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[Continued on next page]

(54) Title: A METHOD OF PREDICTING RISK OF RECURRENCE OF CANCER

Figure 1A

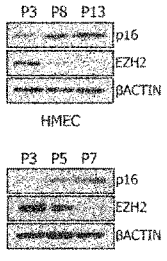


Figure 1B

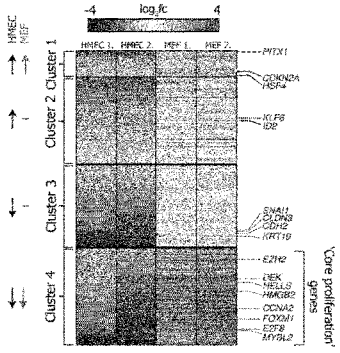


Figure 1C

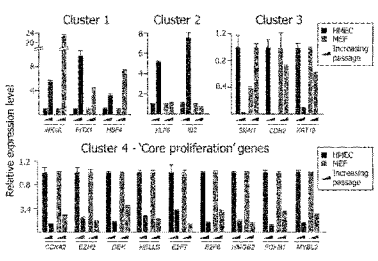
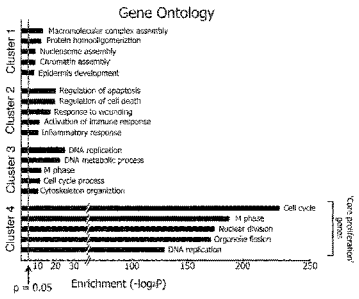


Figure 1D



(57) Abstract: A method for predicting risk of recurrence of cancer in an individual with cancer, the method comprising a step of assaying a cancer sample from the individual for positive expression of at least two genes or proteins encoded by those genes selected from the group consisting of FOXM1, UHRF1, PTTG1, E2F1, MYBL2, HMGB2, ATAD2, E2F8, ZNF367 and TCF19, wherein positive expression of the at least two genes correlates with increased risk of recurrence of cancer compared with an individual who does not exhibit positive expression of the at least two genes or proteins encoded by those genes.

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Title

A method of predicting risk of recurrence of cancer

Field of the Invention

The invention relates to a method of predicting the risk of tumour recurrence in a subject. Specifically, the invention relates to a method of predicting the risk of early-stage node-negative breast cancer, prostate cancer and other tumour recurrence.

Background to the Invention

Breast cancer is a heterogeneous disease which presents challenges for clinicians in predicting the likelihood of disease progression, particularly in patients where the disease is detected in the early stages. For these women, the conventional clinico-pathological parameters (tumour size, lymph node status, patient age, tumour grade, and expression of biomarkers including Estrogen Receptor (ER), Progesterone Receptor (PR), Human Epidermal growth factor Receptor 2 (Her2), Ki67) are not sufficient to characterise disease complexity and accurately predict the likelihood of tumour recurrence following adjuvant treatment or tumour removal by surgery. Therefore, due to inaccurate risk stratification, many of these patients who are inherently at a low risk of recurrence are assigned to receive chemotherapy, when in fact the majority of these women would remain cancer-free even without this toxic treatment.

In fact, it is estimated that, for node-negative, ER-positive disease, up to 85% of patients would be overtreated if given chemotherapy (Fisher *et al.*, 2004). Furthermore, surviving patients treated with chemotherapy face a higher risk of developing a second, independent, primary cancer in unrelated tissues within their lifetime (Boffetta and Kaldor, 1994). Considering the severe side-effects, the public health burden and the future health implications of chemotherapy, the overtreatment of patients represents a major problem in the clinical management of early-stage breast cancer.

The challenge is to develop a method of accurately and reproducibly distinguishing the low-risk from the high-risk patients so that therapy can be assigned accordingly. Current guidelines often lead to differing opinions from breast oncologists as to whether to assign neoadjuvant and/or adjuvant therapy, as many are reluctant to forego neoadjuvant and/or adjuvant therapy without a reliable assessment of recurrence risk. The addition of more accurate and reliable prognostic and predictive biomarkers to the standard clinical assessment would greatly improve the ability of both doctors and patients to make more well-informed treatment decisions. Some progress is being made in this regard with the multigene assays Oncotype Dx® Breast Cancer Assay and

MammaPrint™, which are currently being assessed in the Trial Assigning IndividuaLized Options for Treatment (Rx) (TAILORx) and Microarray In Node-negative and 1 to 3 positive lymph node Disease may Avoid ChemoTherapy (MINDACT) trials, respectively (Cardoso *et al.*, 2008; Sparano, 2006). MammaPrint™ and Prosigna™ are examples of Food and Drug Agency-approved prognostic tests in this arena.

WO 2005/039382 describes a number of gene sets used in predicting the likelihood of breast cancer recurrence, otherwise known as Oncotype Dx® referred to above. The invention is related to a gene set comprising 'one or more' genes from a panel of 50 genes. WO 2104/130825 describes a gene set comprising least 4 genes from a panel of cell cycle genes for detecting risk of lung cancer. US 7914988 describes a gene expression signature to predict relapse in prostate cancer, known as the GEX score. The invention is related to a gene set comprising 'all or a sub-combination of' genes from a panel of 21 genes.

The widespread use of gene expression profiling has led to a rapid expansion in the identification of gene expression signatures found to correlate with different aspects of tumour progression. These include the 'poor prognosis' (van de Vijver *et al.*, 2002; Wang *et al.*, 2005), 'invasiveness' (Liu *et al.*, 2007), and 'genomic grade' (Sotiriou *et al.*, 2006) signatures. US 2008/275652 describes how this genomic grade signature comprises at least 2 or 4 genes selected from a panel of 97 genes. However, despite the ability of these signatures to predict breast cancer prognosis, there is surprisingly little overlap between signatures. The Applicants suggest that many genes in these signatures may be 'passengers', rather than 'drivers' of tumour progression. Recent advances in genome-wide reverse engineering have made it possible to successfully identify regulatory interactions between transcription factors and downstream genes which were causal rather than correlative (Carro *et al.*, 2010). One such algorithm, the Algorithm for the Reconstruction of Accurate Cellular Networks (ARACNe) (Margolin *et al.*, 2006), uses gene interaction networks constructed from transcriptomic datasets to identify 'hubs', usually transcription factors, which are predicted to directly regulate multiple genes in the signature.

It is an object of the present invention to overcome at least one of the above-mentioned problems.

Summary of the Invention

Predicting the risk of tumour recurrence, and thus the need for adjuvant therapy, for lymph node negative breast cancer patients (and early stage, node positive breast cancer) can be a significant

problem for clinicians and patients. A 'core proliferation signature' has been identified herein which is consistently high in proliferating primary cultures, and is downregulated during cellular senescence. This gene signature is also highly expressed in aggressive breast cancers. A hierarchy of several Master Transcriptional Regulators (MTRs – transcription factors responsible for the regulation of this core set of genes) upstream of these core proliferation genes has been identified. Further analysis of the expression of these factors in breast cancer datasets at the mRNA and protein levels reveals a remarkable ability to predict recurrence risk for early-stage breast cancer. Strikingly, combining two of these factors outperforms the currently used clinical biomarkers for breast cancer recurrence risk, as well as recently developed multi-gene prognostic assays such as Oncotype Dx®. The addition of the senescence regulator $p16^{INK4A}$ to the prognostic panel of proliferative factors allows the identification of tumours with a disrupted cellular senescence pathway, further improving the prognostic power of the invention. Furthermore, unbiased survival analysis of several breast cancer datasets has revealed genes involved in alternative breast cancer-associated pathways such as apoptosis-resistance, invasion and immune response, which can be combined with the MTR panel to increase the prognostic power even further. This approach devised by the Applicant has succeeded in identifying 'drivers' of cancer proliferation which, when combined with additional biomarkers, has the potential to become a superior prognostic assay for early-stage cancer. Thus, by identifying the upstream 'drivers' or regulators of key signatures, more accurate and reliable predictors of breast cancer prognosis can be identified. The Applicant has called this 'core proliferation signature' OncoMasTR, and this name will be used herein.

According to the invention, there is provided a method for predicting risk of recurrence of cancer in an individual with cancer, the method comprising a step of assaying a cancer sample from the individual for positive expression of at least two genes (or proteins encoded by those genes) selected from the group consisting of FOXM1, UHRF1, PTTG1, E2F1, MYBL2, HMGB2, ATAD2, E2F8, ZNF367 and TCF19, wherein positive expression of at least two genes, or proteins encoded by said genes, correlates with increased risk of recurrence of cancer compared with an individual with cancer who does not exhibit positive expression of the same genes.

According to the invention, there is provided a method of predicting risk of recurrence of cancer in an individual with cancer following treatment with CDK4/6 inhibitors, the method comprising a step of assaying a cancer sample from the individual for positive expression of at least two genes, or proteins encoded by said genes, selected from the group consisting of FOXM1, UHRF1, PTTG1, E2F1, MYBL2,HMGB2, ATAD2, E2F8, ZNF367 and TCF19,

wherein positive expression of the at least two genes, or proteins encoded by said genes, correlates with increased risk of recurrence of cancer in an individual with cancer following treatment with CDK4/6 inhibitors compared with an individual with cancer who does not exhibit positive expression of the at least two genes or proteins encoded by those genes.

According to the invention, there is provided a method of determining a 5-year survival rate or a 10-year survival rate of an individual diagnosed with breast cancer, the method comprising a step of assaying a cancer tumour sample from the individual for positive expression of at least two genes, or proteins encoded by those genes, selected from FOXM1, UHRF1, PTTG1, E2F1, MYBL2, HMGB2, ATAD2, E2F8, ZNF367 and TCF19, wherein positive expression of the at least two genes, or proteins encoded by those genes, correlates with decreased chance of 5-year survival rate or a 10-year survival rate compared with an individual with cancer who does not exhibit positive expression of the at least two genes or proteins encoded by those genes.

In one embodiment, the method further comprises the step of assaying for the expression of the $p16^{INK4A}$ gene or protein in addition to the at least two genes (or proteins) selected from the group consisting of FOXM1, UHRF1, PTTG1, E2F1, MYBL2, HMGB2, ATAD2, E2F8, ZNF367 and TCF19, wherein dysregulated expression of $p16^{INK4A}$ in combination with positive expression of the at least two genes (or proteins encoded by those genes) selected from the group consisting of FOXM1, UHRF1, PTTG1, E2F1, MYBL2, HMGB2, ATAD2, E2F8, ZNF367 and TCF19, correlates with increased risk of recurrence of cancer, or a decreased chance of a 5-year survival rate or a 10-year survival rate, compared with an individual with cancer who does not exhibit dysregulated expression of $p16^{INK4A}$ and positive expression of the at least two genes (or proteins encoded by those genes). Breast cancer patients with dysregulated expression of $p16^{INK4A}$ and positive expression of the at least two genes (or proteins encoded by those genes) have an increased risk of recurrence of cancer, or a decreased chance of a 5-year survival rate or a 10-year survival rate, compared with patients with cancer that do not exhibit the expression pattern of this combination of genes (or proteins encoded by those genes).

In one embodiment, the at least two genes selected are FOXM1 and UHRF1. In one embodiment, the at least two genes selected are FOXM1 and PTTG1. In one embodiment, the at least two genes selected are FOXM1 and E2F1. In one embodiment, the at least two genes selected are FOXM1 and MYBL2. In one embodiment, the at least two genes selected are FOXM1 and HMGB2. In one embodiment, the at least two genes selected are UHRF1 and PTTG1. In one embodiment, the at least two genes selected are UHRF1 and E2F1. In one

embodiment, the at least two genes selected are UHRF1 and MYBL2. In one embodiment, the at least two genes selected are UHRF1 and HMGB2. In one embodiment, the at least two genes selected are PTTG1 and E2F1. In one embodiment, the at least two genes selected are PTTG1 and MYBL2. In one embodiment, the at least two genes selected are PTTG1 and HMGB2. In one embodiment, the at least two genes selected are E2F1 and MYBL2. In one embodiment, the at least two genes selected are E2F1 and HMGB2. In one embodiment, the at least two genes selected are MYBL2 and HMGB2. In one embodiment, the at least two genes selected are FOXM1 and ATAD2. In one embodiment, the at least two genes selected are FOXM1 and E2F8. In one embodiment, the at least two genes selected are FOXM1 and ZNF367. In one embodiment, the at least two genes selected are FOXM1 and TCF19. In one embodiment, the at least two genes selected are UHRF1 and ATAD2. In one embodiment, the at least two genes selected are UHRF1 and E2F8. In one embodiment, the at least two genes selected are UHRF1 and ZNF367. In one embodiment, the at least two genes selected are UHRF1 and TCF19. In one embodiment, the at least two genes selected are PTTG1 and ATAD2. In one embodiment, the at least two genes selected are PTTG1 and E2F8. In one embodiment, the at least two genes selected are PTTG1 and ZNF367. In one embodiment, the at least two genes selected are PTTG1 and TCF19. In one embodiment, the at least two genes selected are E2F1 and ATAD2. In one embodiment, the at least two genes selected are E2F1 and E2F8. In one embodiment, the at least two genes selected are E2F1 and ZNF367. In one embodiment, the at least two genes selected are E2F1 and TCF19. In one embodiment, the at least two genes selected are MYBL2 and ATAD2. In one embodiment, the at least two genes selected are MYBL2 and E2F8. In one embodiment, the at least two genes selected are MYBL2 and ZNF367. In one embodiment, the at least two genes selected are MYBL2 and TCF19. In one embodiment, the at least two genes selected are HMGB2 and ATAD2. In one embodiment, the at least two genes selected are HMGB2 and E2F8. In one embodiment, the at least two genes selected are HMGB2 and ZNF367. In one embodiment, the at least two genes selected are HMGB2 and TCF19. In one embodiment, the at least two genes selected are E2F8 and ATAD2. In one embodiment, the at least two genes selected are E2F8 and TCF19. In one embodiment, the at least two genes selected are E2F8 and ZNF367. In one embodiment, the at least two genes selected are ZNF367 and ATAD2. In one embodiment, the at least two genes selected are ZNF367 and TCF19. In one embodiment, the at least two genes selected are TCF19 and ATAD2. Preferably, the at least two genes selected above are combined with $p16^{INK4A}$.

