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PRECLINICAL STUDY

RecurrenceOnline: an online analysis tool to determine breast cancer recurrence and hormone receptor status using microarray data

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Abstract In the last decades, several gene expression-based predictors of clinical behavior were developed for breast cancer. A common feature of these is the use of multiple genes to predict hormone receptor status and the probability of tumor recurrence, survival or response to chemotherapy. We developed an online analysis tool to compute ER and HER2 status, Oncotype DX 21-gene recurrence score and an independent recurrence risk classification using gene expression data obtained by interrogation of Affymetrix microarray profiles. We implemented rigorous quality control algorithms to promptly exclude any biases related to sample processing, hybridization and scanning. After uploading the raw microarray data, the system performs the complete evaluation automatically and provides a report summarizing the results. The system is accessible online at http://www.recurrenceonline.com. We validated the system using data from 2,472 publicly available microarrays. The validation of the prediction of the 21-gene recurrence score was significant in lymph node negative patients (Cox-Mantel, $P = 5.6\text{E-}16$, HR = 0.4, CI = 0.32–0.5). A correct classification was obtained for 88.5% of ER- and 90.5% of ER + tumors ($n = 1{,}894$). The prediction of recurrence risk in all patients by using the mean of the independent six strongest genes ($P < 1\text{E-}16$, HR = 2.9, CI = 2.5–3.3), of the four strongest genes in lymph node negative ER positive patients ($P < 1\text{E-}16$, HR = 2.8, CI = 2.2–3.5) and of the three genes in lymph node positive patients ($P = 3.2\text{E-}9$, HR = 2.5, CI = 1.8–3.4) was highly significant. In summary, we integrated available knowledge in one platform to validate currently used predictors and to provide a global tool for the online determination of different prognostic parameters simultaneously using genome-wide microarrays.

Keywords Survival analysis · Breast cancer · Prognosis · Bioinformatics · Microarray · Recurrence score · Recurrence risk · Lymph node status

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Introduction

Recently available multigenic prognostic biomarkers promise to provide a prediction efficiency superior to monogenic tests, enabling better patient tailored therapy in the treatment of breast cancer. In essence, development is carried out on two major platforms, including RT-PCR-based Oncotype DX [1], Theros Breast Cancer Index [2], Breast bioclassifier [3], Celera metastatic score [4], 8-gene score [5] and microarray-based Mammaprint [6], MapQuant Dx [7], BLN assay [8], Invasive Gene Signature [9] and Wound Response Indicator [10]. In addition, the FISH-based eXagen test [11] and the IHC-based Mammostrat

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Table 1 Summary of multigenic tests developed for breast cancer

Name	Company	Available	No of genes	Sample	Technique	Diagnostic aim
Oncotype Dx	Genomic Health	EU, USA	21	FFP	Q-RT-PCR	Prognosis, recurrence after tamoxifen therapy
Theros Breast Cancer Index	Biotheranostics	USA	2(5)	FFP	Q-RT-PCR	Prognosis, recurrence after endocrine therapy
Breast Bioclassifier	ARUP	USA	55	FFP	RT-PCR	Prognosis
Celera Metastatic Score	Applera	–	14	FFP	RT-PCR	Prognosis, recurrence after tamoxifen therapy
eXagen	eXagen diagnostics	–	3	FFP	FISH	Prognosis
Mammostrat	Applied genomics	USA	5		IHC	Prognosis
ProEX™ Br	TriPath	–	5		IHC	Prognosis
MammaPrint	Agendia	EU, USA	70	F/F	Microarray	Prognosis in patients over 61 years
MapQuant Dx	Ipsoggen	EU	97	F/F	Microarray	Prognosis
Breast Lymph Node (BLN) Assay	GeneSearch Veridex	UK	76	F/F	Microarray	Intraoperative metastasis identification
Invasive Gene Signature	–	–	186	F/F	Microarray	Prognosis
Wound Response Indicator	–	–	512	F/F	Microarray	Prognosis

F/F fresh/frozen, *FFP* formalin-fixed paraffin-embedded, *IHC* immunohistochemistry, *FISH* fish fluorescent in situ hybridization

[12] are available (see Table 1). Most of these datasets were summarized and evaluated in our recent meta-analysis [13].

