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Original article

Prediction of Oncotype DX and TAILORx risk categories using histopathological and immunohistochemical markers by classification and regression tree (CART) analysis

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ABSTRACT

Oncotype DX is an RT-PCR assay used to predict which patients with ER-positive node-negative (NN) disease will benefit from chemotherapy. Each patient is stratified into a risk category based on a recurrence score (RS) and the TAILORx trial is determining the benefit of chemotherapy for patients with mid-range RSs.

We tested if Oncotype DX and TAILORx risk categories could be predicted by standard pathological features and protein markers corresponding to 10 genes in the assay (ER, PR, Ki67, HER2, BCL2, CD68, Aurora A kinase, survivin, cyclin B1 and BAG1) on 52 patients who enrolled on TAILORx. Immunohistochemistry for the protein markers was performed on whole tissue sections.

Classification and regression tree (CART) analysis correctly classified 69% of cases into Oncotype DX risk categories based on the expression of PR, survivin and nuclear pleomorphism. All tumours with PR staining (Allred score $\geq$2) and marked nuclear pleomorphism were in the high-risk category. No case with PR $<$2, low survivin ($\leq$15.5%) and nuclear pleomorphism $<$3 was high-risk. Similarly, 77% of cases were correctly classified into TAILORx categories based on nuclear pleomorphism, survivin, BAG1 and cyclin B1. Ki67 was the only variable that predicted the absolute RS with a cut-off for positivity of 15% ($p = 0.003$).

In conclusion, CART revealed key predictors including proliferation markers, PR and nuclear pleomorphism that correctly classified over two thirds of ER-positive NN cancers into Oncotype DX and TAILORx risk categories. These variables could be used as an alternative to the RT-PCR assay to reduce the number of patients requiring Oncotype DX testing.

Introduction

A major challenge in the management of breast cancer is to determine the most appropriate treatment for early-stage hormone receptor-positive disease. Approximately 50% of patients present with node-negative (NN) ER-positive disease and 80% of these remain disease-free at 15 years after adequate loco-regional treatment and tamoxifen.[1] Chemotherapy can reduce recurrence risk by an additional 2–10%.[2] Currently, we rely heavily upon traditional predictive tools to decide whether these patients are offered chemotherapy or not and consequently many women with early-stage disease are over-treated.

Oncotype DX is a reverse transcriptase polymerase chain reaction (RT-PCR) assay that predicts the likelihood of 10-year recurrence in tamoxifen-treated, ER-positive, NN breast cancer patients.[3,4] It analyses the expression of 16 genes from formalin-fixed paraffin-embedded tissue and generates a recurrence score (RS) corresponding to the recurrence risk. Patients are classified as low- (RS $<$ 18), intermediate- (RS 18–30) or high-risk (RS $>$ 30) with a risk of distant recurrence of 6.8%, 14.3% and 30.5% respectively. Those in the high-risk category benefit from chemotherapy and those in the low-risk group do not, while the advantage of chemotherapy to those in the intermediate category is uncertain.[4] The Trial Assigning IndividuaLised Options for Treatment (TAILORx) trial was established in 2006 to evaluate the ability of the RS

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to guide therapeutic decisions in patients with an intermediate RS. TAILORx defined risk categories slightly differently from the original Oncotype DX criteria (low-risk, RS < 11; intermediate-risk, RS 11–25; high-risk, RS > 25). In TAILORx, patients with RSs of 11–25 were randomised to treatment because a RS of 11 is associated with a risk of local and distant relapse of 10% and this threshold is used for recommending adjuvant chemotherapy.[5]

Oncotype DX costs approximately $3800 per assay and its routine use would have major cost implications for health services. The assay is incorporated into the National Comprehensive Cancer Network management guidelines[6] and oncologists refer patients with equivocal clinicopathological variables for testing.[7] In a recent study, the assay stratified 40% and 66% of cases to intermediate-risk category by Oncotype Dx and TAILORx respectively[8] and it is unclear if any useful information is provided by the assay in these cases. TAILORx results are expected in 2015 and should provide data to guide treatment of these patients.

The 16 genes in the Oncotype DX assay are related to critical cancer properties: oestrogen activity (ER, PR, BCL2, SCUBE2), HER2 activation (HER2, GRB7), proliferation (Ki67, Aurora A kinase, survivin, cyclin B1, MYBL2), invasion (MMP11, CTSL2) and others (GSTM1, CD68, BCL2-associated athanogene 1 (BAG1)). ER, PR, and HER2 are examined routinely by immunohistochemistry (IHC) and fluorescent in situ hybridisation (HER2) and robust antibodies are available for BCL2 and Ki67. Surrogates for proliferation (mitotic activity, tumour grade) are also routinely examined. Consequently, many have investigated if combinations of these variables can predict the RS.[9–13] The aim of our study was to investigate if Oncotype DX and TAILORx risk categories could be predicted by a combination of clinicopathological parameters and protein markers. This pilot project, to the best of our knowledge, is the first to test cyclin B1, Aurora A kinase, survivin, BAG1 and CD68 in parallel with more routine variables (ER, PR, HER2, BCL2, Ki67) as predictors of the RS.