In one embodiment, at least three genes are selected and the genes selected are FOXM1, UHRF1 and PTTG1. In one embodiment, the genes selected are FOXM1, UHRF1 and E2F1. In one embodiment, the genes selected are FOXM1, UHRF1 and MYBL2. In one embodiment,

the genes selected are FOXM1, UHRF1 and HMGB2. In one embodiment, the genes selected are FOXM1, PTTG1 and E2F1. In one embodiment, the genes selected are FOXM1, PTTG1 and MYBL2. In one embodiment, the genes selected are FOXM1, PTTG1 and HMGB2. In one embodiment, the genes selected are FOXM1, E2F1 and MYBL2. In one embodiment, the genes selected are FOXM1, E2F1 and HMGB2. In one embodiment, the genes selected are FOXM1, MYBL2 and HMGB2. In one embodiment, the genes selected are UHRF1, PTTG1 and E2F1. In one embodiment, the genes selected are UHRF1, PTTG1 and MYBL2. In one embodiment, the genes selected are UHRF1, PTTG1 and HMGB2. In one embodiment, the genes selected are PTTG1, E2F1 and MYBL2. In one embodiment, the genes selected are PTTG1, E2F1 and HMGB2. In one embodiment, the genes selected are E2F1, MYBL2 and HMGB2. In one embodiment, the genes selected are FOXM1, UHRF1 and ATAD2. In one embodiment, the genes selected are FOXM1, UHRF1 and E2F8. In one embodiment, the genes selected are FOXM1, UHRF1 and ZNF67. In one embodiment, the genes selected are FOXM1, UHRF1 and TCF19. In one embodiment, the genes selected are FOXM1, PTTG1 and ATAD2. In one embodiment, the genes selected are FOXM1, PTTG1 and E2F8. In one embodiment, the genes selected are FOXM1, PTTG1 and ZNF367. In one embodiment, the genes selected are FOXM1, PTTG1 and TCF19. In one embodiment, the genes selected are FOXM1, E2F1 and ATAD2. In one embodiment, the genes selected are FOXM1, E2F1 and E2F8. In one embodiment, the genes selected are FOXM1, E2F1 and ZNF367. In one embodiment, the genes selected are FOXM1, E2F1 and TCF19. In one embodiment, the genes selected are FOXM1, MYBL2 and ATAD2. In one embodiment, the genes selected are FOXM1, MYBL2 and E2F8. In one embodiment, the genes selected are FOXM1, MYBL2 and ZNF367. In one embodiment, the genes selected are FOXM1, MYBL2 and TCF19. In one embodiment, the genes selected are UHRF1, PTTG1 and ATAD2. In one embodiment, the genes selected are UHRF1, PTTG1 and E2F8. In one embodiment, the genes selected are UHRF1, PTTG1 and ZNF367. In one embodiment, the genes selected are UHRF1, PTTG1 and TCF19. In one embodiment, the genes selected are PTTG1, E2F1 and ATAD2. In one embodiment, the genes selected are PTTG1, E2F1 and E2F8. In one embodiment, the genes selected are PTTG1, E2F1 and ZNF367. In one embodiment, the genes selected are PTTG1, E2F1 and TCF19. In one embodiment, the genes selected are E2F1, MYBL2 and ATAD2. In one embodiment, the genes selected are E2F1, MYBL2 and E2F8. In one embodiment, the genes selected are E2F1, MYBL2 and ZNF367. In one embodiment, the genes selected are E2F1, MYBL2 and TCF19. In one embodiment, the genes selected are FOXM1, HMGB2 and ATAD2. In one embodiment, the genes selected are FOXM1, HMGB2 and E2F8. In one embodiment, the genes selected are FOXM1, HMGB2 and ZNF67. In one embodiment, the genes selected are FOXM1, HMGB2 and TCF19. In one embodiment, the genes selected are HMGB2, PTTG1 and ATAD2. In one

embodiment, the genes selected are HMGB2, PTTG1 and E2F8. In one embodiment, the genes selected are HMGB2, PTTG1 and ZNF367. In one embodiment, the genes selected are HMGB2, PTTG1 and TCF19. In one embodiment, the genes selected are HMGB2, E2F1 and ATAD2. In one embodiment, the genes selected are HMGB2, E2F1 and E2F8. In one embodiment, the genes selected are HMGB2, E2F1 and ZNF367. In one embodiment, the genes selected are HMGB2, E2F1 and TCF19. In one embodiment, the genes selected are HMGB2, MYBL2 and ATAD2. In one embodiment, the genes selected are HMGB2, MYBL2 and E2F8. In one embodiment, the genes selected are HMGB2, MYBL2 and ZNF367. In one embodiment, the genes selected are HMGB2, MYBL2 and TCF19. In one embodiment, the genes selected are UHRF1, HMGB2 and ATAD2. In one embodiment, the genes selected are UHRF1, HMGB2 and E2F8. In one embodiment, the genes selected are UHRF1, HMGB2 and ZNF367. In one embodiment, the genes selected are UHRF1, HMGB2 and TCF19. In one embodiment, the genes selected are E2F8, ZNF367 and ATAD2. In one embodiment, the genes selected are E2F8, ZNF367 and TCF19. In one embodiment, the genes selected are ATAD2, E2F8 and TCF19. Preferably, the at least three genes selected above are combined with $p16^{INK4A}$.

In one embodiment, at least four genes are selected and the genes selected are FOXM1, UHRF1, PTTG1 and E2F1. In one embodiment, the genes selected are FOXM1, UHRF1, PTTG1 and MYBL2. In one embodiment, the genes selected are FOXM1, UHRF1, PTTG1 and HMGB2. In one embodiment, the genes selected are FOXM1, UHRF1, E2F1 and MYBL2. In one embodiment, the genes selected are FOXM1, UHRF1, E2F1 and HMGB2. In one embodiment, the genes selected are FOXM1, UHRF1, MYBL2 and HMGB2. In one embodiment, the genes selected are FOXM1, PTTG1, E2F1 and MYBL2. In one embodiment, the genes selected are FOXM1, PTTG1, E2F1 and HMGB2. In one embodiment, the genes selected are FOXM1, E2F1, MYBL2 and HMGB2. In one embodiment, the genes selected are UHRF1, PTTG1, E2F1 and MYBL2. In one embodiment, the genes selected are UHRF1, PTTG1, E2F1 and HMGB2. In one embodiment, the genes selected are PTTG1, E2F1, MYBL2 and HMGB2. In one embodiment, the genes selected are FOXM1, UHRF1, PTTG1 and ATAD2. In one embodiment, the genes selected are FOXM1, UHRF1, PTTG1 and E2F8. In one embodiment, the genes selected are FOXM1, UHRF1, PTTG1 and ZNF367. In one embodiment, the genes selected are FOXM1, UHRF1, PTTG1 and TCF19. In one embodiment, the genes selected are FOXM1, UHRF1, E2F1 and ATAD2. In one embodiment, the genes selected are FOXM1, UHRF1, E2F1 and E2F8. In one embodiment, the genes selected are FOXM1, UHRF1, E2F1 and ZNF367. In one embodiment, the genes selected are FOXM1, UHRF1, E2F1 and TCF19. In one embodiment, the genes selected are FOXM1, UHRF1,

MYBL2 and ATAD2. In one embodiment, the genes selected are FOXM1, UHRF1, MYBL2 and E2F8. In one embodiment, the genes selected are FOXM1, UHRF1, MYBL2 and ZNF367. In one embodiment, the genes selected are FOXM1, UHRF1, MYBL2 and TCD1. In one embodiment, the genes selected are FOXM1, UHRF1, HMGB2 and ATAD2. In one embodiment, the genes selected are FOXM1, UHRF1, HMGB2 and E2F8. In one embodiment, the genes selected are FOXM1, UHRF1, HMGB2 and ZNF37. In one embodiment, the genes selected are FOXM1, UHRF1, HMGB2 and TCF19. In one embodiment, the genes selected are FOXM1, PTTG1, E2F1 and ATAD2. In one embodiment, the genes selected are FOXM1, PTTG1, E2F1 and E2F8. In one embodiment, the genes selected are FOXM1, PTTG1, E2F1 and ZNF367. In one embodiment, the genes selected are FOXM1, PTTG1, E2F1 and TCF19. In one embodiment, the genes selected are FOXM1, PTTG1, MYBL2 and ATAD2. In one embodiment, the genes selected are FOXM1, PTTG1, MYBL2 and E2F8. In one embodiment, the genes selected are FOXM1, PTTG1, MYBL2 and ZNF367. In one embodiment, the genes selected are FOXM1, PTTG1, MYBL2 and TCF19. In one embodiment, the genes selected are FOXM1, PTTG1, HMGB2 and ATAD2. In one embodiment, the genes selected are FOXM1, PTTG1, HMGB2 and E2F8. In one embodiment, the genes selected are FOXM1, PTTG1, HMGB2 and ZNF367. In one embodiment, the genes selected are FOXM1, PTTG1, HMGB2 and TCF19. In one embodiment, the genes selected are FOXM1, E2F1, MYBL2 and ATAD2. In one embodiment, the genes selected are FOXM1, E2F1, MYBL2 and E2F8. In one embodiment, the genes selected are FOXM1, E2F1, MYBL2 and ZNF367. In one embodiment, the genes selected are FOXM1, E2F1, MYBL2 and TCF19. In one embodiment, the genes selected are FOXM1, E2F1, HMGB2 and ATAD2. In one embodiment, the genes selected are FOXM1, E2F1, HMGB2 and E2F8. In one embodiment, the genes selected are FOXM1, E2F1, HMGB2 and ZNF367. In one embodiment, the genes selected are FOXM1, E2F1, HMGB2 and TCF19. In one embodiment, the genes selected are FOXM1, MYBL2, HMGB2 and ATAD2. In one embodiment, the genes selected are FOXM1, MYBL2, HMGB2 and E2F8. In one embodiment, the genes selected are FOXM1, MYBL2, HMGB2 and ZNF367. In one embodiment, the genes selected are FOXM1, MYBL2, HMGB2 and TCF19. In one embodiment, the genes selected are UHRF1, PTTG1, E2F1 and ATAD2. In one embodiment, the genes selected are UHRF1, PTTG1, E2F1 and E2F8. In one embodiment, the genes selected are UHRF1, PTTG1, E2F1 and ZNF367. In one embodiment, the genes selected are UHRF1, PTTG1, E2F1 and TCF19. In one embodiment, the genes selected are UHRF1, PTTG1, MYBL2 and ATAD2. In one embodiment, the genes selected are UHRF1, PTTG1, MYBL2 and E2F8. In one embodiment, the genes selected are UHRF1, PTTG1, MYBL2 and ZNF36. In one embodiment, the genes selected are UHRF1, PTTG1, MYBL2 and TCF19. In one embodiment, the genes selected are UHRF1, PTTG1, HMGB2 and ATAD2. In one

embodiment, the genes selected are UHRF1, PTTG1, HMGB2 and E2F8. In one embodiment, the genes selected are UHRF1, PTTG1, HMGB2 and ZNF367. In one embodiment, the genes selected are UHRF1, PTTG1, HMGB2 and TCF19. In one embodiment, the genes selected are PTTG1, E2F1, MYBL2 and ATAD2. In one embodiment, the genes selected are PTTG1, E2F1, MYBL2 and E2F8. In one embodiment, the genes selected are PTTG1, E2F1, MYBL2 and ZNF367. In one embodiment, the genes selected are PTTG1, E2F1, MYBL2 and TCF19. In one embodiment, the genes selected are PTTG1, E2F1, HMGB2 and ATAD2. In one embodiment, the genes selected are PTTG1, E2F1, HMGB2 and E2F8. In one embodiment, the genes selected are PTTG1, E2F1, HMGB2 and ZNF367. In one embodiment, the genes selected are PTTG1, E2F1, HMGB2 and TCF19. In one embodiment, the genes selected are E2F1, MYBL2, HMGB2 and ATAD2. In one embodiment, the genes selected are E2F1, MYBL2, HMGB2 and E2F8. In one embodiment, the genes selected are E2F1, MYBL2, HMGB2 and ZNF367. In one embodiment, the genes selected are E2F1, MYBL2, HMGB2 and TCF19. In one embodiment, the genes selected are ATAD2, EDF8, ZNF367 and TCF19. Preferably, the at least four genes selected above are combined with $p16^{INK4A}$.

In one embodiment, at least five genes are selected and the genes selected are FOXM1, UHRF1, PTTG1, E2F1 and MYBL2. In one embodiment, the genes selected are FOXM1, UHRF1, PTTG1, E2F1 and HMGB2. In one embodiment, the genes selected are FOXM1, PTTG1, E2F1, MYBL2 and HMGB2. In one embodiment, the genes selected are UHRF1, PTTG1, E2F1, MYBL2 and HMGB2. In one embodiment, the genes selected are FOXM1, UHRF1, PTTG1, E2F1 and ATAD2. In one embodiment, the genes selected are FOXM1, UHRF1, PTTG1, E2F1 and E2F8. In one embodiment, the genes selected are FOXM1, UHRF1, PTTG1, E2F1 and ZNF367. In one embodiment, the genes selected are FOXM1, UHRF1, PTTG1, E2F1 and TCF19. In one embodiment, the genes selected are FOXM1, UHRF1, PTTG1, MYBL2 and ATAD2. In one embodiment, the genes selected are FOXM1, UHRF1, PTTG1, MYBL2 and EFF8. In one embodiment, the genes selected are FOXM1, UHRF1, PTTG1, MYBL2 and ZNF367. In one embodiment, the genes selected are FOXM1, UHRF1, PTTG1, MYBL2 and TCF19. In one embodiment, the genes selected are FOXM1, UHRF1, PTTG1, HMGB2 and ATAD2. In one embodiment, the genes selected are FOXM1, UHRF1, PTTG1, HMGB2 and E2F8. In one embodiment, the genes selected are FOXM1, UHRF1, PTTG1, HMGB2 and ZNF367. In one embodiment, the genes selected are FOXM1, UHRF1, PTTG1, HMGB2 and TCF19. In one embodiment, the genes selected are UHRF1, PTTG1, E2F1, MYBL2 and ATAD2. In one embodiment, the genes selected are UHRF1, PTTG1, E2F1, MYBL2 and E2F8. In one embodiment, the genes selected are UHRF1, PTTG1, E2F1, MYBL2 and ZNF367. In one embodiment, the genes selected are UHRF1, PTTG1, E2F1,

MYBL2 and TCF19. In one embodiment, the genes selected are UHRF1, PTTG1, E2F1, HMBG2 and ATAD2. In one embodiment, the genes selected are UHRF1, PTTG1, E2F1, HMBG2 and E2F8. In one embodiment, the genes selected are UHRF1, PTTG1, E2F1, HMBG2 and ZNF367. In one embodiment, the genes selected are UHRF1, PTTG1, E2F1, HMBG2 and TCF19. In one embodiment, the genes selected are PTTG1, E2F1, MYBL2, HMGB2 and ATAD2. In one embodiment, the genes selected are PTTG1, E2F1, MYBL2, HMGB2 and E2F8. In one embodiment, the genes selected are PTTG1, E2F1, MYBL2, HMGB2 and ZNF367. In one embodiment, the genes selected are PTTG1, E2F1, MYBL2, HMGB2 and TCF19. In one embodiment, the genes selected are ATAD2, E2F8, ZNF367, TCF19 and FOXM1. In one embodiment, the genes selected are ATAD2, E2F8, ZNF367, TCF19 and UHRF1. In one embodiment, the genes selected are ATAD2, E2F8, ZNF367, TCF19 and PTTG1. In one embodiment, the genes selected are ATAD2, E2F8, ZNF367, TCF19 and E2F1. In one embodiment, the genes selected are ATAD2, E2F8, ZNF367, TCF19 and MYBL2. In one embodiment, the genes selected are ATAD2, E2F8, ZNF367, TCF19 and HMGB2. Preferably, the at least five genes selected above are combined with $p16^{INK4A}$.

In one embodiment, the at least two genes comprise FOXM1, and at least one further gene selected from UHRF1, PTTG1, E2F1, MYBL2, HMGB2, ATAD2, E2F8, ZNF367 and TCF19. Preferably, the at least two genes is further combined with $p16^{INK4A}$.

In one embodiment, the at least two genes comprise UHRF1, and at least one further gene selected from FOXM1, PTTG1, E2F1, MYBL2, HMGB2, ATAD2, E2F8, ZNF367 and TCF19. Preferably, the at least two genes is further combined with $p16^{INK4A}$.

In one embodiment, the at least two genes comprise PTTG1, and at least one further gene selected from FOXM1, UHRF1, E2F1, MYBL2, HMGB2, ATAD2, E2F8, ZNF367 and TCF19. Preferably, the at least two genes is further combined with $p16^{INK4A}$.

In one embodiment, the at least two genes comprise E2F1, and at least one further gene selected from FOXM1, PTTG1, UHRF1, MYBL2, HMGB2, ATAD2, E2F8, ZNF367 and TCF19. Preferably, the at least two genes is further combined with $p16^{INK4A}$.

In one embodiment, the at least two genes comprise MYBL2, and at least one further gene selected from FOXM1, PTTG1, E2F1, UHRF1, HMGB2, ATAD2, E2F8, ZNF367 and TCF19. Preferably, the at least two genes is further combined with $p16^{INK4A}$.

In one embodiment, the at least two genes comprise HMGB2, and at least one further gene selected from FOXM1, PTTG1, E2F1, MYBL2, UHRF1, ATAD2, E2F8, ZNF367 and TCF19. Preferably, the at least two genes is further combined with $p16^{INK4A}$.

In one embodiment, the genes selected are FOXM1, UHRF1, PTTG1, E2F1, MYBL2 and HMGB2. Preferably, the genes selected are further combined with $p16^{INK4A}$.