Of the above mentioned tests, the Oncotype DX assay is by far the most widely used with over 135,000 tests performed already (Genomic Health Annual report, 2009). Here, FFPE samples are sent to a single laboratory, where the isolation of RNA and analysis are performed. Twenty-one genes are quantified in the test to predict distant recurrence in breast cancer patients with lymph-node negative, estrogen-receptor (ER) positive tumors. Patients are classified into high/intermediate/low risk of recurrence based on the recurrence score, which is computed using an precisely defined mathematical formula [1]. The assay not only provides prediction of relapse-free survival, but also predicts the risk of locoregional recurrence [14]. It might also support the treatment decision whether a patient should receive adjuvant chemotherapy [15]. The benefit of adjuvant chemotherapy over and above endocrine therapy differs greatly in the assay-determined risk categories [16]. The effect of Oncotype Dx on physicians' decision-making for systemic adjuvant therapy ranged from 25% [17] to 44% [18]. The actual cost of the assay itself seems to be well justified by saving the cost of unnecessary chemotherapy [19]. However, significant weaknesses of the test are the lack of any independent quality control and the slow processing requiring several weeks.

Determining estrogen receptor status accurately is essential to identify patients eligible for endocrine therapy in breast cancer. Another receptor of significant clinical importance is HER2 (ERBB2), which is over-expressed in over 25% of invasive ductal breast cancers [20]. The elevated levels of HER2 are associated with increased proliferation and poor prognosis [21]. Immunhistochemistry based HER2 positive patients are eligible for first-line trastuzumab treatment in combination with chemotherapy [22] and for monotherapy in patients who progressed after chemotherapy [23]. Trastuzumab treatment results in increased response rate and longer survival [22].

A genome-wide Affymetrix microarray measures over 22,000 genes including the hormone receptors and those genes used in different multigenic breast cancer classifications. Here, we report the development of an online analysis tool which is capable of computing ER and HER2 status and the risk of recurrence using gene expression data obtained by interrogating Affymetrix HGU133A and HGU133Aplus2 microarrays. Furthermore, we implemented rigorous quality control algorithms to promptly exclude any biases related to sample processing, hybridization and scanning. We performed a validation of the system in 2,472 breast cancer specimens obtained from microarray datasets published in GEO.

Methods

Server set-up

All computations on the raw microarray data are performed in real time (Fig. 1). Recurrence online is set up using a

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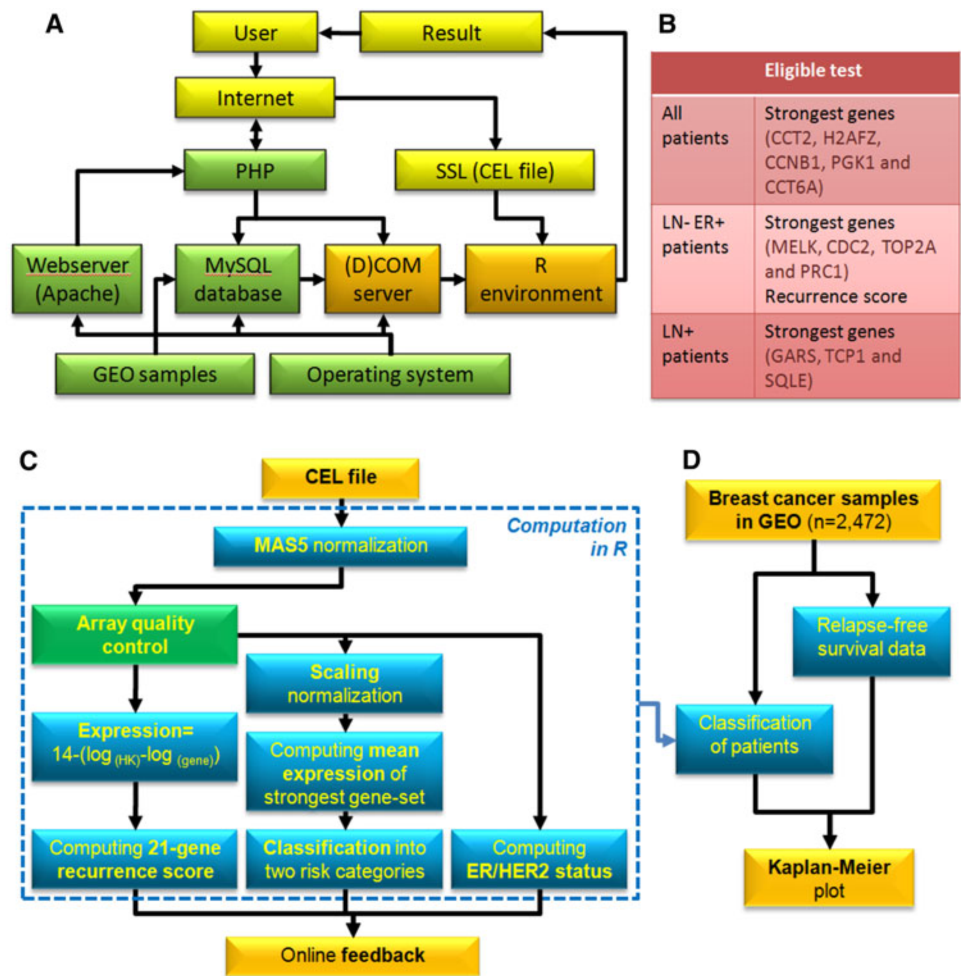