Methods

Case selection

Fifty-two of the 72 patients enrolled onto TAILORx between December 2007 and October 2009 at our institute were eligible for this study. Cases were excluded because a representative tumour block or slides were unavailable ($n = 17$) or consent was withdrawn ($n = 3$). Patients ranged from 32 to 73 years of age (mean 58 years). Tumour type, grade, size, presence of lymphovascular invasion (LVI) were recorded from pathology reports. Oncotype DX RS, ER, PR and HER2 scores (where available) were recorded from the Oncotype DX report from Genomic Health (GH).

Immunohistochemistry

A representative tumour block was selected from each case. Where possible, the block for Oncotype DX analysis was used ($n = 34$). Whole tissue sections (WTSs) were cut at 3 μm onto glass and SuperFrost® Plus slides (Thermo Scientific) for IHC. Immunostaining protocols are summarised in Table 1. A standard diaminobenzidine method was used to detect the reactions and sections were counterstained with haematoxylin.

Nuclear staining for ER and PR was evaluated by the Allred method (0–8).[14] ER and PR were also recorded as positive (Allred score >2) and negative (Allred scores of 0 or 2). Membranous staining for HER2 was evaluated by the Dako Herceptest protocol. For BCL2, the percentage of cells with cytoplasmic staining was scored in a minimum of 200 cells in 3 high-power fields (hpfs). The percentage of cells with nuclear staining for Ki67 in a minimum of 300 cells was counted across the tumour section including "hot spots" of positivity. Cytoplasmic staining for CD68 was evaluated in tumour cells and tumour-associated macrophages (TAMs) (Fig. 1a). For tumour cells, the percentage of positive cells was scored and the average percentage of TAMs in 3 hpfs was recorded.

The percentage of cells with nuclear staining for BAG1 and survivin was scored in tumour and benign tissue (Fig. 1b and c). Intensity of cytoplasmic staining (0–3) was also recorded. The percentage of tumour cells showing either cytoplasmic or nuclear staining for cyclin B1 was recorded (Fig. 1d). Staining was predominantly cytoplasmic and six cases showed either associated nuclear or only nuclear staining. Cyclin B1 staining was not seen in normal breast tissue. For Aurora A kinase, the percentage of tumour cells showing staining (nuclear or cytoplasmic) was recorded (Fig. 1e). A minimum of 200 cells in 3 hpfs were counted for survivin, BAG1, cyclin B1 and Aurora A kinase.

Twenty percent of cases stained with routine antibodies were double-scored (HI and GC) and concordance was excellent (ER, PR, HER2, BCL2, CD 68, 100%; Ki67, 94%). All novel antibodies were double scored (HI and MW). Discrepant cases were reviewed by both assessors and a final score agreed.

Statistics and data analysis

Classification and regression tree (CART) analysis was used to examine if combinations of variables could predict the risk

Table 1
Antibodies: sources and optimisation protocols.

Antibody	Source	Clone	Dilution	Antigen retrieval	Incubation
ER	Neomarkers	SP1	1:100	Automated; as per Benchmark® XT CC1[a] protocol for 30 min	Automated
PR	Leica	16	1:200	Automated; as per Benchmark® XT CC1[a] protocol for 30 min	Automated
Ki67	Dako	MIB-1	1:200	Automated; as per Benchmark® XT CC1[a] protocol for 30 min	Automated
HER2	Dako		Neat (pre-filled)	Automated; as per HercepTest™ program	Automated
BCL2	Novocastra	bcl-2/100/D5	1:200	Automated; as per Leica BOND-MAX™ ER2[b] protocol for 20 min	Automated
CD68	Dako	EBM11	1:500	Automated; as per Leica BOND-MAX™ ER1 protocol for 10 min	Automated
BAG 1	Santa Cruz	3.10G3E2	1:500	MW for 10 min, HIER buffer pH9	1hr.; RT
Survivin	Abcam	Polyclonal	1:1000	MW for 15 min, HIER buffer pH6	Overnight; RT
Cyclin B1	Abcam	Polyclonal	1:500	MW for 15 min, HIER buffer pH9	Overnight; RT
Aurora A Kinase	Novocastra	JLM28	1:100	MW for 20 min, HIER buffer pH9	1 h; RT

Abbreviations: CC: cell conditioning; ER: epitope retrieval; HIER: heat-induced epitope retrieval; MW: microwave; RT: room temperature.
[a] Universal buffer pH8.4, contains EDTA.
[b] Citrate-based buffer pH6.