In one embodiment, the genes selected are FOXM1, UHRF1, PTTG1, E2F1, MYBL2, HMGB2, and one or more or all of ATAD2, E2F8, ZNF367 and TCF19. Preferably, the genes selected are further combined with $p16^{INK4A}$.

In one embodiment, the genes selected consist essentially of FOXM1, UHRF1, PTTG1, E2F1, MYBL2, and HMGB2. Preferably, the genes are further combined with $p16^{INK4A}$. The term "consist essentially of" should be understood to mean all six genes, or five genes, or four genes, or three genes, or two genes selected from FOXM1, UHRF1, PTTG1, E2F1, MYBL2, and HMGB2.

In one embodiment, the cancer is selected from the group comprising node-negative, ER-positive breast cancer; early stage, node positive breast cancer; multiple myeloma, prostate cancer, glioblastoma, lymphoma, fibrosarcoma; myxosarcoma; liposarcoma; chondrosarcoma; osteogenic sarcoma; chordoma; angiosarcoma; endotheliosarcoma; lymphangiosarcoma; lymphangioendotheliosarcoma; synovioma; mesothelioma; Ewing's tumour; leiomyosarcoma; rhabdomyosarcoma; colon carcinoma; pancreatic cancer; breast cancer; ovarian cancer; squamous cell carcinoma; basal cell carcinoma; adenocarcinoma; sweat gland carcinoma; sebaceous gland carcinoma; papillary carcinoma; papillary adenocarcinomas; cystadenocarcinoma; medullary carcinoma; bronchogenic carcinoma; renal cell carcinoma; hepatoma; bile duct carcinoma; choriocarcinoma; seminoma; embryonal carcinoma; Wilms' tumour; cervical cancer; uterine cancer; testicular tumour; lung carcinoma; small cell lung carcinoma; bladder carcinoma; epithelial carcinoma; glioma; astrocytoma; medulloblastoma; craniopharyngioma; ependymoma; pinealoma; hemangioblastoma; acoustic neuroma; oligodendroglioma; meningioma; melanoma; retinoblastoma; and leukemias. Suitably, the cancer is an epithelial cancer.

In one embodiment, the cancer is preferably breast cancer or prostate cancer. Ideally, the breast cancer is early stage, typically node-negative breast cancer or early stage, node positive breast

cancer. Ideally, the breast cancer is early stage, node-negative or early stage, node positive, ER-positive breast cancer.

In one embodiment, the recurrence is development of a secondary tumour.

In one embodiment, the recurrence is developing a further, independent primary cancer unrelated to the sampled cancer.

In one embodiment of the invention, there is provided a method of predicting the risk of recurrence of breast cancer in an early stage, node-negative breast cancer patient, or an early stage, node positive breast cancer patient, the method comprising a step of assaying a cancer tumour sample from the breast cancer patient for positive expression of at least two genes (or proteins encoded by those genes) selected from the group consisting of FOXM1, UHRF1, PTTG1, E2F1, MYBL2, HMGB2, ATAD2, E2F8, ZNF367, and TCF19, wherein positive expression of the at least two genes (or proteins encoded by those genes) correlates with increased risk of recurrence of cancer compared with an individual with cancer who does not exhibit positive expression of the at least two genes (or proteins encoded by those genes).

In one embodiment, the method further comprises the step of assaying for the expression of the $p16^{INK4A}$ gene (or a protein encoded by said gene) in addition to the at least two genes (or proteins encoded by those genes) selected from the group consisting of FOXM1, UHRF1, PTTG1, E2F1, MYBL2, HMGB2, ATAD2, E2F8, ZNF367 and TCF19, wherein dysregulated expression of $p16^{INK4A}$ in combination with positive expression of a combination of the at least two of genes (or proteins encoded by those genes), correlates with increased risk of recurrence of cancer compared with an individual with cancer who does not exhibit dysregulated expression of $p16^{INK4A}$ and positive expression of the at least two genes (or proteins encoded by those genes). Breast cancer patients with dysregulated $p16^{INK4A}$ and positive expression of the at least two genes (or proteins encoded by those genes) have an increased risk of recurrence of cancer compared with individuals with cancer that do not exhibit the combination of positive expression of the at last two genes and dysregulated expression of $p16^{INK4A}$.

In one embodiment of the invention, there is provided a method of identifying a cancer patient that is suitable for treatment with a therapy for preventing recurrence or progression of the cancer, the method comprising a step of assaying a cancer sample from the cancer patient for positive expression of at least two genes (or proteins encoded by those genes) selected from the group consisting of FOXM1, UHRF1, PTTG1, E2F1, MYBL2, HMGB2, ATAD2, E2F8,

ZNF367 and TCF19, wherein positive expression of the at least two genes (or proteins encoded by those genes) compared with an individual with cancer who does not exhibit positive expression of the at least two genes (or proteins encoded by those genes), is indicative that the cancer patient is suitable for treatment with a therapy for preventing recurrence or progression of the cancer.

In one embodiment, the therapy is a neoadjuvant therapy. In the specification, the term "neoadjuvant therapy" should be understood to mean treatment given before primary treatment to increase the chances of long-term survival. Primary treatment is generally surgery. Neoadjuvant therapy are generally selected from chemotherapy, hormonal therapy, targeted therapy, radiation therapy, immunotherapy or a combination thereof.

In one embodiment, the therapy is an adjuvant therapy. In the specification, the term "adjuvant therapy" should be understood to mean any treatment given after primary treatment to increase the chances of long-term survival. Primary treatment is generally surgery. Adjuvant therapy are generally selected from chemotherapy, hormonal therapy, targeted therapy, radiation therapy, immunotherapy or a combination thereof.

In one embodiment, the therapy can be a combination of neoadjuvant and adjuvant therapy. It should be understood that in the specification, the "neoadjuvant" and "adjuvant" therapies can be used interchangeably.

In one embodiment, the method further comprises the step of assaying for the expression of the $p16^{INK4A}$ gene (or a protein encoded by said gene) in addition to the at least two genes (or proteins encoded by those genes) selected from the group consisting of FOXM1, UHRF1, PTTG1, E2F1, MYBL2, HMGB2, ATAD2, E2F8, ZNF367 and TCF19, wherein dysregulated expression of $p16^{INK4A}$ in combination with positive expression of a combination of at least two of the genes (or proteins encoded by those genes), when compared with an individual with cancer who does not exhibit dysregulated expression of $p16^{INK4A}$ and positive expression of the at least two genes, is indicative that the cancer patient is suitable for treatment with an adjuvant therapy for preventing recurrence or progression of the cancer. Breast cancer patients with dysregulated $p16^{INK4A}$ expression and positive expression of the at least two genes (or proteins encoded by those genes) may be suitable for treatment with an adjuvant therapy for preventing recurrence or progression of the cancer.

In one embodiment, the cancer patient may be suitable for treatment with a neoadjuvant therapy for preventing recurrence or progression of the cancer.

In one embodiment, the cancer is early stage, node-negative breast cancer or early stage, node positive breast cancer. Ideally, breast cancer is early stage, node-negative, ER-positive breast cancer or early stage, node positive, ER-positive breast cancer.

In one embodiment, the adjuvant therapy and neoadjuvant therapy is chemotherapeutic therapy. In one embodiment, the adjuvant therapy and neoadjuvant therapy is a CDK4/6 inhibitor therapy such as palbociclib therapy (PD 0332991, Pfizer), Abemaciclib (LY2835219; Lilly, USA), or LEE011 (Novartis, Switzerland).

In one embodiment of the invention, there is provided a system for obtaining data from at least one test sample obtained from at least one individual, the system comprising:

a determination module configured to receive at least one test sample and perform at least one test analysis on the test sample to assay for expression of at least two genes (or proteins encoded by those genes) selected from the group consisting of FOXM1, UHRF1, PTTG1, E2F1, MYBL2, HMGB2, ATAD2, E2F8, ZNF367 and TCF19;

optionally, a storage system for storing expression data generated by the determination module; and

a display module for displaying a content based in part on the data output from said determination module, wherein the content comprises a signal indicative of the expression of the at least two genes.

In one embodiment, the determination module is further configured to perform at least one test analysis on the test sample for dysregulation of $p16^{INK4A}$ in combination with the test analysis on the at least two genes (or proteins encoded by those genes).

In one embodiment, the system comprises a correlation module for correlating the expression data of the at least two genes (or proteins encoded by those genes) from the determination module with recurrence potential of cancer, wherein the expression data of each gene (or a protein encoded by the gene) is compared with a reference value for the gene (or a protein encoded by the gene) to determine positive expression of the gene (or a protein encoded by the gene), and wherein positive expression of the at least two genes (or proteins encoded by those genes) correlates with increased potential for recurrence compared with an individual with cancer who does not exhibit positive expression of the at least two genes (or proteins encoded by those genes), and wherein the display module displays a content based in part on the data

from the correlation system, the content optionally comprising a signal indicative of the recurrence potential of the cancer.

In one embodiment, the correlation module further correlates the expression data of the at least two genes (or proteins encoded by those genes) from the determination module with recurrence potential of cancer, together with the expression data of $p16^{INK4A}$, wherein the expression data of each gene (or a protein encoded by the gene) and $p16^{INK4A}$ is compared with a reference value for each gene (or a protein encoded by the gene) and $p16^{INK4A}$, respectively, to determine positive expression of the gene (or a protein encoded by the gene) and dysregulation of $p16^{INK4A}$, and wherein positive expression of the at least two genes (or proteins encoded by those genes) and dysregulation of $p16^{INK4A}$ correlates with increased potential for recurrence compared with an individual with cancer who does not exhibit positive expression of the at least two genes (or proteins encoded by those genes) and dysregulation of $p16^{INK4A}$, and wherein the display module displays a content based in part on the data from the correlation system, the content optionally comprising a signal indicative of the recurrence potential of the cancer.

Suitably, the determination system may be selected from an immunohistochemical detection apparatus, a Western Blot, a Northern Blot, a Southern Blot, quantitative polymerase chain reaction (PCR), reverse transcriptase PCR (RT-PCR), quantitative real time RT-PCR (qRT-PCR), an enzyme-linked immunosorbent assay (ELISA), protein determination on polyacrylamide gels, and such methods known to those skilled in the art. Ideally, the determination system comprises an immunohistochemical detection apparatus.

In one embodiment of the invention, the content based on the comparison result or the determination system is displayed on a computer monitor. In one embodiment of the invention, the content based on the comparison result or determination system is displayed through printable media. The display module can be any suitable device configured to receive from a computer and display computer readable information to a user. Non-limiting examples include, for example, general-purpose computers such as those based on Intel PENTIUM-type processor, Motorola PowerPC, Sun UltraSPARC, Hewlett-Packard PA-RISC processors, any of a variety of processors available from Advanced Micro Devices (AMD) of Sunnyvale, California, or any other type of processor, visual display devices such as flat panel displays, cathode ray tubes and the like, as well as computer printers of various types.

In one embodiment, a World Wide Web browser is used for providing a user interface for display of the content based on the comparison result. It should be understood that other modules of the invention can be adapted to have a web browser interface. Through the Web

browser, a user may construct requests for retrieving data from the comparison module. Thus, the user will typically point and click to user interface elements such as buttons, pull down menus, scroll bars and the like conventionally employed in graphical user interfaces.

In one embodiment of the invention, there is provided a method for monitoring the effectiveness of treatment of cancer in an individual with cancer, the method comprising a step of assaying a cancer sample from the individual with cancer for expression of at least two genes selected from the group consisting of FOXM1, UHRF1, PTTG1, E2F1, MYBL2, HMGB2, ATAD2, E2F8, ZNF367 and TCF19, wherein higher expression of at least two genes selected from the group consisting of FOXM1, UHRF1, PTTG1, E2F1, MYBL2, HMGB2, ATAD2, E2F8, ZNF367 and TCF19 correlates with ineffective treatment and poor outcome compared with an individual with cancer who has lower expression of the at least two genes.

In one embodiment, the method further comprises the step of assaying the cancer sample for expression of the $p16^{INK4A}$ gene (or a protein encoded by said gene) in combination with assaying the at least two genes (or proteins encoded by said genes), whereby dysregulated expression of $p16^{INK4A}$ correlates with ineffective treatment and poor outcome compared with an individual with cancer who has moderate expression of $p16^{INK4A}$.

In one embodiment of the invention, there is provided a method for treating cancer comprising the steps of:

identifying an individual with increased potential for recurrence of cancer by assaying a cancer sample from the individual for expression of at least two genes selected from the group consisting of FOXM1, UHRF1, PTTG1, E2F1, MYBL2, HMGB2, ATAD2, E2F8, ZNF367 and TCF19, wherein higher expression of at least two genes selected from the group consisting of FOXM1, UHRF1, PTTG1, E2F1, MYBL2, HMGB2, ATAD2, E2F8, ZNF367 and TCF19 correlates with increased potential for recurrence of cancer compared with an individual with cancer who has lower expression of the at least two genes; and

treating the individual with a therapeutically effective amount of an adjuvant therapy.

In one embodiment, the individual is treated with a therapeutically effective amount of a neoadjuvant therapy.

In one embodiment of the invention, there is provided a method for treating cancer comprising the steps of:

identifying an individual with increased potential for recurrence of cancer by assaying a cancer sample from the individual for expression of at least two genes selected from the group consisting of FOXM1, UHRF1, PTTG1, E2F1, MYBL2, HMGB2, ATAD2, E2F8, ZNF367 and TCF19, wherein higher expression of at least two genes selected from the group consisting of FOXM1, UHRF1, PTTG1, E2F1, MYBL2, HMGB2, ATAD2, E2F8, ZNF367 and TCF19 correlates with increased potential for recurrence of cancer compared with an individual with cancer who has lower expression of the at least two genes; and

treating the individual with a therapeutically effective amount of a neoadjuvant therapy.

In one embodiment, the individual is treated with a therapeutically effective amount of an adjuvant therapy.

In one embodiment, the method further comprises the step of assaying the cancer sample for expression of the $p16^{INK4A}$ gene (or a protein encoded by said gene) in combination with assaying the at least two genes (or proteins encoded by said genes), whereby dysregulated expression of $p16^{INK4A}$ correlates with potential for recurrence of cancer when compared with an individual with cancer who has moderate expression of $p16^{INK4}$.

In one embodiment, the neoadjuvant therapy and adjuvant therapy is an agent selected from, but not limited to, trastuzumab (Herceptin®), lapatinib (Tykerb®), neratinib, afatinib (Tovok®), pertuzumab, CDK4/6 inhibitors (such as palbociclib (PD 0332991, Pfizer), Abemaciclib (LY2835219; Lilly, USA), and LEE011 (Novartis, Switzerland)), cyclophosphamide, methotrexate, 5-fluorouracil, gemcitabine, adriamycin (doxorubicin), epirubucin, docetaxel (Taxotere®), paclitaxel (Taxol®), capecitabine (Xeloda®), and tamoxifen.

The invention also relates to a method of treating an individual to prevent or inhibit recurrence of the cancer comprising a step of identifying a cancer patient at risk of recurrence using a method of the invention, and then treating the cancer patient with an agent or agents to prevent or inhibit recurrence of the cancer. Typically, the agent or agents comprise adjuvant or neoadjuvant therapy, or a combination of both.

In one embodiment, there is provided a method of predicting risk of recurrence of cancer in an individual with cancer, the method comprising a step of assaying a cancer sample from the individual for positive expression of at least four genes, or proteins encoded by said genes, selected from FOXM1, UHRF1, PTTG1, E2F1, MYBL2, HMGB2, ATAD2, E2F8, ZNF367 and TCF19, wherein positive expression of the at least four genes, or proteins encoded by said

genes, correlates with increased risk of recurrence of cancer compared with an individual with cancer who does not exhibit positive expression of the at least four genes or proteins encoded by those genes.