Fig. 1 Overview of the server (**a**), the eligible tests (**b**), the applied analysis corridor in the online computations (**c**) and the implementation of the validation study (**d**)

central server accessible via the internet. The central server runs an Apache webserver and a (D)COM server which provides statistical computations in R. After a secure upload of the .CEL file, data is loaded into the R statistical environment, where the calculations are performed. The package "affy" is used for normalization. For the validation, the background database is handled by a MySQL server, which integrates gene expression and clinical data simultaneously. The homepage was constructed using a modular online system built in the freely available drupal (www.drupal.org) environment. The user receives feedback from the webpage.

We focused on the two most widely used human microarray platforms, the Affymetrix HGU133A (GEO platform ID: GPL96) and the Affymetrix HGU133plus2 (GEO platform ID: GPL570). The final output of a hybridization of an Affymetrix microarray is the acquisition of a .CEL file, which contains all the raw results of the microarray. The implemented various metrics of our tool rely on these raw .CEL files. The input window of recurrenceonline.com is presented in Fig. 2a.

Array quality control

Heber and Sick [24] suggested eight quality metrics as a basic quality assessment for Affymetrix microarrays. First, we have implemented their methods and tested them on an extended version of our previously published database [25]. The distribution of the arrays was assessed and outliers were identified as those having a parameter value outside of the range of 95% of samples. Then, the "Array quality control" parameter implemented in www.recurrenceonline.com was set to give a warning in cases in which the thresholds published by Heber et al. are surpassed or outliers are detected as compared to our meta-analysis. The cutoff values are summarized in Table 2.

Computation of the recurrence score

After the quality control the raw Affymetrix .CEL files are MAS5 normalized in *R* using the affy Bioconductor library. MAS5 can be applied to individual chips, making comparison to the validation data and future extension of the

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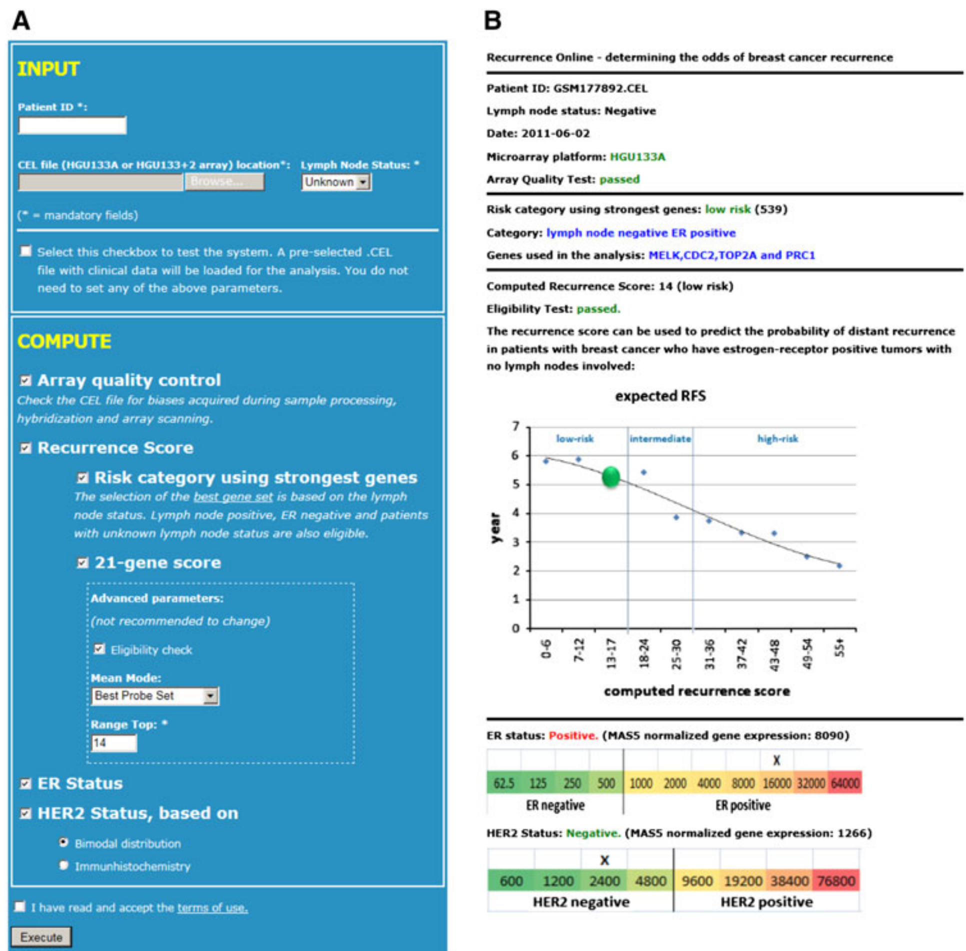

