platinum-based therapy.

The precise mechanism of XIST-mediated X inactivation is still under debate (42). It was suggested that BRCA1 supports the localization of XIST RNA to the Xi, as the BRCA1-deficient cells or tumors examined had lost localized XIST RNA (43–45). However, this hypothesis was challenged by others (46, 47). The recent finding that BRCA1 maintains heterochromatin integrity (48) supports the idea that BRCA1 contributes to X inactivation after XIST-induced chromatin condensation. The contribution is not a simple one, however. Despite the large Brca1 deletion present in all mammary tumors of our mouse model, Xist gene expression varies considerably. Variability of XIST expression was also present in those human breast cancers in which a BRCA1 mutation was found, or which were classified as BRCA1-like by aCGH (25).

Our study shows that GEMMs that resemble breast cancer in humans are useful to investigate chemotherapy response prediction. Tools to identify predictive markers can be tested under controlled conditions, and targeted ablation of genes helps to dissect mechanisms of resistance. Ultimately, predictive markers identified in GEMMs may improve the clinical success rate for chemotherapy in humans.

Disclosure of Potential Conflicts of Interest
M. Jonkers and M. Lodén are employees of MRC-Holland BV that markets the RT-MLPA tests used in this article.

References

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