In one embodiment, there is provided a method of predicting risk of recurrence of cancer in an individual with cancer following treatment with CDK4/6 inhibitors, the method comprising a step of assaying a cancer sample from the individual for positive expression of at least four genes, or proteins encoded by said genes, selected from FOXM1, UHRF1, PTTG1, E2F1, MYBL2, HMGB2, ATAD2, E2F8, ZNF367 and TCF19, wherein positive expression of the at least four genes correlates with increased risk of recurrence of cancer in an individual with cancer following treatment with CDK4/6 inhibitors compared with an individual with cancer who does not exhibit positive expression of the at least four genes or proteins encoded by those genes.

In one embodiment, there is provided a method of predicting risk of recurrence of breast cancer in an early stage, node negative breast cancer patient, the method comprising a step of assaying a cancer tumour sample from the patient for positive expression of at least four genes, or proteins encoded by those genes, selected from FOXM1, UHRF1, PTTG1, E2F1, MYBL2, HMGB2, ATAD2, E2F8, ZNF367 and TCF19, wherein positive expression of the at least four genes, or proteins encoded by those genes, correlates with increased risk of recurrence of cancer compared with a patient with cancer who does not exhibit positive expression of the at least four genes or proteins encoded by those genes.

In one embodiment, there is provided method of determining a 5-year survival rate or a 10-year survival rate of an individual diagnosed with breast cancer, the method comprising a step of assaying a cancer tumour sample from the individual for positive expression of at least four genes, or proteins encoded by those genes, selected from FOXM1, UHRF1, PTTG1, E2F1, MYBL2, HMGB2, ATAD2, E2F8, ZNF367 and TCF19, wherein positive expression of the at least four genes, or proteins encoded by those genes, correlates with decreased chance of 5-year survival rate or 10-year survival rate compared with an individual with cancer who does not exhibit positive expression of the at least four genes or proteins encoded by those genes.

In one embodiment, the methods further comprising the step of assaying for the expression of $p16^{INK4A}$ gene or a protein encoded by said gene, wherein dysregulated expression of $p16^{INK4A}$, in combination with positive expression of the at least four genes or proteins encoded by those genes, correlates with increased risk of recurrence of cancer or a decreased chance of 5-year

survival rate or 10-year survival rate compared with an individual with cancer who does not exhibit dysregulated expression of $p16^{INK4A}$ and positive expression of the at least four genes or proteins encoded by those genes.

In one embodiment, there is provided a method of identifying a cancer patient that is suitable for treatment with a therapy for preventing recurrence or progression of the cancer, the method comprising a step of assaying a cancer sample from the cancer patient for positive expression of at least four genes selected from FOXM1, UHRF1, PTTG1, E2F1, MYBL2, HMGB2, ATAD2, E2F8, ZNF367 and TCF19, wherein positive expression of the at least four genes or proteins encoded by those genes compared with an individual with cancer who does not exhibit positive expression of the at least two genes or proteins encoded by those genes, is indicative that the cancer patient is suitable for treatment with a therapy for preventing recurrence or progression of the cancer.

In one embodiment, there is provided a system for obtaining data from at least one test sample obtained from at least one individual, the system comprising a determination module configured to receive at least one test sample and perform at least one test analysis on the test sample to assay for expression of at least four genes or proteins encoded by those genes selected from FOXM1, UHRF1, PTTG1, E2F1, MYBL2, HMGB2, ATAD2, E2F8, ZNF367 and TCF19; optionally, a storage system for storing expression data generated by the determination module; and a display module for displaying a content based in part on the data output from said determination module, wherein the content comprises a signal indicative of the expression of at the least two genes or proteins encoded by those genes.

In one embodiment, there is provided a method for monitoring the effectiveness of treatment of cancer in an individual with cancer, the method comprising a step of assaying a cancer sample from the individual with cancer for expression of at least four genes or proteins encoded by said genes selected from FOXM1, UHRF1, PTTG1, E2F1, MYBL2 and HMGB2, wherein higher expression of at least four genes selected from FOXM1, UHRF1, PTTG1, E2F1, MYBL2, HMGB2, ATAD2, E2F8, ZNF367 and TCF19 correlates with ineffective treatment and poor outcome compared with an individual with cancer who has lower expression of the at least four genes or proteins encoded by those genes.

In one embodiment, there is provided a method of predicting risk of recurrence or progression of breast cancer in a patient, and treating the patient with a therapy for preventing recurrence of the cancer, the method comprising a step of assaying a cancer sample from the patient for

positive expression of at least four genes selected from FOXM1, UHRF1, PTTG1, E2F1, MYBL2, HMGB2, ATAD2, E2F8, ZNF367 and TCF19, wherein positive expression of the at least four genes, or proteins encoded by those genes, correlates with increased risk of recurrence or progression of cancer compared with a patient with cancer who does not exhibit positive expression of the at least four genes, or proteins encoded by those genes; and administering a neoadjuvant or an adjuvant therapy, or a combination of both, to the patient to prevent recurrence or progression of the cancer.

In one embodiment, the at least four genes, or proteins encoded by said genes, are FOXM1, PTTG1, UHRF1 and HMGB2.

Brief Description of the Drawings

The invention will be more clearly understood from the following description of an embodiment thereof, given by way of example only, with reference to the accompanying drawings, in which:-

Figure 1 illustrates the Identification of master transcriptional regulators (MTRs) of breast cell proliferation. (A) Western blot analysis of the proliferation marker EZH2 and the cellular senescence marker p16INK4A in growing (low passage) and senescent (high passage) human mammary epithelial cells (HMECs) and mouse embryonic fibroblasts (MEFs). β-actin was used as a loading control. (B) Duplicate transcriptomic profiling experiments in growing and senescent HMEC and MEF cultures were aligned in order to identify genes expressed at a consistently high level in proliferating cells. Heat-map analysis depicts all genes up- or down-regulated by more than two-fold in HMECs, and the corresponding change in MEFs. (Cluster 1 = 58 genes; Cluster 2 = 193 genes; Cluster 3 = 184 genes; Cluster 4 = 214 genes). Cluster 4 represents a 'core proliferation' signature comprising the genes most significantly and consistently downregulated during serial passaging of both HMECs and MEFs. (C) Quantitative real-time PCR validation of gene expression changes of representative genes from each of the gene clusters shown in panel B. The ribosomal RNA gene, RPLPO, was used for normalization of these data. (D) Gene ontology analysis of individual gene clusters. Red line indicates a p-value of 0.05. (E) Gene enrichment analysis of clusters 1-4 in the MammaPrint signature and the Genomic Grade signature. The fold change of the observed overlap versus what would be expected by chance is represented on the Y-axis. The number of 'core proliferation' genes (top number) present in each 'poor prognosis' signature (bottom number) is shown.

Figure 2 illustrates that E2F1, FOXM1 and MYBL2 bind core proliferative genes in HMECs. (A) Reverse engineering analysis using ARACNe predicts 6 upstream Master Transcriptional Regulators (MTRs) of the 'core proliferation' signature. Shown is a

representative ARACNe network of the HMEC/MEF 'core proliferation' signature (Cluster 4) within the NKI dataset (van de Vijver *et al.*, 2002). MTRs are highlighted in red, and cluster 4 genes are highlighted in green. (B) Validation of MTR binding to genes within the 'core proliferation' signature by ChIP-qRT-PCR. Precipitated DNA was analyzed by qRT-PCR using primers directed towards the promoters of the indicated genes (SEQ ID NOs: 1 to 38). Anti-HA antibody was used as a negative control for ChIP, and the β-ACTB and CHD5 promoters were used as negative promoter controls for qRT-PCR. ChIP enrichments are presented as the percentage of protein bound, normalised to input. The error bars indicate standard deviation of three technical replicates. (C) Heat-map analysis showing ChIP-seq data for FOXM1, MYBL2 and E2F1,in HMEC-Tert cells. Binding at the promoters of genes from Clusters 1-4 is indicated by increasing signal for each factor, FOXM1 (red), MYBL2 (green) and E2F1 (blue). The region between -2 and +2 of the transcriptional start site (TSS) of these genes is shown. (D) Representative ChIP-seq tracks of the indicated genes, with FOXM1, MYBL2 and E2F1 bound at their promoters in HMEC-Tert cells. RNA-seq data from both low and high passage HMECs is also depicted for each gene. The KRT2 gene is included as a negative control.

Figure 3 illustrates that master transcriptional regulators predict patient outcome. (A) Master transcriptional regulators are predicted to be upstream of the 'Genomic Grade' poor prognosis signature. Shown is a representative ARACNe network of the 'Genomic Grade' signature (Sotiriou *et al.*, 2006) within the Loi dataset (Loi *et al.*, 2007). MTRs are highlighted in red, and Genomic Grade signature genes are highlighted in green. (B) Kaplan-Meier analyses demonstrate that the combination of the 6 MTRs (upper) exhibit superior prognostic value than Ki67 (lower) in node negative samples without adjuvant chemotherapy in the combined microarray dataset in terms of recurrence-free survival (Loi et al., 2007; Miller et al., 2005; and van de Vijver et al., 2002) (n=457). The MTR combined score and Ki67 gene expression data were split as 2 (Lo/Hi) and 3 (Lo/Med/Hi) groups. (C) Representative examples of immunohistochemical staining for the indicated factors in low and high-risk tumors on a breast cancer tissue microarray. Low risk tumors were defined as those that did not recur within the study timeframe, whereas high risk tumors did recur. (D) Kaplan-Meier survival curves for FOXM1, UHRF1, HMGB2 and PTTG1 combined, compared to Ki67 and the St. Gallen criteria in TMA samples (n=408) in terms of recurrence-free survival. (E) Heat map illustrating the prognostic power of FOXM1, UHRF1, HMGB2 and PTTG1 and the 4 MTRs combined on the breast tumours from the TMA cohort (n=408) in terms of recurrence-free survival. Ki67 staining results and St. Gallen criteria were included for comparison. The scale represents –log10 of the p-values calculated using log-rank test.

Figure 4 illustrates that absent and high CDKN2A mRNA and p16 protein levels predict poor prognosis in breast cancer. (A) Correlations of the mRNA expression levels of

CDKN2A with gene copy number alterations (CNA) in the RB1 and CDKN2A gene loci using the GISTIC tool on data 457 breast cancers from TCGA (TCGA, 2012, Nature, 490, 61–70). (B) Kaplan-Meier survival curves for CDKN2A mRNA in node negative breast cancers without adjuvant chemotherapy in the combined microarray dataset (n=457) in terms of recurrence-free survival. Samples were stratified into 3 groups based on CDKN2A mRNA expression levels, cut at the 33rd and 66th percentile. Additionally, the undetected and high expression groups were combined and compared to the moderate expression group. Chi2 values and p-values were calculated using log-rank test. (C) Representative examples of immunohistochemical staining for p16 on low and high-risk tumors. Low risk tumors were defined as those which did not recur within the study timeframe, whereas high risk tumors did recur. (D) Kaplan-Meier survival curves for p16 protein levels in the TMA cohort (n = 408) measuring recurrence-free survival. Patients were stratified by p16 protein levels into negative, moderate (<50% positive cells) and high expression (>50% positive cells) groups. Chi2 values and p-values were calculated using log-rank test. (E) Kaplan-Meier survival curves for p16 protein levels in the TMA cohort (n = 408) measuring breast cancer-specific survival. Patients were stratified as in panel C.

Figure 5 illustrates that combined measurements of MTR and p16(INK4A) levels outperforms estimates of currently used strategies. (A) Kaplan-Meier survival curves comparing the prognostic value of the OncoMasTR RNA score (combination of CDKN2A and 6 MTRs) with estimates of the Oncotype Dx (21-gene) and Mammaprint (70-gene) signatures in node negative samples without adjuvant chemotherapy in the combined microarray dataset (n=457) in terms of recurrence-free survival. Both low/moderate/high and low/high splits were used to facilitate comparison to existing prognostic signatures. (B) Heat maps illustrating the prognostic value of CDKN2A alone, 6 MTRs combined, OncoMasTR RNA score, 70-gene signature, 21-gene signature and Ki67 in node negative samples without adjuvant chemotherapy in three individual breast cancer microarray datasets (Loi et al., 2007; Miller et al., 2005; and van de Vijver et al., 2002) and the combined dataset (n=457) in terms of recurrence-free survival. The 70-gene and 21-gene signature predicted risk groups were estimated based on gene expression data using the genefu package in R. The scale represents –log10 of the p-values calculated using log-rank test. Both 2 and 3 group splits were used to facilitate comparison to existing prognostic signatures. (C) Kaplan-Meier survival curves illustrating the combined score of 4 MTRs (FOXM1, UHRF1, HMGB2, PTTG1) and p16 (OncoMasTR IHC score) in all samples (left, n=408) and node negative samples (right, n=222) from the TMA cohort using recurrence-free survival data. The prognostic values of the 4 MTRs alone, p16 alone, the OncoMasTR IHC score, Ki67 and St. Gallen criteria were represented as a heat map based on the –log10 of p-values calculated using the log-rank test. (D) Kaplan-Meier survival as in panel C, using breast cancer specific survival data.

Figure 6 illustrates the performance of the OncoMasTR RNA score in ER-positive patients. (A) Kaplan-Meier survival curves comparing the prognostic value of the OncoMasTR RNA score (6 MTRs and CDKN2A) with the 21-gene and 70-gene signatures in ER-positive patients who did not receive adjuvant chemotherapy, in the combined microarray dataset (n=536) in terms of recurrence-free survival. (B) Kaplan-Meier survival curves as in panel A. in lymph-node negative, ER-positive patients who did not receive adjuvant chemotherapy, in the combined microarray dataset (n=366).

Figure 7 illustrates the performance of the OncoMasTR RNA score as measured by Taqman qRT-PCR. Kaplan-Meier survival curves demonstrating the prognostic value of the OncoMasTR RNA score (4 MTRs +/- CDKN2A) as indicated in ER-positive, lymph-node negative patients in the NKI dataset who did not receive adjuvant chemotherapy (n = 151), in terms of distant metastasis-free survival. Patients were divided into Low and High risk groups, and Low, Moderate and High risk groups as indicated. To do this, expression data for each MTR gene was used to split patients into low/high groups at the median. The sum of the 6 MTR (+/- CDKN2A) were taken and further split by median (2 groups) or by 33th and 66th percentile (3 groups). The end point is DMFS (censored at 10 years).

Figure 8 illustrates the performance of the OncoMasTR IHC score in terms of Distant Metastasis-free survival. Kaplan-Meier survival curves demonstrating the prognostic value of the OncoMasTR IHC score (4 MTRs +/- CDKN2A) as indicated in lymph-node negative patients (LN-) (n = 220), ER-positive patients (ER+) (n = 331), and LN-ER+ patients (n = 187), who did not receive adjuvant chemotherapy, in terms of distant metastasis-free survival.

Figure 9 illustrates the prognostic value of additional MTRs – ATAD2 and TCF19. Kaplan-Meier survival curves demonstrating the prognostic value of ATAD2 and TCF19 within ER-positive, lymph-node negative patients in the combined microarray dataset (n = 375), in terms of distant metastasis-free survival, censored at 10 years. The gene expression values for ATAD2 and TCF19 were split into low/high groups by the median within each of the three datasets. There are no probes mapping to E2F8 and ZNF367 in the NKI dataset.

Figure 10 illustrates Kaplan-Meier survival curves for 6 MTRs (FOXM1, UHRF1, MYBL2, HMGB2, E2F1, PTTG1) in The Cancer Genome Atlas (TGCA) prostate cancer transcriptomic dataset (n=150) in terms of metastasis-free survival.

Figure 11 illustrates a Forest plot of the top 100 combinations of MTRs from the list of 10 MTRs described here, with at least 4 MTRs in each combination.

Figure 12 illustrates Kaplan-Meier plots of the top 24 MTR combinations. In each case, the black line refers to high expression of the marker combination and grey refers to low expression of the marker combination.

Figure 13 illustrates the MTR10 and CDKN2A signature score in pablociclib treated

human cell lines.

Detailed Description of the Drawings

Definitions

In this specification, the term "cancer sample" should be understood to mean tumour cells, tumour tissue, or other biological material derived from a tumour, for example conditioned media.