Fig. 2 **a** One-step online interface is used for input (**a**). The analysis results window **b** delivers a graphical assessment of the tested parameters

Table 2 Parameters implemented in the array quality control metrics

Parameter	Recommendation by Heber et al. for good quality arrays	Range of 95% of arrays in 2,472 breast cancer samples	www.recurrenceonline.com gives warning if result is
Background	Between 10 and 100 normal	40–114	Larger than 100
Scaling factor	Smaller than 3	0.3–2.3	Larger than 3
Percent present calls	In the range 20–50%	42–58%	Smaller than 30%
bioB-, bioC-, bioD- and cre-spikes	Always present	Not applicable	Not present
dap-, lys-, phe-, thr-, tryp-spikes	lys < phe < thr < dap	Not applicable	Not "lys < phe < thr < dap"
GAPDH 3′ to 5′ ratio	Close to one	0.28–5	Larger than 4 or smaller than 0.25
Beta-actin 3′ to 5′ ratio	Smaller than 3	0–4.7	Larger than 3

validation datasets easily feasible. Moreover, MAS5 ranked among the best normalization methods when compared to the results of RT-PCR measurements in our recent study [26]. Then, the differences of the log-transformed expression of the 16 genes and the housekeeping genes ACTB, GAPDH, RPLP0, GUS, and TFRC are subtracted from the "range top" (adjustable parameter) to emulate RT-PCR results. For genes with multiple probe sets represented on the Affymetrix microarrays the probe set with the highest average expression or the average expression of available probe sets can be used (adjustable parameter). Then, the recurrence score is computed as described by Paik et al. 2004. Finally, samples are classified as being in the high/intermediate/low group based on their recurrence score.

Assessment of ER and HER2 status

Gong et al. [27] demonstrated the possibility to determine estrogen receptor and ERBB2 status reliably and reproducibly using Affymetrix microarrays. We implemented their

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approach using the suggested cutoff values of 500 (in the probe set 205225_at) for estrogen receptor and 1,150 (in the probe set 216836_s_at) for ERBB2 receptor. In addition, for the ERBB2 receptor the bimodal distribution of the validation datasets was decomposed into two Gaussian distributions (which correspond to two specific ERBB2 expression statuses) as described earlier [28]. Briefly, based on the two inferred distributions a cohort-specific cut-off value for ERBB2 using Mahalanobis distance—which minimizes the estimated false positive rate (FPR) and the false negative rate (FNR)—was derived. The actual cutoff for ERBB2 is user selectable: "bimodal distribution" uses 4,800 as cutoff, while "immunhistochemistry" uses the 1,150 cutoff suggested by Gong et al.

Validation

The validation was performed on microarrays which were previously published in GEO in following datasets: GSE1456, GSE2034, GSE2990, GSE3494, GSE4922, GSE6532, GSE7390, GSE11121, GSE12093, GSE5327, GSE9195, GSE16391, GSE12276, GSE2603, GSE17705, and GSE21653. The datasets were generated using HGU133A or HGU133Aplus2 microarrays which possess overlapping probe set identifiers and are also used in www.recurrenceonline.com analyses. The construction of the database was performed as described earlier [25]. After classification of the samples as having high/intermediate/low recurrence scores, the groups were compared using Kaplan–Meier survival plots in WinSTAT 2007 for Excel (Robert K. Fitch Software, Germany) and using the "survplot" package (http://www.cbs.dtu.dk/~eklund/survplot/) in R.

Computation of risk category using the "strongest genes"

Using our validation database we identified the genes with the strongest predictive power in all patients, in lymph node positive, and in lymph node negative ER positive and ER negative patients. First, a filtering was performed to select only those probe sets which reliably work on the microarray. Probe sets were retained having a median expression over 890 (the whole-array median) or having a median expression of at least 445 and covering at least 20% of the gene and not mapping to multiple genes. After this, the gene with the lowest p value and the highest HR value in the given cohort of patients was selected. Then, the second probe set was added, and the mean expression of the two probe sets was used for classification. This was repeated as long as the predictive power of the mean of the used probe sets increased. A leave-one-out cross validation (LOOCV) was performed in each of the three cohorts to measure the robustness as whether the same genes will be selected by excluding any of the samples. Finally, the classification was implemented in RecurrenceOnline as the "Risk category using strongest genes" option.

Comparison of classification performance by using ROC

Although we do not had access to the Oncotype DX scores for the same samples, we contrasted area under the curve (AUC) measures using our methods and published AUC measures available for the Oncotype DX [29]. ROC analysis was performed using MedCalc 11.6. (MedCalc Software, Mariakerke, Belgium).

Results

Online system

We have established an online platform which enables the computation of four different features using Affymetrix HGU133A or HGU133plus2 microarray data. An example of a complete analysis is depicted in Fig. 2b.

On these microarrays, some genes are measured by several probe sets. For the analysis of ERBB2 status and ER status, we used the probe sets with the highest average expression. For the prediction of the recurrence score, one can decide how to handle these probe sets for each gene. The "Average" computes the average expression of all available probe sets for each gene. The "Best probe set" uses only the best probe as measured by average expression of each probe set and mapping to the gene by blasting to Ensembl genes. Finally, as one of the housekeeping genes is related to proliferation (GAPDH) [30], this gene can be excluded from the analysis using the "Best probe-GAPDH" option.

The ER and ERBB2 status are computed using the MAS5 normalized expression values. The computation of the recurrence score is preceded by a transformation of the linear MAS5 expression values to logarithmic scale. In contrast to RT-PCR, where larger values mean lower expression, larger values mean higher expression on microarrays. Therefore, an inversion is necessary, and the range top parameter defines in this context the top of the dynamic range on the microarray.

Validation cohort

All together 2,472 microarray measurements were entered into the validation database. The average relapse-free survival is 6.39 ± 4.0 years with 869/2,239 relapses. Only lymph node negative patients with available relapse-free survival times (n = 1,509) were included in the basic classification

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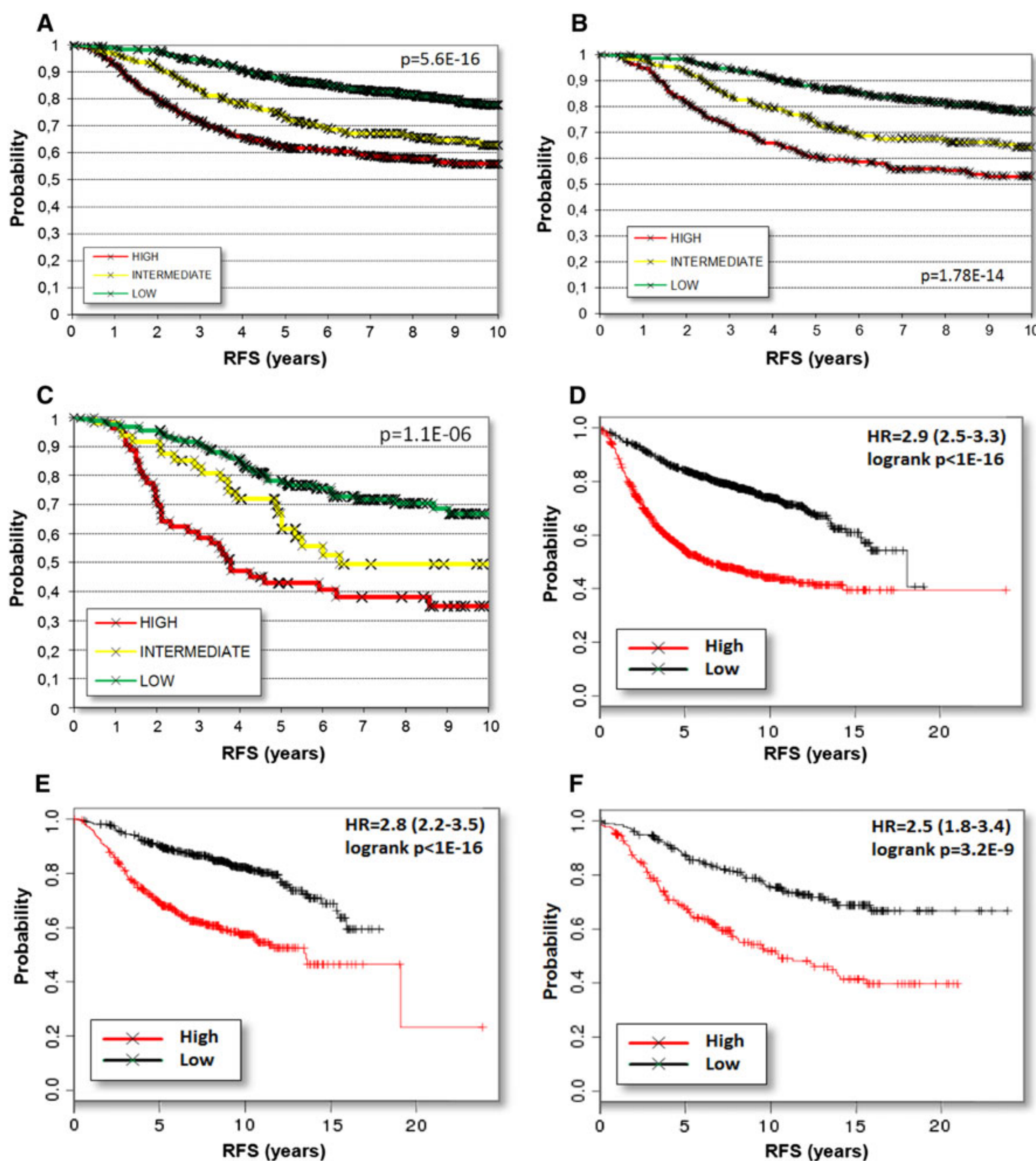