In the specification, the term "Master Transcriptional Regulators (MTRs)" should be understood to mean a specific set of Transcription Factors (TFs) that are upstream of, and have been shown to regulate, core proliferation genes involved in cancer progression and metastasis. In other words, these specific MTRs regulate cancer and in particular, breast cancer progression.

In the specification, the term "positive expression" as applied to a gene or a protein encoded by that gene should be understood to mean a level of expression of the gene or protein encoded by that gene that is increased above an average level of expression of the same gene or protein encoded by that same gene found in a cohort of matched control individuals with cancer (the "control group"). The cohort of matched individuals may consist of individuals who did not experience a recurrence of a cancer following surgery to remove the cancer. In relation to controls, the usual practise for one skilled in the art would be to use a 'standard' control, for example, for Immunohistochemistry (IHC), a cell line or cell lines where the expression level of the biomarker is known, or for qPCR (quantitative Polymerase Chain Reaction), a similar standard control or a pool of a number of samples is known.

In the specification, the term "dysregulated expression" as applied to $p16^{INK4A}$ expression should be understood to mean a level of expression of $p16^{INK4A}$ that is negative, increased above or decreased below a level of expression of the $p16^{INK4A}$ found in a cohort of matched individuals with cancer that did not recur following surgery to remove the cancer.

The terms "normal expression" or "moderate expression" as applied to a gene or protein should be understood to mean a level of expression of the gene (or protein encoded by that gene) that is equivalent to a level of expression of the same gene or protein encoded by that same gene found in a cohort of matched control individuals with cancer. The cohort of matched individuals may consist of individuals who did not experience a recurrence of a cancer following surgery to remove the cancer.

The method used to set thresholds is different for the microarray analysis, qRT-PCR analysis, and protein expression. For microarrays, the threshold is relative (samples were split into three equal groups, so the threshold is dataset dependent), and for the qPCR and protein expression it is set at specific points. For RNA (microarrays), expression levels of 'low', 'moderate' and 'high' refer to expression values that fall within the lower, middle or upper third of the expression range; or alternatively, 'low' and 'high' expression can refer to expression values that fall within the lower or upper half of the expression range. For qRT-PCR and protein expression levels, specific thresholds have been set, but in general, the term "dysregulated" refers to tumours with expression values falling above or below set values in the range of expression. For the terms "moderate" and "normal", the terms refer to tumours with expression values falling within set values in the range of expression. For example, for $p16^{INK4A}$, the normalised qRT-PCR thresholds for 'moderate' expression are 0.7 and 1.99. The normalised protein thresholds (using IHC) are 1% and 50% of positive cells. That is, a moderate score here refers to a tumour with >1% and <50% tumour cells positive for $p16^{INK4A}$. These values may be adjusted based on any new data but the same theory applies for the terms "normal", "moderate" and "dysregulated" with respect to expression levels of $p16^{INK4A}$.

In the specification, the term "adjuvant therapy" should be understood to mean any treatment given after primary treatment to increase the chances of long-term survival. In the specification, the term "neoadjuvant therapy" should be understood to mean treatment given before primary treatment to increase the chances of long-term survival. Primary treatment is generally surgery. Adjuvant therapy and neoadjuvant therapy are generally selected from chemotherapy, hormonal therapy, targeted therapy, radiation therapy, immunotherapy or a combination thereof.

In the specification, the term "sample" should be understood to mean tumour cells, tumour tissue, non-tumour tissue, conditioned media, blood or blood derivatives (serum, plasma *etc*), urine, or cerebrospinal fluid.

Detection of expression generally involves immunohistological staining of a tumour biopsy tissue or a control biopsy tissue using suitable means such as immunohistochemical staining; however, many other means of detecting the biomarkers of the invention will be apparent to those skilled in the art. For example, quantitative polymerase chain reaction (PCR), reverse transcriptase PCR (RT-PCR), quantitative real time RT-PCR (qRT-PCR), ELISA, Western Blot, protein determination on polyacrylamide gels, and the like.

In this specification, the term "cancer" should be understood to mean a cancer that is treated by chemotherapeutic regimens. An example of such a cancer include multiple myeloma, prostate cancer, glioblastoma, lymphoma, fibrosarcoma; myxosarcoma; liposarcoma; chondrosarcom; osteogenic sarcoma; chordoma; angiosarcoma; endotheliosarcoma; lymphangiosarcoma; lymphangioendotheliosarcoma; synovioma; mesothelioma; Ewing's tumour; leiomyosarcoma; rhabdomyosarcoma; colon carcinoma; pancreatic cancer; breast cancer; node-negative, ER-positive breast cancer; early stage, node positive breast cancer; early stage, node positive, ER-positive breast cancer; ovarian cancer; squamous cell carcinoma; basal cell carcinoma; adenocarcinoma; sweat gland carcinoma; sebaceous gland carcinoma; papillary carcinoma; papillary adenocarcinomas; cystadenocarcinoma; medullary carcinoma; bronchogenic carcinoma; renal cell carcinoma; hepatoma; bile duct carcinoma; choriocarcinoma; seminoma; embryonal carcinoma; Wilms' tumour; cervical cancer; uterine cancer; testicular tumour; lung carcinoma; small cell lung carcinoma; bladder carcinoma; epithelial carcinoma; glioma; astrocytoma; medulloblastoma; craniopharyngioma; ependymoma; pinealoma; hemangioblastoma; acoustic neuroma; oligodendroglioma; meningioma; melanoma; retinoblastoma; and leukemias.

In this specification, the term "early stage" as applied to a cancer, especially a breast cancer, should be understood to mean tumours which are locally invasive but have not spread to the regional axillary lymph nodes or any other region of the body outside the breast tissue. That is, the cancer has not spread beyond the breast or the lymph nodes in the armpit on the same side of the body nor to any other part of the body.

In the specification, the term "early stage, node positive breast cancer" should be understood to mean tumours which are locally invasive and have spread to between 1-3 regional axillary lymph nodes, but not to any other region of the body outside the breast tissue.

In this specification, the term "node-negative" as applied to a cancer, especially a breast cancer, should be understood to mean tumours which have not spread to the regional axillary lymph nodes or any region outside the breast tissue.

In the specification, the terms "breast cancer patient" or "patient" means a patient who has a primary breast cancer tumour and awaits treatment for the cancer or has already undergone or is undergoing treatment for the primary tumour. The term should also be understood to include a patient who has had a primary breast cancer and is in remission, for example remission following treatment including one or more of tumour resection, first line chemotherapy,

radiotherapy, hormonal therapy, other targeted therapy, or a combination of the above.. Usually, the patient will be a breast cancer patient who has, or is undergoing, treatment for a primary tumour and who has been identified as having potential for developing a metastatic phenotype. In one embodiment, the patient has an ER-positive, node negative breast cancer.

In the specification, the term "recurrence" should be understood to mean the recurrence of the cancer which is being sampled in the patient, in which the cancer has returned to the sampled area after treatment, for example, if sampling breast cancer, recurrence of the breast cancer in the (source) breast tissue. The term should also be understood to mean recurrence of a primary cancer whose site is different to that of the cancer initially sampled, that is, the cancer has returned to a non-sampled area after treatment, such as non-locoregional recurrences.

In this specification, the term "poor outcome" should be understood to mean that the chances of disease free survival are low.

In the specification, the term "survival rate" should be understood to mean the period of time during which a patient diagnosed with cancer such as breast cancer, will likely survive. The survival rate is expressed as a 5-year survival rate, a 10-year survival rate, a 15-year survival rate, a 20-year survival rate, a 25-year survival rate, a 30-year survival rate, a 35-year survival rate, a 40-year survival rate, a 45-year survival rate, or a 50-year survival rate. Ideally, the survival rate is expressed as a 5-year survival rate or a 10-year survival rate.

In this specification, the term "treatment" should be understood to mean its generally accepted meaning which encompasses prohibiting, preventing, restraining, and slowing, stopping or reversing progression or severity of a metastatic, recurrent or existing breast cancer phenotype or other cancer phenotype.

In this specification, the term "at least two" should be understood to mean and encompass that at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine or all genes can be selected from the group consisting of FOXM1, UHRF1, PTTG1, E2F1, MYBL2, HMGB2, ATAD2, E2F8, ZNF367 and TCF19.

The computer readable storage media can be any available tangible media that can be accessed by a computer. Computer readable storage media includes volatile and non-volatile, removable and non-removable tangible media implemented in any method or technology for storage of information such as computer readable instructions, data structures, program modules or other

data. Computer readable storage media includes, but is not limited to, RAM (random access memory), ROM (read only memory), EPROM (erasable programmable read only memory), EEPROM (electrically erasable programmable read only memory), flash memory or other memory technology, CD-ROM (compact disc read only memory), DVDs (digital versatile disks) or other optical storage media, magnetic cassettes, magnetic tape, magnetic disk storage or other magnetic storage media, other types of volatile and non-volatile memory, and any other tangible medium which can be used to store the desired information and which can accessed by a computer including and any suitable combination of the foregoing.

Computer-readable data embodied on one or more computer-readable storage media may define instructions, for example, as part of one or more programs that, as a result of being executed by a computer, instruct the computer to perform one or more of the functions described herein, and/or various embodiments, variations and combinations thereof. Such instructions may be written in any of a plurality of programming languages, for example, Java, J#, Visual Basic, C, C#, C++, Fortran, Pascal, Eiffel, Basic, COBOL assembly language, and the like, or any of a variety of combinations thereof. The computer-readable storage media on which such instructions are embodied may reside on one or more of the components of either of a system, or a computer readable storage medium described herein, may be distributed across one or more of such components.

The computer-readable storage media may be transportable such that the instructions stored thereon can be loaded onto any computer resource to implement the aspects of the present invention discussed herein. In addition, it should be appreciated that the instructions stored on the computer-readable medium, described above, are not limited to instructions embodied as part of an application program running on a host computer. Rather, the instructions may be embodied as any type of computer code (*e.g.*, software or microcode) that can be employed to program a computer to implement aspects of the present invention. The computer executable instructions may be written in a suitable computer language or combination of several languages. Basic computational biology methods are known to those of ordinary skill in the art and are described in, for example, Setubal and Meidanis et al., Introduction to Computational Biology Methods (PWS Publishing Company, Boston, 1997); Salzberg, Searles, Kasif, (Ed.), Computational Methods in Molecular Biology, (Elsevier, Amsterdam, 1998); Rashidi and Buehler, Bioinformatics Basics: Application in Biological Science and Medicine (CRC Press, London, 2000) and Ouelette and Bzevanis Bioinformatics: A Practical Guide for Analysis of Gene and Proteins (Wiley & Sons, Inc., 2nd ed., 2001).

The functional modules of certain embodiments of the invention include at minimum a determination system, a storage device, optionally a comparison module, and a display module. The functional modules can be executed on one, or multiple, computers, or by using one, or multiple, computer networks. The determination system has computer executable instructions to provide *e.g.*, expression levels of at least two genes (or a protein encoded by said genes) selected from the group consisting of FOXM1, UHRF1, PTTG1, E2F1, MYBL2 and HMGB2, and optionally including $p16^{INK4A}$, in computer readable form.

The determination system, can comprise any system for assaying a breast cancer tumour sample for expression of genes (or proteins encoded by said genes) selected from the group consisting of FOXM1, UHRF1, PTTG1, E2F1, MYBL2, HMGB2, ATAD2, E2F8, ZNF367, TCF19 and $p16^{INK4A}$. Standard procedures, such as immunohistochemistry, a Western Blot, a Northern Blot, a Southern Blot, quantitative polymerase chain reaction (PCR), reverse transcriptase PCR (RT-PCR), quantitative real time RT-PCR (qRT-PCR), an enzyme-linked immunosorbent assay (ELISA), protein determination on polyacrylamide gels, RNA sequencing, RNA microarrays and other RNA hybridisation or amplification techniques, and such methods known to those skilled in the art, may be employed.

The information determined in the determination system can be read by the storage device. As used herein the "storage device" is intended to include any suitable computing or processing apparatus or other device configured or adapted for storing data or information. Examples of an electronic apparatus suitable for use with the present invention include a stand-alone computing apparatus, data telecommunications networks, including local area networks (LAN), wide area networks (WAN), Internet, Intranet, and Extranet, and local and distributed computer processing systems. Storage devices also include, but are not limited to: magnetic storage media, such as floppy discs, hard disc storage media, magnetic tape, optical storage media such as CD-ROM, DVD, electronic storage media such as RAM, ROM, EPROM, EEPROM and the like, general hard disks and hybrids of these categories such as magnetic/optical storage media. The storage device is adapted or configured for having recorded thereon nucleic acid sequence information. Such information may be provided in digital form that can be transmitted and read electronically, e.g., via the Internet, on diskette, via USB (universal serial bus) or via any other suitable mode of communication.

As used herein, "stored" refers to a process for encoding information on the storage device. Those skilled in the art can readily adopt any of the presently known methods for recording information on known media to generate manufactures comprising information relating to

FOXM1, UHRF1, PTTG1, E2F1, MYBL2, HMGB2, ATAD2, E2F8, ZNF367, TCF19 and $p16^{INK4A}$ expression in a sample.

In one embodiment the reference data stored in the storage device to be read by the comparison module is compared.

The "comparison module" can use a variety of available software programs and formats for the comparison operative to compare FOXM1, UHRF1, PTTG1, E2F1, MYBL2, HMGB2, ATAD2, E2F8, ZNF367, TCF19 and $p16^{INK4A}$ expression information data determined in the determination system to reference samples and/or stored reference data. In one embodiment, the comparison module is configured to use pattern recognition techniques to compare information from one or more entries to one or more reference data patterns. The comparison module may be configured using existing commercially-available or freely-available software for comparing patterns, staining, and may be optimized for particular data comparisons that are conducted. The comparison module provides computer readable information related to the expression levels of FOXM1, UHRF1, PTTG1, E2F1, MYBL2, HMGB2, ATAD2, E2F8, ZNF367, TCF19 and $p16^{INK4A}$ of the sample.

The comparison module, or any other module of the invention, may include an operating system (e.g., UNIX) on which runs a relational database management system, a World Wide Web application, and a World Wide Web server. World Wide Web application includes the executable code necessary for generation of database language statements (e.g., Structured Query Language (SQL) statements). Generally, the executables will include embedded SQL statements. In addition, the World Wide Web application may include a configuration file which contains pointers and addresses to the various software entities that comprise the server as well as the various external and internal databases which must be accessed to service user requests. The Configuration file also directs requests for server resources to the appropriate hardware--as may be necessary should the server be distributed over two or more separate computers. In one embodiment, the World Wide Web server supports a TCP/IP protocol. Local networks such as this are sometimes referred to as "Intranets." An advantage of such Intranets is that they allow easy communication with public domain databases residing on the World Wide Web (e.g., the GenBank or Swiss Pro World Wide Web site). Thus, in a particular preferred embodiment of the present invention, users can directly access data (via Hypertext links for example) residing on Internet databases using a HTML interface provided by Web browsers and Web servers.

The comparison module provides a computer readable comparison result that can be processed in computer readable form by predefined criteria, or criteria defined by a user, to provide a content based in part on the comparison result that may be stored and output as requested by a user using a display module.

The methods described herein therefore provide for systems (and computer readable media for causing computer systems) to perform methods as described in the Statements of Invention above, for example methods for diagnosing metastatic potential or recurrence potential of a breast cancer or a non-breast cancer in an individual or methods for identifying a breast cancer patient or a non-breast cancer patient suitable for treatment or prevention of metastatic or recurrent cancer with a suitable chemotherapeutic adjuvant or non-adjuvant therapeutic.

Systems and computer readable media described herein are merely illustrative embodiments of the invention for performing methods of diagnosis in an individual, and are not intended to limit the scope of the invention. Variations of the systems and computer readable media described herein are possible and are intended to fall within the scope of the invention.

The modules of the machine, or those used in the computer readable medium, may assume numerous configurations. For example, function may be provided on a single machine or distributed over multiple machines.

Materials and Methods

Cell Culture

Primary HMEC cells were grown as described (Garbe et al., 2009). HMEC-tert cells were immortalised using a pBABE-hTERT-hygro construct. Mouse embryonic fibroblasts (MEFs) were derived from embryonic day 13.5 C57BL6 mouse embryos and maintained in DMEM media supplemented with 10% (v/v) FBS (Hyclone), 100U/ml penicillin and 100U/ml streptomycin (Gibco).

RNA sequencing

Total RNA was extracted from proliferating and senescent HMECs using the RNeasy kit (Qiagen). Polyadenylated RNA species were enriched from 5µg total RNA, and sequencing libraries were prepared from PolyA+ RNA using the TruSeq Sample Prep kit (Illumina). Libraries were used directly for cluster generation and sequencing analysis using the Genome Analyser II (Illumina) following the protocol of the manufacturer. Base calling and mapping to the human genome (build hg19) were performed using the BWA sequence alignment tool. The

mRNA fold changes were calculated based on the total number of sequence reads mapped per gene in the two experiments.

DNA microarray analysis

Total RNA was extracted from proliferating and senescent MEFs using the RNeasy kit (Qiagen). For each time point, RNA was prepared from three independent MEF cultures and pooled to reduce experimental variation. Cy3 labeled cRNA, for use with a custom designed 44k microarray (Agilent), was prepared and hybridized to the supplier's instructions. Microarrays were scanned using Agilent's DNA microarray scanner and data analysed as previously described (Hokamp et al., 2004). Gene ontology analysis was carried out using the DAVID bioinformatics resource (http://david.abcc.ncifcrf.gov/). Publicly available breast cancer microarray datasets were downloaded from Rosetta Inpharmatics and Gene Expression Omnibus (GSE6532 and GSE3494). Within each dataset, the expression data of each gene was divided at the median into two groups, or at the 33rd and 66th percentile into 3 groups, depending on the analysis. To generate a combined MTR score, the gene expression values for each of the 6 genes were divided at the median, given a score of 1 or 2 based on the expression level, and the sum of these scores was then divided, as above, to create 2 or 3 groups. *INK4A* gene expression was divided into 3 groups (low, moderate and high) at the 33rd and 66th percentile. The moderate group was given a score of 1 and the low and high groups were combined and given a score of 2. To generate the OncoMasTR RNA score, the combined MTR score and the *INK4A* score were summed together and the final scores were divided into 2 or 3 groups. Duplicate samples were removed in the combined microarray dataset. The genefu package in R was used to estimate the risk groups which approximate the Oncotype Dx® assay (based on 21-gene signature), and the MammaPrint assay (based on 70-gene signature) (Haibe-Kains et al). For the Van de Vijver dataset, the previously defined 70-gene risk groups were used (van de Vijver et al., 2002).

Real-Time Quantitative PCR

Total RNA was extracted from cells using the RNeasy kit (Qiagen) according to manufacturer's protocol. 1ug RNA was used to generate cDNA by reverse transcriptase PCR using the TaqMan Reverse Transcription kit (Applied Biosytems). Relative mRNA expression levels were determined using the SYBR Green I detection chemistry (Applied Biosystems) on the ABI Prism 7500 Fast Real-Time PCR System. The ribosomal constituent RPLPO was used as a control gene for normalization (SEQ ID NO: 39 (Forward - TTCATTGTGGGAGCAGAC) and SEQ ID NO: 40 (Reverese - CAGCAGTTTCTCCAGAGC)). Primer sequence pairs used are as follows (For = Forward Primer; Rev = Reverse Primer):

SEQ ID NO: 1 For: AGACCGTCCTCAACCAGCTCTTC and SEQ ID NO: 2 Rev: GAAGTGCTTGGAGATCACCGG;
SEQ ID NO: 3 For: CAA CAA TAG CCT ATC CAA CAT CCA G and SEQ ID NO: 4 Rev: GGA GCC CAG TCC ATC AGA ACT C;
SEQ ID NO: 5 For: CTGCCTGAAGAGCACCAGATTG and SEQ ID NO: 6 Rev: CAAGGATCATGAGAGGCACTCC;
SEQ ID NO: 7 For: CACTGACCAGCAATGCCAGTAC and SEQ ID NO: 8 Rev: CCCCTTGACAAGGTCTGGATTC;
SEQ ID NO: 9 For: GCTCCTAAAAGGCCACCATCTG and SEQ ID NO: 10 Rev: TGATCTTTGGGCGATGTTCAG;
SEQ ID NO: 11 For: TGT CAG GAC CTT CGT AGC ATT G and SEQ ID NO: 12 Rev: GGG CTT TGA TCA CCA TAA CCA TC;
SEQ ID NO: 13 For: CAA TCT CAA CAA AAC CCT TGG C and SEQ ID NO: 14 Rev: CTC GGC GTA CTT ATT CTC CTC C;
SEQ ID NO: 15 For: AGAGGATTTGAGGGACAGGGTC and SEQ ID NO: 16 Rev: CCTCTTTCTTCCTCCGGTGC;
SEQ ID NO: 17 For: ATGGAGCTGGGTGCTGAGAAC and SEQ ID NO: 18 Rev: CCTTCTTCAACTCCATGAGCCC;
SEQ ID NO: 19 For: ACA AAG AAG GAA ATA GAG GGA CCG and SEQ ID NO: 20 Rev: GAT GAG TGG GAG ACT TGG GTT C;
SEQ ID NO: 21 For: CAGCCCGAGCTTTTGTTACAAC and SEQ ID NO: 22 Rev: TTCGCTGCTGACATCTGAGTTC;
SEQ ID NO: 23 For: AAGGTGAGCAAGATGGAAATCC and SEQ ID NO: 24 Rev: CGATCTGCAGGTCCAAGATGTAG
SEQ ID NO: 25 For: CTCTCTGAGGCCAAGGATCTCC and SEQ ID NO: 26 Rev: CCTTGTTGCAGTATTTGCAGTTG;
SEQ ID NO: 27 For: TGAGCCTGCAGATTTTAAGGTG and SEQ ID NO: 28 Rev: TGGAAAGCTTCTCACGGCATAC;
SEQ ID NO: 29 For: AGCTGGCCTGAATCATTAATACG and SEQ ID NO: 30 Rev: GGTGAAGGTCCATGAGACAAGG;
SEQ ID NO: 31 For: GGGACAGTAAAAATGTGTCCTGC and SEQ ID NO: 32 Rev: TGCCAGCAATAGATGCTTTTTG;
SEQ ID NO: 33 For: CAT TCC CGC TCT CCT TCC C and SEQ ID NO: 34 Rev: GCT CGG CTC CCC AGA ATC;
SEQ ID NO: 35 For: CCTCACTGGAGGAGTGATGCG and SEQ ID NO: 36 Rev: AAGCATCCTAAGCCATTCCATG;
SEQ ID NO: 37 For: CCA TTG AAA ACA AGG ACG ATG C and SEQ ID NO: 38 Rev: CTG TCC CCA ACA ACA TCA AGC.

ChIP and ChIP-sequencing

ChIP analyses were performed as described previously (Bracken et al., 2006). For ChIP-SEQ, DNA from 10 independent ChIP experiments was pooled and quantified using a Qubit fluorometer (Invitrogen). Sequencing libraries were generated using 100ng of immunoprecipitated DNA using the ChIP-SEQ Sample Prep Kit (Illumina). Amplified library DNA was purified by gel isolation and quality checked to unsure the absence of adaptor dimer contamination using the Bioanalyzer 2100 and DNA High Sensitivity Chip assay (Agilent). DNA libraries were quantified and diluted to 10 pM. Diluted libraries were used directly for cluster generation and sequencing analysis using the Genome Analyser II (Illumina) following the protocol of the manufacturer. Base calling and mapping to the human genome (hg19) of the 42-bp sequences were done using the Bowtie alignment tool allowing for up to 2 mismatches in each read. To avoid any PCR bias only two reads per chromosomal position were allowed, thus eliminating spurious spikes. Peak detection was performed using MACs, and Input DNA was used as a control for normalization.

ARACNe analysis

Breast cancer transcriptional networks were generated by ARACNe (Margolin et al., 2006), using published breast cancer datasets (ExPO; Loi et al., 2007; van de Vijver et al., 2002), and queried using in-house or published gene signatures. For the ExPO and Loi networks, ARACNe was run on the complete expression datasets, whereas for the NKI network, a filtering step was

applied prior to ARACNe to remove uninformative probes. The 70 gene Mammaprint signature was derived though supervised classification of DNA microarray data from 78 lymph node-negative patients, and predicts a short time to distant metastasis (van 't Veer et al., 2002). The larger 231-gene signature from which the 70-gene signature was derived was used for this analysis. The Genomic Grade signature was developed from a training dataset of 64 ER-positive breast tumors, and is composed of genes differentially expressed between low and high histologic grade. The larger 207-gene set list from which the 97-gene Genomic Grade Index was derived was used for ARACNe analysis (Sotiriou et al., 2006).

Statistical analysis

Kaplan-Meier survival curves were used for survival analysis and Chi square and p-values were calculated using log-rank test. Multivariate Cox proportional hazards analysis was used to evaluate the added prognostic value of individual genes and combined scores, on top of a standard clinical model including age (<50, >=50 years), nodal status (positive or negative), tumour size (<2cm, >=2cm), tumour grade (1 vs. 2 and 3), treatment status, and ER and HER2 status. Multivariate analysis was also carried out using the standard clinical model above, plus the 21-gene signature predicted risk group. The contribution of each marker was assessed by the change in likelihood ratio (LR-Chi, *df*=1) and p-values were calculated. A p-value of less than 0.05 was considered significant. The primary clinical endpoint used for analysis for the microarray and TMA data was recurrence-free survival (RFS). All statistical analysis was carried out using the R programming language (version 2.15.0). Heatmaps were created using an online tool (http://chibi.ubc.ca/matrix2png). Enrichment analysis was carried out by calculating the number of unique 'poor prognosis' genes present in the 'core proliferation' signature, compared to what would be expected across the genome (Observed/Expected). Unique genes in the 'poor prognosis' signatures were n=61 for the MammaPrint signature, and n=207 for the Genomic Grade signature, and analysis was normalised based on the experimental platform used to derive the signature.

TMA Cohort

The tissue microarray (TMA) used in this study was derived from a reference cohort of 512 consecutive invasive breast cancer cases diagnosed at the Department of Pathology, Malmo University Hospital, Malmo, Sweden, between 1988 and 1992, and has been previously described (Svensson et al., 2005). In brief, the median age was 65 years (range 27–96) and median follow-up time regarding disease-specific and overall survival was 11 years (range 0–17). Patients with recurrent disease and previous systemic therapies were excluded, as well as a number of misclassified ductal carcimona *in situ* (DCIS) cases. Two hundred and sixty-three

patients were dead at the last follow-up (December 2004), 90 of which were classified as breast cancer-specific deaths. Tissue cores (1mm) from areas representative of invasive cancer were extracted from donor blocks and arrayed in duplicate. This study has been approved by the Ethics Committee at Lund University and Malmo University Hospital.

Immunohistochemistry

TMA slides were deparaffinised in xylene and rehydrated in descending gradient alcohols. Heat-mediated antigen retrieval was performed using 10 mM sodium citrate buffer (pH 6.0) in a PT module (LabVision, UK) for 15 min at 95°C. The LabVision IHC kit (LabVision, UK) was used for staining. Endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide for 10 min. Sections were blocked for 10 min in UV blocking agent and the relevant primary antibody was incubated for 1 hr. Sections were washed in phosphate buffered saline with 0.1% Tween 20 (PBS-T), following which primary antibody enhancer was applied for 20 min, and sections were washed in PBS-T. Sections were then incubated with HRP polymer for 15 min, washed in PBS-T and then developed for 10 min using diaminobenzidine (DAB) solution (LabVision, UK). All incubations and washing stages were carried out at room temperature. The sections were counterstained in haematoxylin, dehydrated in alcohol and xylene and mounted using DPX mounting medium. As a negative control, the primary antibody was substituted with PBS-T.

Primary antibodies used were HMGB2 (Abcam; 1:1500), UHRF1 (BD Biosciences; 1:1000), PTTG1 (Invitrogen; 1:500), FOXM1 (Santa Cruz, C20; 1:300), and p16 (Clone JC8; 1:5000). TMA sections had been previously been stained in the Ventana Benchmark (Ventana Medical Systems Inc, USA) using prediluted antibodies to ER (clone 6F11, Ventana), PR (clone 16, Ventana) and Her2 (Pathway CB-USA 760-2694), or in the Dako Techmate 500 (Dako, Denmark) for Ki-67 (1:200, M7240, Dako).

TMA analysis

Slides were scanned at 20× magnification using a ScanScope XT slide scanner (Aperio Technologies, CA). For manual scoring, staining of tumor cells was evaluated by a pathologist on the basis of intensity, on a scale of negative (0), weak (1), moderate (2) and strong (3); and percentage, on a scale of 0-6 (0 = 0-1%; 1 = 1-10%; 2 = 10-25%: 3 = 25-50%; 4 = 50-75%; 5 = 75-90%; 6 = 90-100%). Staining for the factors HMGB2 and UHRF1 was predominantly nuclear, whereas PTTG1, FOXM1 and $p16^{INK4A}$ stained both the nuclear and cytoplasmic compartments and were scored accordingly. For UHRF1, PTTG1 and $p16^{INK4A}$, the percentage of positive tumor nuclei was the most significant variable in relation to outcome and was used

in all further analysis. For HMGB2, a modified Allred score (intensity plus percentage) was used and, for FOXM1, the percentage of cytoplasmic positivity within tumor cells was the most significant variable. For analysis of the four MTRs, a threshold for positivity was applied independently for each variable, to create a binary score with low (0) and high (1) expression. For $p16^{INK4A}$, the 'negative' (0% positive cells) and 'high' (>50% positive cells) expression groups were combined and given a score of 1, and compared to the 'moderate' group with a score of 0. To generate a combined MTR score at the protein level, the sum of the binary scores for all four MTRs was generated. Tumors with high expression of >1 MTR were classified as having a high MTR score. To generate the combined 4MTR+$p16^{INK4A}$ score (OncoMasTR IHC score), the binary 4MTR score was combined with the binary $p16^{INK4A}$ score, and divided into two groups with a threshold of >2.

Results

Identification of a 'core proliferation' gene expression signature.

The applicant set out to identify a set of 'core proliferation' genes that are consistently highly expressed in actively growing cells in a lineage-independent fashion. To do this, the applicant isolated human mammary epithelial cells (HMECs) and mouse embryonic fibroblasts (MEFs) and passaged them towards cellular senescence, as characterised by an increase in the levels of $p16^{INK4A}$ (Zindy et al., 1997), and a decrease in the levels of the E2F target gene, EZH2 (Bracken et al., 2003) (Figure 1A). The applicant next performed a genome-wide mRNA expression analysis on proliferating and senescing HMEC and MEF cultures and identified four differentially expressed gene clusters (Figure 1B). The expression changes of representative genes from each cluster were validated by quantitative RT-PCR (Figure 1C). The Cluster 3 genes, which were down-regulated during serial passaging of HMEC cells, included several genes involved in mammary epithelial cell-specific processes, such as the luminal cytokeratin KRT19 and the tight junction protein CLDN3. This is consistent with the fact that the proportion of luminal and myoepithelial cells shifts during serial passaging of HMEC cells (Garbe et al., 2009). Therefore, the applicant reasoned that many of the genes within Cluster 3 were down-regulated independently of the progressive decrease in proliferation rate. Consistent with this, a gene ontology analysis for each of the four gene clusters revealed a greater enrichment of functional categories linked to cell cycle and proliferation in Cluster 4, compared to Cluster 3 (Figure 1D). Therefore, the strategy to combine the expression changes of both serially passaged MEF and HMEC allowed the identification of a 'core proliferation' genes in mammary epithelial cells.