Fig. 3 Kaplan–Meier plots of survival based on classification using the recurrence score in all lymph node negative patients ($n = 1{,}509$) (**a**), and in lymph node negative estrogen receptor positive patients (**b**, $n = 1{,}028$, ER status is IHC based). The recurrence-score-classification is also effective in only tamoxifen treated (**d**, $n = 251$) estrogen receptor positive, lymph node negative patients. Classification using the mean expression of the six independent strongest genes in all patients regardless of lymph node and ER status (**d**, $n = 2{,}316$), the strongest four genes in lymph node negative ER positive patients (**e**, $n = 1{,}077$, ER status is array-based); and the strongest three genes in lymph node positive patients (**f**, $n = 482$)

using the recurrence score algorithm. The classification resulted in 46% ($n = 701$) of patients classified into the low, 19% ($n = 279$) into the intermediate and 35% ($n = 527$) into the high-risk group, the Kaplan–Meier plots for all three categories are depicted in Fig. 3a. In these patients, the difference in the survival curves were significant ($P = 5.6\text{E}{-}16$, Hazard Ratio = 0.40, Confidence Interval = 0.32–0.50). The significance was slightly reduced when only ER positive samples were included for the recurrence score ($P = 2.1\text{E}{-}15$, HR = 0.36, CI = 0.28–0.47 (see Fig. 3b). The classification of the 1,509 lymph node negative patients using the available clinical variables resulted in much lower significances (ER status: $P = 0.0002$, HR = 0.66, CI = 0.51–0.86, grade: P = not significant). In many previous publications, authors reported not only the overall significance, but also the significance between the worst and the best performing

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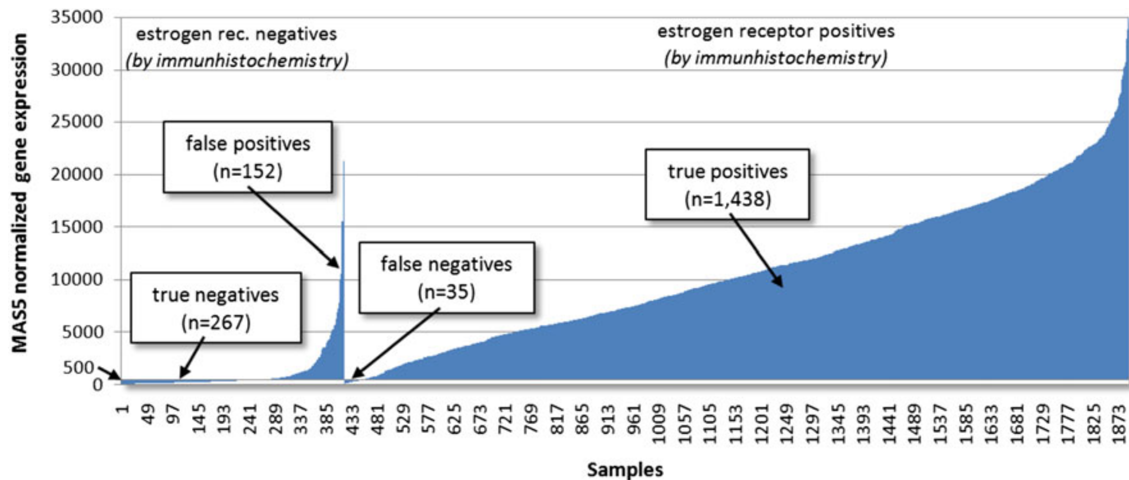

Fig. 4 Validation of the estrogen receptor status determination in 1,894 patients with available immunhistochemistry results. The patients are ordered on the basis of the expression of the probe set 205225_at, which corresponds to the ESR1 gene. The horizontal axis crosses the vertical axis at 500, which is used as a threshold for determining the estrogen receptor state as positive or negative. By using this threshold, over 90% of patients are correctly classified

groups. Therefore, in a similar setting, we excluded the median 10% of the samples from the analysis of the lymph node negative, estrogen receptor positive samples ($n = 1{,}077$ remaining after reduction). In this setting, the recurrence score yielded a slightly higher discriminative power ($P = 2.1\text{E}{-}15$, HR = 0.34, CI = 0.26–0.45) (graph not shown).

In an additional analysis option, we assessed only those patients whose treatment protocols were published. The survival plot showing the discriminative power in only tamoxifen-treated patients ($n = 251$) is depicted in Fig. 3c. Classification was not significant in lymph node positive ($n = 482$) and in lymph node negative estrogen receptor negative patients ($n = 199$).

Computation of risk category using the strongest genes

Using our validation database we selected the most informative genes with the highest predictive value in all patients with available relapse-free survival time, in lymph node positive patients, and in lymph node negative ER positive patients. Due to the low number of patients in the lymph node negative ER negative group ($n = 199$), we have not derived a discriminative signature for these patients. In "all patients", the genes CCT2, H2AFZ, RACGAP1, CCNB1, PGK1, and CCT6A are used ($n = 2316$, classification power using the mean expression of 2,600: $P < 1\text{E}{-}16$, HR = 2.9, CI = 2.5–3.3, see Fig. 3d). In "lymph node negative ER positive patients", the mean expression of the genes MELK, CDC2, TOP2A, and PRC1 are used (cutoff = 600, $n = 1077$, $P < 1\text{E}{-}16$, HR = 2.8, CI = 2.2–3.5, see Fig. 3e). In "lymph node positive patients" the genes GARS, TCP1, and SQLE are used for classification ($n = 482$; cutoff = 2600, $P = 3.2\text{E}{-}9$, HR = 2.5, CI = 1.8–3.4, see Fig. 3f).

In the leave-one-out cross validation of the cohort containing "all patients", and of the "lymph node negative ER positive patients" the set of the top genes was confirmed in 100% of the analyses. In the "lymph node positive" cohort, the selected genes were confirmed in 91.9% of the analyses. One gene, GATAD2A, surpassed SQLE in 8.1% of the LOOCV tests. However, addition of GATAD2A did not increase predictive power ($P = 1\text{E}{-}8$, HR = 2.4, CI = 1.8–3.3.).

Validation of ER-status determination

Estrogen receptor status based on immunohistochemical assessment (IHC) was available for 1,894 patients. Of these, 267 were classified as negative and 1,438 as positive by both IHC and microarray. Positive IHC with negative microarray results were obtained in 35 samples and positive microarray with negative IHC results in 152 patients. In summary, 88.5% of ER− and 90.5% of ER+ samples were classified correctly (see Fig. 4).

Another important feature of the ER status is the fact, that only ER positive patients are eligible for the original recurrence score analysis. Therefore, we used our ER-status determination and computed the recurrence score separately (in lymph node negative patients with available survival) for patients having positive IHC results ($n = 1{,}028$) and for patients having positive microarray results ($n = 1{,}090$). While the Kaplan–Meier survival plots delivered a significant difference in both cases, the group having the array-based ER-status determination had a stronger predictive power

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($P = 2.1\text{E}{-}15$, HR = 0.36, CI = 0.28–0.47 vs. $P < 1\text{E}{-}16$, HR = 0.36, CI = 0.28–0.46).

Comparison of classification performances

The AUC for the mean of strongest genes in all patients was 0.695 (sensitivity = 63%, specificity = 67%). For the strongest genes in the lymph node negative ER positive patients the AUC was 0.613 (sensitivity = 67%, specificity = 56%), and for the mean of the strongest genes of lymph node positive patients the AUC was 0.569 (sensitivity = 45%, specificity = 69%).

The AUC of RecurrenceOnline in the ROC analysis using the 21-gene score for all lymph node negative and ER positive patients was 0.637 (sensitivity = 61%, specificity = 62%). The previous AUC for Oncotype DX was 0.59 (sensitivity = 68%, specificity = 50%) [29].

The AUC for the estrogen status determination in the ROC analysis was 0.807 (sensitivity = 97%, specificity = 64%). The ROC plots are depicted on Fig. 5.

Array quality control

All together 77 samples (out of 2,472) failed at least one of the quality control thresholds, 7 samples failed more than one quality parameter. Of the first, 46 were lymph node negative and of these, 39 were ER positive. Discrimination of these patients by Kaplan–Meier survival analysis using the computed RS score was impossible ($P = 0.11$).