The applicant next wished to determine how enriched the Cluster 4 'core proliferation' genes were in two of the best known 'breast cancer poor prognosis' signatures, the MammaPrint 70-gene signature and the 'Genomic Grade' signature (Sotiriou et al., 2006; van 't Veer et al., 2002). This revealed a significant enrichment of Cluster 4 genes, but not genes from Clusters 1-3, in both poor prognosis signatures (Figure 1E), supporting the, perhaps unsurprising, view that a major contributor to the prognostic power of these two signatures is their ability to simply measure tumor cell proliferation (Mosley and Keri, 2008; Wirapati et al., 2008).

Identification of upstream master transcriptional regulators (MTRs) of the 'core proliferation' signature.

Interestingly, despite the ability of several established poor prognostic signatures to predict breast cancer outcome, there is surprisingly little overlap between the signatures themselves (Fan et al., 2006; Haibe-Kains et al., 2008). The applicant reasoned that the proliferative genes within these signatures, several of which are 'core proliferation' genes in the analysis presented herein (Figure 1E), may in fact be just passengers, rather than drivers of tumour cell proliferation. Therefore, the applicant hypothesised that the upstream transcriptional regulators of the 'core proliferation' genes would be more reliable predictors of breast cancer prognosis.

Considering the hierarchical nature of gene expression regulation, the applicant wished to identify the key transcriptional regulators upstream of the core proliferation signature. To identify the upstream master transcriptional regulators (MTRs) of the 'core proliferation' genes, a bioinformatic approach called ARACNe was used (Carro et al., 2010; Margolin et al., 2006). This approach uses interaction networks constructed from gene expression datasets to infer direct transcriptional interactions. ARACNe was applied to three publicly available breast cancer gene-expression datasets (ExPO; Loi et al., 2007; van de Vijver et al., 2002) and predicted several upstream MTRs of the 'core proliferation' genes in breast cancer (Figure 2A and Table 1). Among the top scoring MTRs were Forkhead Box M1 (FOXM1), ubiquitin-like PHD and RING finger 1 (UHRF1), Securin or Pituitary Tumour-Transforming Gene 1 (PTTG1), E2F Transforming Factor 1 (E2F1), v-myb myeloblastosis viral oncogene homolog (avian)-like 2 (MYBL2) and High Mobility Group Box 2 (HMGB2), which were relatively consistent across the three independent breast cancer datasets, supporting the idea that the MTRs would prove to be more reliable indicators of tumor cell proliferation than their downstream target genes. Four additional genes were also identified consistently across datasets as being upstream of the 'core proliferation' genes. These are ATAD2, E2F8, ZNF367 and TCF19.

Table 1: Top ranking master transcriptional regulators of the indicated expression signatures as predicted by ARACNe

Rank	Core Proliferation signature	Poor Prognosis signature	Genomic grade signature
1	FOXM1	PTTG1	PTTG1
2	PTTG1	FOXM1	FOXM1
3	UHRF1	UHRF1	UHRF1
4	MYBL2	ATAD2	MYBL2
5	HMGB2	MYBL2	ATAD2
6	ATAD2	ZNF367	HMGB2
7	E2F1	HMGB2	ZBTB20
8	E2F8	TCF19	E2F1
9	ZNF367	E2F8	E2F8
10	TCF19	E2F1	ZNF367

The applicant next wished to determine if some of the MTRs directly bind to the promoters of Cluster 4, 'core proliferation' genes, as predicted. Chromatin immunoprecipitations (ChIPs) followed by quantitative Real Time PCR (qPCR) confirmed the direct binding of four of the MTRs (FOXM1, MYBL2, E2F1 and HMGB2) to the promoters of 'core proliferation' genes in HMEC-Tert cells (Figure 2B). To gain a broader view on MTR binding throughout the genome, ChIP followed by high-throughput sequencing (ChIP-seq) was performed on HMEC-Tert cells for E2F1, MYBL2 and FOXM1. This revealed that all three MTRs primarily associate with the promoters of the Cluster 4, 'core proliferation' genes, and to a lesser extent, some Cluster 3 genes (Figure 2C). The ChIP-seq tracks of three representative genes show peaks depicting binding of E2F1, MYBL2 and FOXM1 on the *CCNB1*, *UBE2C* and *CENPA* gene promoters (Figure 2D), but not on the promoter of a gene not expressed in HMECs, *KRT2*. The applicant was not able to investigate the genome-wide binding patterns of PTTG1 or UHRF1 due to the lack of suitable high quality ChIP-grade antibodies. However, the fact that PTTG1 has been reported to have a role in the transcriptional activation of cell cycle genes, supports the ARACNe predictions (Tong and Eigler, 2009; Tong et al., 2007). On the other hand, UHRF1 is generally considered to be a transcriptional repressor, being required for the maintenance of DNA methylation during cell division (Bostick et al., 2007). Therefore, UHRF1 is unlikely to directly regulate core proliferation genes, and is more likely to be a co-regulated proliferative gene. Supporting this possibility, E2F1, MYBL2, and FOXM1 also bind the promoter of the *UHRF1* gene in HMEC cells.

In parallel with the identification of these MTRs, the Applicant also carried out unbiased survival analysis of 565 node-negative patients from four independent breast cancer gene expression datasets (Buffa *et al.*, 2011; Ivshina *et al.*, 2006; Loi et al., 2007; van de Vijver *et al.*, 2002), in order to identify the genes associated with patient survival in ranked order (Table 2).

Strikingly, this analysis identified several of the proliferation MTRs as among the top 20 genes associated with breast cancer outcome in these node-negative patients, with several of these proliferation MTRs scoring higher than conventional clinical biomarkers (ER, PR, Ki67) or genes incorporated into the Oncotype Dx® assay (BIRC5, CCNB1, BCL2, CTSL2). This result illustrated the power of these MTRs as prognostic biomarkers, and inspired us to investigate them further.

Table 2: Unbiased survival analysis of all genes across four breast cancer datasets (Van de Vijver *et al*, Loi *et al*, Ivshina *et al*, Buffa *et al.*,). Gene expression values were divided at the median, analysed in relation to overall survival using the log rank test, and ranked in order of prognostic power. (italics = MTRs; bold = conventional clinical biomarkers; shaded = OncoMasTR pathway genes)

Rank	Gene	Function
1	PRC1	Cell cycle
2	*UHRF1*	*Proliferation MTR*
3	ZWINT	Cell cycle
4	IGBP1	Signal transduction
5	RPL29	Ribosomal protein
6	CCNB2	Proliferation
7	TRIP13	DNA repair
8	CDC45L	Cell cycle
9	TROAP	Cell adhesion
10	TACC3	Proliferation
11	LRP2	Lipoprotein/Hormone signalling/Stress response
12	MAD2L1	Cell cycle
13	BLM	DNA replication and repair
14	CDKN3	Cell cycle
15	SEC14L2	Cholesterol Biosynthesis
16	*MYBL2*	*Proliferation MTR (and Oncotype Dx)*
17	**BIRC5**	**Oncotype Dx® (Anti-apoptosis)**
18	*PTTG1*	*Proliferation MTR*
19	H2AFZ	Chromatin remodeling
20	TK1	DNA replication
21	FBXO5	Ubiquitin pathway
22	EIF2C2	RNAi pathway
23	EBP	Cholesterol Biosynthesis
24	PLP2	Endoplasmic reticulum protein
25	EZH2	Proliferation/Polycomb protein
26	*FOXM1*	*Proliferation MTR*
27	PDZK1	Scaffolding protein/Cholesterol metabolism
28	FEN1	DNA repair
29	TXNRD1	Oxidative stress
30	COL4A1	Basement membrane component

31	STC2	Calcium homeostasis/Estrogen signalling
32	GPR56	Cell signalling
33	SQLE	Sterol Biosynthesis
34	EXO1	DNA repair
35	YWHAZ	Anti-apoptosis
36	GATA3	Hormone Response
37	KIF4A	Cell cycle
38	ADM	Hormone sigalling
39	CREBL2	Cell cycle
40	TTK	Proliferation
41	BUB1	Cell cycle/Apoptosis
42	CTPS	DNA synthesis
43	CHST3	Cell migration/Wound response
44	CAMLG	Apoptosis/Calcium homeostasis
45	PSMD1	Proteasome component
46	KIF13B	DNA damage pathway
47	NRM	Nuclear membrane protein
48	STXBP2	Vesicle trafficking
49	GALT	Glycoprotein metabolism
50	GPI	Glycogen metabolism/Angiogenesis
51	POLD1	DNA replication
52	RRM2	DNA replication
53	MYB	Proliferation/Differentiation
54	CDC20	Cell cycle
55	SERPINH1	Inflammatory response/Protolysis
56	SERPINA3	Proteolysis
57	HMMR	Cell motility
58	PDCD4	Invasion/Apoptosis
59	PGK1	Glucose metabolism
60	RQCD1	Cell differentiation
61	NDRG1	Stress response/Apoptosis
62	SLU7	mRNA splicing
63	**ESR1**	**Oncotype Dx® (Hormone Response)**
64	SPARCL1	Cell migration/Invasion
65	NME5	Anti-apoptosis
66	BTG2	Anti-proliferative
67	WDR5	Histone modification
68	HMGCL	Ketogenesis
69	SERPINE1	Cell migration/invasion
70	BTN2A1	Lipid metabolism
71	CELSR2	Cell-cell adhesion/signalling
72	PKM2	Glucose metabolism
73	ORC1L	DNA replication
74	FANCA	DNA repair
75	FLT3	Angiogenesis
76	TYMS	
77	SIRT1	
78	GARS	

79	XPOT	
80	FUT8	Protein glycosylation
81	BTD	
82	LZTFL1	
83	STIP1	
84	ME1	
85	UCP2	
86	RPL14	
87	NP	
88	CIRBP	
89	ORC6L	
90	PSMD7	
91	CCNE2	
92	CENPA	
93	CDC25B	
94	*E2F1*	*Proliferation MTR*
95	**CCNB1**	**Proliferation (Oncotype Dx®)**
96	H2AFX	
97	RAD54L	
98	ADAMTS7	
99	LEPR	
100	KIAA1609	
101	KIAA1407	
102	CCNA2	Cell cycle
103	PFKL	
104	KIAA0999	
105	SLC23A2	
106	FUCA1	
107	RFC2	
108	CCNI	
109	NEK2	
110	HS3ST1	
111	DYSF	
112	AGTR1	
113	VAV3	
114	PDE6B	
115	POLA2	
116	ATP5G3	
117	KIAA0831	
118	PTMA	
119	GSTM3	
120	PHB	
121	MAP4K4	
122	**PGR**	**Oncotype Dx® (Hormone Response)**
123	**BCL2**	**Oncotype Dx® (Anti-apoptosis)**
124	IGFBP4	
125	CENPE	
126	CYC1	

127	CDO1	
128	MYCBP	
129	SKP2	
130	RAB3D	
131	DHCR7	
132	KIAA1324	
133	ATP11A	
134	BECN1	
135	HDGF	
136	PCYT1A	
137	TNNC1	
138	CENPF	
139	ADCY1	ATP metabolism
140	**MKI67**	**Oncotype Dx® (Proliferation)**
141	KIAA0101	
142	KCNN3	
143	SLC19A1	
144	EPHA4	Cell adhesion/signalling/migration/invasion
145	CDC25C	
146	NFATC1	
147	PDE5A	
148	ABCF1	
149	CKS2	
150	PRRG2	CalciumVitamin K signalling
151	CLDN4	Cell adhesion
152	GTSE1	
153	RAI2	
154	PRLR	Hormone signalling
155	SEMA7A	
156	CPT1A	
157	PDHA1	
158	RAB27B	
159	MCM2	
160	FLNB	
161	SLC2A3	Glucose transport/metabolism
162	IMPDH2	
163	*HMGB2*	*Proliferation MTR*
164	HOXB13	Homeobox protein
165	NFRKB	
166	RPS6KA5	
167	CRIP2	
168	BTF3	
169	MAGED1	
170	NAPG	
171	ASNS	
172	PTTG2	
173	TPST1	Wound response
174	RPLP1	

175	GLTSCR2	
176	PLA2R1	
177	POLQ	
178	CSTB	
179	CALU	Calcium-dependent signalling
180	PPARD	
181	TXN	
182	NAT1	
183	MYO7A	
184	EIF4G1	
185	SHMT2	
186	PTDSS1	
187	LHX2	
188	PLA2G10	
189	ANLN	
190	ATP5J	
191	POLR2D	
192	SERF1A	
193	EPHB4	
194	CDC23	
195	PTPN14	
196	PEX12	
197	PPP1R11	
198	CSPG5	
199	DONSON	
200	**CTSL2**	**Oncotype Dx® (Invasion)**

Proliferative MTRs are excellent predictors of breast cancer prognosis on the RNA and protein levels.

Next, the potential clinical significance of the MTRs as prognostic markers in breast cancer was explored. The applicant began by performing an unbiased ARACNe analysis of the MammaPrint and Genomic Grade signatures, both of which have been shown to predict clinical outcome in breast cancer patients (Sotiriou et al., 2006; van 't Veer et al., 2002). Remarkably, across the three independent datasets analysed (ExPO; Loi et al., 2007; van de Vijver et al., 2002), FOXM1, E2F1, MYBL2, UHRF1, PTTG1, HMGB2, ATAD2, E2F8, ZNF367, and TCF19 were predicted to be among the top upstream regulators of both 'poor prognosis' signatures (Figure 3A and Table 1). This suggests that these MTRs directly regulate the expression of many genes within both the MammaPrint and Genomic Grade prognostic signatures.

The applicant next wished to explore the possibility that the MTRs may themselves be reliable predictors of poor prognosis. The association of each individual MTR with patient survival was

examined in a combined dataset of three published microarray studies representing the genome-wide mRNA expression of 457 lymph node-negative breast tumours untreated by chemotherapy (Loi et al., 2007; Miller et al., 2005; van de Vijver et al., 2002). This revealed that high mRNA expression levels of any of FOXM1, E2F1, MYBL2, UHRF1, PTTG1, HMGB2 in breast tumours was significantly associated with reduced recurrence-free survival time, and a combination of all six MTRs was more powerful at stratifying the patients compared to any MTR alone (Figure 3B). Significantly, using either a low/high or a low/moderate/high categorisation strategy, the six MTR combination was better at predicting recurrence-free survival than the established proliferation marker Ki67 (Figure 3B). These six MTRs now form the 'core' panel of the method or assay of the present invention, also called the OncoMasTR assay. High mRNA expression levels of ATAD2 and TCF19 in breast tumours was also significantly associated with reduced recurrence-free survival time in this cohort (Figure 9). Expression information was not available in this cohort for E2F8 and ZNF367.

Next, the protein levels of the MTRs were examined in an independent breast cancer patient cohort via immunohistochemistry (IHC). Antibodies were screened for all 6 MTRs and four identified that specifically recognised FOXM1, HMGB2, PTTG1 and UHRF1. Tissue microarrays (TMAs) representing 512 invasive breast tumours were evaluated for the protein levels of each of these MTRs (Figure 3C). The stained TMAs were manually scored and the results analysed in relation to recurrence-free survival for the 430 tumours with information on all four MTRs (Figure 3D). Each MTR was individually associated with poor prognosis, and the combination of all four MTRs was more powerful at stratifying the patients in relation to survival, compared to existing prognostic indicators such as Ki67 or the St. Gallen criteria, a prognostic index based on age, nodal status, tumour size, ER/PR status and tumour grade (Goldhirsch et al., 2001) (Figure 3D). The results from this Kaplan-Meier analysis were also represented in a heat-map format to indicate the strength of the association with recurrence-free survival (Figure 3E). To the knowledge of the inventors, this heat-map arrangement has not been previously used to present large-scale survival analysis, and provides an intuitive way of determining the best prognostic combination in any particular dataset.