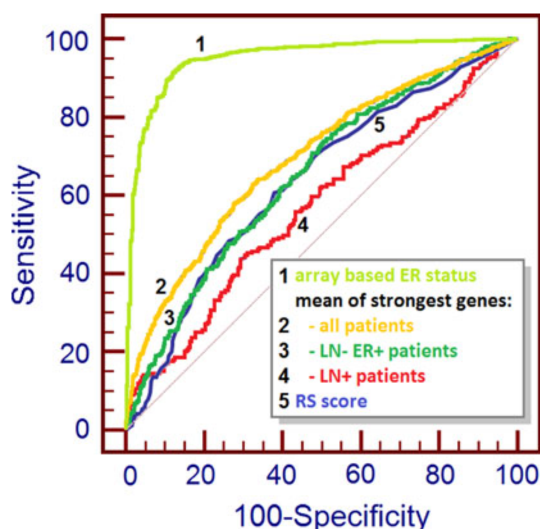

Fig. 5 ROC plots for the implemented risk category prediction sets: the array-based ER status determination vs. IHC based actual ER status (**1**), the predictive power of the strongest genes identified in all patients (**2**), LN-ER+ patients (**3**), LN+ patients (**4**) and the predictive power of the recurrence-online computed recurrence score (**5**)

Discussion

In our study, we aimed to prepare a tool capable to perform different assays simultaneously by the use of genome-wide microarrays. The approach is based on the fact that microarray facilities are available at virtually all university research centers. Thus, the microarray profiles of individual tumor specimens obtained at these centers can be compared to and evaluated in the context of a large number of clinical cases, which have been carefully characterized in previous publications and, hence, can be considered as a global reference. This can be performed in a shared resource setting by www.recurrenceonline.com.

We successfully validated recurrenceonline.com using 2,472 independent gene expression profiles of breast cancer specimens, available in GEO with clinical follow-up. The predictive power of our study is higher as compared to the original classifications [1]. Most probably there are several reasons for this improvement: RNA is prepared from fresh material prior to microarray analysis. This procedure allows improves expression measurements compared to paraffin embedded tissue as the source of RNA. The second reason is the use of multiple probe sets for the measurement of the genes on the microarrays. This enables us to select the best probe sets which in turn will enable higher prediction accuracy. Finally, we used a much larger set of patients for validation than previous studies in combination. Unfortunately, direct comparison of recurrence online with Oncotype Dx using tumour samples was not possible as we do not had access to samples for which both Oncotype Dx assay and Affymetrix microarrays have been performed.

We also show the capability to classify the patients regardless of lymph node and ER status into high-risk and low-risk cohorts using three independent sets of predictive genes. As today all lymph node positive patients routinely receive chemotherapy, a more in-depth analysis of these patients will be needed for estimation of treatment consequences.

The determination of ER status relies on a study of Gong et al [27], who effectively used microarrays for ER and HER2 receptor status determination. We implemented their system, but had only enough data to validate the ER-status prediction. The prediction achieved a very high success rate of ~90%. In addition, the measurement of the ER status by microarray further improved the recurrence score designed only for ER positive patients. For the HER2 determination, we also implemented a cut-off value based on the expected bimodal distribution of the receptor expression values as published by Li et al [28].

One might consider quality issues related to the use of microarrays for the measurement of gene expression. Reproducibility of the gene expression measurements was

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already confirmed by the MAQC consortia [31]. However, the high data volume and data complexity in microarray experiments carry many potential sources of unwanted variation that could compromise the results if left uncontrolled. This growing concern and awareness of the importance of assessing the quality of generated microarray data was assessed in a review published by Heber and Sick [24]. Therefore, we implemented a quality metrics measurement based on their suggested parameters. Particularly, seven different quality parameters related to biases in sample processing, hybridization and scanning are assessed by www.recurrenceonline.com. The value of this added analysis is outlined by the fact, that the classification of the samples with failed quality control was not successful. We have set up the system to leave the decision to the investigator, and the report gives a warning in case that the quality of the microarray used is low.

Besides the validation of proprietary analysis pipelines, our system can accelerate prognosis prediction by enabling rapid evaluation of locally processed samples. Theoretically, the local diagnostic pathway can be completed in $\sim$24 h, thereby reducing the time currently needed for such an analysis by over 95%. The computational analysis itself is completed in less than 10 min. The speed could bring additional benefits for the patients, who could receive the most appropriate treatment within a shorter time.

In summary, we developed an online classification system capable of using common genome-wide microarrays to assess hormone receptor status and to compute the risk of recurrence. The system performs all bioinformatic steps automatically and is therefore also suitable for users with negligible statistical knowledge. As the present scheme can be extended with additional gene sets and be applied to other cancer types, one might envision a future single step approach, which permits diagnosis of different tumor types in the same automated pipeline.

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