To further refine the prediction method of the claimed invention and complement the approach taken by the Applicant, the other crucial pathways, besides proliferation control, involved in breast cancer progression were taken into account. Additional genes from the unbiased analysis of four independent breast cancer datasets (described above and in Table 2) were selected, which strongly correlate with survival, and represent other aspects of tumour progression as distinct from proliferation, such as migration/invasion, apoptosis and hormone signalling

pathways (Table 3). When combined with the proliferation MTRs, these genes add a further layer of information, and increase the predictive power of the gene combination even further. These genes form the basis of the OncoMasTR pathway panel which, when combined with the OncoMasTR core genes, further improve the prognostic power of the method.

Table 3: Summary of OncoMasTR Core and Pathway gene panels

OncoMasTR Core Panel	**OncoMasTR Pathway panel**			
Proliferation	Migration/ Invasion	Apoptosis	Hormone/Growth Factor signalling	Other function
UHRF1 **FOXM1** **MYBL2** **PTTG1** **E2F1** **HMGB2**	EPHA4 HOXB13 CLDN4 SERPINE1	BIRC5 BCL2 TXNRD1 NDRG1	CAMLG PRLR ADM PRRG2	IGBP1 FUT8 CALU ADCY1
	Estrogen signalling	Inflammation/ Wound response	Angiogenesis	Metabolic pathways
	GATA3 PDZK1	TPST1 SERPINA3	FLT3	SLC2A3 LRP2

Disruption of cellular senescence pathways can be inferred using a combination of MTRs and $p16^{INK4A}$ levels and is a strong predictor of poor outcome in breast cancer

The applicant next wished to examine if the levels of $p16^{INK4A}$, a potential proxy for bypass of the cellular senescence checkpoint in cancer, could add to the prognostic power of the MTRs. First, to confirm that deregulated *CDKN2A* mRNA levels correlated with genetic perturbation of the cellular senescence checkpoint, The Cancer Genome Atlas (TCGA) breast cancer dataset (Cancer Genome Atlas, 2012) was analysed, and found that high levels of *CDKN2A* mRNA levels correlated with deletion of *RB1*, as previously reported in other studies (Hara et al., 1996; Kotake et al., 2007; Li et al., 1994; Tam et al., 1994), while deletion of *CDKN2A* correlated with decreased mRNA levels (Figure 4A). Strikingly, moderate mRNA levels of *INK4A* were found to correlate with improved recurrence-free survival in 457 lymph node-negative breast cancer patients, while either very low or very high levels correlated with shorter recurrence-free survival (Loi et al., 2007; Miller et al., 2005; van de Vijver et al., 2002) (Figure 4B). The applicant next performed IHC for the $p16^{INK4A}$ protein on the same breast cancer TMAs used previously (Figure 4C). This confirmed that either very high or very low $p16^{INK4A}$ protein levels

also correlated with both shorter recurrence free and breast cancer-specific survival, whereas moderate levels correlated with extended survival (Figure 4D-E).

Based on these observations, the applicant reasoned that the breast cancers with either very high or very low $p16^{INK4A}$ protein levels had bypassed the cellular senescence checkpoint, and this could potentially explain their poor prognosis. The breast cancers with low $p16^{INK4A}$ protein levels were most likely to have a deletion in the *INK4A* gene locus, while those with aberrantly high levels likely had mutations in the *INK4A* gene or deregulation of downstream E2F-pRB pathway members such as Cyclin D1 or pRB. In contrast, the tumors with moderate expression of *INK4A* were most likely enriched in cells that had not bypassed the cellular senescence checkpoint and, therefore, had a more favourable prognosis.

Previous studies of $p16^{INK4A}$ expression in relation to breast cancer prognosis have reported conflicting results - while $p16^{INK4A}$ was found to be associated with poor prognosis in some cohorts (Hui et al., 2000; Milde-Langosch et al., 2001), other studies showed an association with improved outcome (Peurala et al., 2013). These studies have generally split expression values into two groups, low/negative and high, for analysis. However, based on what is known of the biology of $p16^{INK4A}$ and the p16-Rb pathway in cancer, the Applicant proposes that the best approach may be to examine $p16^{INK4A}$ expression in three groups, low/negative, moderate and high expression. This may separate tumors which are likely to have deleted or inactivated $p16^{INK4A}$ (low expressers) and those which have aberrantly high levels of $p16^{INK4A}$ and are likely to have a dysregulated p16-Rb pathway (high expressers) from the tumors with a functioning senescence response (moderate expressers).

A combination of measuring proliferative MTRs and $p16^{INK4A}$ levels (OncoMasTR score) outperforms currently used approaches for predicting breast cancer prognosis.

The prognostic ability of a combination of $p16^{INK4A}$ and the proliferative MTRs were evaluated next. To do this, a score encompassing both proliferative MTRs and $p16^{INK4A}$ expression was developed, termed the 'OncoMasTR RNA score', and compared with estimates of other leading multi-gene prognostic assays (Figure 5A). This revealed that the OncoMasTR RNA score compared favourably to surrogate estimations of the MammaPrint™ and OncotypeDx® signatures, using low/high categories for comparison with MammaPrint™, and low/moderate/high categories for comparison with Oncotype Dx®. In order to further demonstrate the prognostic capability of the OncoMasTR RNA score, the applicant analysed each individual dataset and the combined dataset, and represented the results in a heat-map format (Figure 5B). This extended analysis revealed that, while the MammaPrint™ 70-gene

signature performed best in the dataset which included samples used in its derivation (van 't Veer et al., 2002; van de Vijver et al., 2002), the OncoMasTR RNA score outperformed estimates of both the MammaPrint™ and Oncotype Dx® assays overall when all three datasets were combined.

Next, to validate these observations at the protein level, the applicant combined the $p16^{INK4A}$ protein and the IHC-based 4-MTR panel, called the 'OncoMasTR IHC score', and tested this combination in all patients and in lymph node-negative patients, in relation to both recurrence-free survival (Figure 5C) and breast cancer-specific survival (Figure 5D). This revealed that when $p16^{INK4A}$ is added to the IHC-based MTR panel, the combination of high levels of proliferative MTR proteins and either low or aberrantly high $p16^{INK4A}$ protein was strongly associated with poor prognosis, and there was a striking improvement in the ability to predict patient survival in comparison to the four MTRs without $p16^{INK4A}$, either on all patients (Figure 5C) or on a lymph node-negative sub-cohort (Figure 5D).

The OncoMasTR RNA score outperforms surrogate estimates of MammaPrint™ and Oncotype Dx® in ER-positive patients

In order to further evaluate the potential clinical utility of the OncoMasTR RNA score, its prognostic power was examined in 366 ER-positive, lymph node-negative patients, which reflects the inclusion criteria for the Oncotype Dx® assay. The OncoMasTR RNA score outperformed surrogate estimates of both the MammaPrint™ (low/high groups), and Oncotype Dx® (low/mod/high groups) assays in both the entire cohort (Figure 6A), and lymph node-negative patient cohort (Figure 6B). The OncoMasTR RNA score was also assessed using a Taqman® qRT-PCR approach in 151 ER-positive, lymph node-negative patients using DMFS as an endpoint, matched to the cohort used for IHC validation (Figure 7). This demonstrated that the OncoMasTR RNA score, when measured by Taqman® qRT-PCR analysis, showed analogous performance to the microarray-based analysis. Furthermore, the OncoMasTR IHC score also demonstrated utility in this group of patients, using either recurrence-free survival, or distant metastasis-free survival (Figure 8) as an endpoint.

The OncoMasTR score has independent prognostic value in all patients and lymph node-negative patients

Next, in order to determine if the MTR and *INK4A*/$p16^{INK4A}$ combination can provide additional prognostic information independent of standard clinicopathological variables, the applicant performed multivariate analysis using Cox proportional hazards models. The OncoMasTR score was found to contribute added prognostic information to a standard clinicopathological variable

model, in terms of recurrence-free survival, at both mRNA (Table 4) and protein (Table 5) levels. This was also observed in the lymph node-negative patient cohort. The added prognostic value of the OncoMasTR score on top of the standard clinical model is superior to all other prognostic indicators, including Ki67, the 70-gene signature (MammaPrint™) and the 21-gene signature (Oncotype Dx®). Furthermore, the OncoMasTR RNA score was found to provide significant additional prognostic information to a model comprising the standard clinical variables together with the Oncotype Dx® surrogate estimation.

Table 4: Multi-variate Cox regression analysis using a standard clinical variable model* in the combined microarray datasets

		All patients (n = 567)		Node negative patients (n=410)	
	Variable	Chi2**	p-value	Chi2	p-value
Lo/Med/Hi	FOXM1	24.14	<0.001	26.59	<0.001
	E2F1	25.28	<0.001	15.56	<0.001
	HMGB2	10.89	<0.001	7.47	0.006
	MYBL2	25.43	<0.001	15.91	<0.001
	PTTG1	12.37	<0.001	10.16	0.001
	UHRF1	22.71	<0.001	17.61	<0.001
	CDKN2A	2.23	0.135	13.82	<0.001
	6MTR	33.80	<0.001	20.27	<0.001
	OncoMasTR RNA score	43.87	<0.001	44.04	<0.001
	21 gene	29.02	<0.001	38.03	<0.001
	Ki67	8.30	0.004	7.45	0.006
Lo/Hi	6MTR	23.82	<0.001	29.32	<0.001
	OncoMasTR RNA score	29.62	<0.001	32.20	<0.001
	70 gene	30.20	<0.001	28.88	<0.001
	Ki67	5.52	0.018	8.99	0.003

*Clinical variables used: Age (>=50 years), Nodal status, Tumor size (>=2cm), Tumor grade (>1), treatment (endocrine therapy) and ER status.
**Added prognostic value of each variable, represented by change in the Chi2 value from the model of only clinical variables to the model of clinical variable + marker in the three combined microarray datasets. Recurrence-free survival was used as the endpoint for this analysis.

Table 5: Multi-variate Cox regression analysis using a standard clinical variable model* in tissue microarrays

		All patients (n = 272)		Node negative patients (n=171)	
	Variable	Chi2**	p-value	Chi2	p-value
Lo/Hi	FOXM1	1.60	0.207	0.49	0.485
	HMGB2	0.05	0.819	2.53	0.112
	PTTG1	4.03	0.044	0.17	0.677
	UHRF1	4.53	0.033	0.77	0.379
	p16	6.73	0.009	7.23	0.007
	4 MTRs	12.24	<0.001	0.28	0.597
	OncoMasTR IHC score	24.86	<0.001	7.28	0.007
	Ki67	5.23	0.022	3.42	0.064

*Clinical variables used: Age (>=50 years), Nodal status, Tumor size (>=2cm), tumor grade (>1), treatment (chemotherapy, endocrine therapy, radiotherapy), ER and HER2 status.
**Added prognostic value of each variable, represented by change in the Chi2 value from the model of only clinical variables to the model of clinical variable + marker in the tissue microarray datasets. Recurrence-free survival was used as the endpoint for this analysis.

Prognostic power in a Prostate Cancer Cohort

This current project describes the validation of the OncoMasTR panel as a breast cancer prognostic on independent cohorts, however the panel may also be used for other cancer types such as those listed above. For example, a publically available prostate cancer transcriptomic dataset was analysed (Taylor et al., 2010), revealing that the OncoMasTR panel showed prognostic capability in terms of metastasis-free survival in this cancer type (see Figure 10). Prostate cancer patients with high expression of the 6 MTR panel (FOXM1, E2F1, MYBL2, UHRF1, PTTG1, HMGB2) were found to have a poor outcome in comparison to patients with low expression of these genes.

A method of prediction based on the expression of these MTRs and $p16^{INK4A}$ will be capable of addressing the unmet need of early stage breast cancer patients, and provide them with the necessary tools to make better informed treatment decisions. The addition of additional pathway genes, or novel MTRs such as ATAD2, E2F8, ZNF367 and TCF19, some of which have been demonstrated to predict poor prognosis in breast cancer patients (Figure 9); may also improve the prognostic capability of this assay even further. Such a test will improve on what is currently available based on the fact that each of these MTRs is upstream of many genes involved in breast cancer proliferation and thus, by measuring these MTRs, one is effectively measuring the status of a much larger 'proliferation signature'. The predictive power of this panel of proliferation MTRs has been augmented by the addition of the senescence regulator

$p16^{INK4A}$. By combining these 'core' genes with selected 'pathway' genes, one can thoroughly dissect the molecular complexities of breast cancer, and accurately determine the likelihood of recurrence.

The prognostic potential of these 10 MTRs, in combination with $p16^{INK4A}$, were subsequently individually analysed using BreastMark (Madden, S. F. *et al.* BreastMark: an integrated approach to mining publicly available transcriptomic datasets relating to breast cancer outcome. *Breast Cancer Res* **15**, R52, doi:10.1186/bcr3444 (2013)), an integrated approach for performing cross-dataset survival analysis in breast cancer (Table 6). This algorithm integrates gene expression and survival data from 26 datasets on 12 different microarray platforms corresponding to approximately 17,000 genes in up to 4,738 samples. The breakdown of the individual clinical information available with each dataset is described in detail in the original manuscript, along with the methods used for analysing/normalising the gene expression data. Cross-dataset survival analysis across multiple disparate microarray platforms is facilitated by gene centring the data to remove probe specific information and dichotomising the samples within each dataset before combining them to perform a global pooled survival analysis. In the analysis presented herein, disease free survival (DFS) was chosen as the survival endpoint and median gene expression was used to dichotomise the data.

There are over a 1,000 combinations of MTRs with four or more genes that can be chosen from the list of 10 MTRs described herein, each of which can be combined and assessed for their prognostic potential. In order to identify the optimal combination of these MTRs, BreastMark was adapted in the following way. For each combination of MTRs, the processed datasets from BreastMark were taken and, within each dataset, the expression data of each MTR was divided at the median into two groups. Once the samples have been dichotomised, the gene expression data is no longer used, allowing comparisons across different datasets/platforms. To generate a combined master transcriptional regulator (MTR) score, the gene expression values for each of the MTR in a particular combination were divided at the median, given a score of 1 or 2 based on the expression level. This results in each sample in a particular dataset getting a MTR score based on the sum of its individual MTR scores. For example, if a particular MTR combination contained 6 genes, and each gene in a particular sample was expressed at a level below the median expression of that gene in that dataset, the MTR score would be 6, the sum of the score of 1 for each of the 6 MTR. This results in a range of MTR scores between 6 (all MTRs are lowly expressed) and 12 (all MTRs are highly expressed), which can then be dichotomised based on the median MTR score for that dataset and combined with the DFS information to

identify if this combination of MTRs is prognostic (a significant p-value) and how prognostic it is (the hazard ratio).

The top 100 combination of MTRs can be seen in the forest plot in Figure 11, and the individual Kaplan-Meier plots for the top 24 combinations can be seen in Figure 12. The samples were ranked based on the size of the hazard ratio once significance had been established (adjusting for multiple testing using the Benjamini and Hochberg method (Benjamini, Y., Drai, D., Elmer, G., Kafkafi, N. & Golani, I. Controlling the false discovery rate in behavior genetics research. Behavioural brain research 125, 279-284 (2001)). It should be noted that the sample sizes vary depending on the combination of MTRs used as not all MTRs are present in all 26 BreastMark datasets, *e.g.* ZNF367 is only present in four datasets totalling 295 samples.

Table 6. Individual breast cancer survival analysis of the top ten Master Transcriptional Regulators identified by ARACNe, using the BreastMark algorithm.

Transcription Factor	Entrez Gene ID	Hazard Ratio	P-value	Sample Number
ATAD2	29028	1.378 (1.224 - 1.552)	1.03E-07	2576
E2F1	1869	1.301 (1.15 - 1.472)	2.92E-05	2357
E2F8	79733	1.375 (1.214 - 1.558)	4.74E-07	2281
FOXM1	2305	1.578 (1.392 - 1.788)	5.45E-13	2357
HMGB2	3148	1.271 (1.122 - 1.439)	0.0001493	2357
MYBL2	4605	1.506 (1.339 - 1.694)	7.08E-12	2652
PTTG1	9232	1.586 (1.402 - 1.794)	1.25E-13	2437
TCF19	6941	1.27 (1.097 - 1.471)	0.00136	1378
UHRF1	29128	1.318 (1.144 - 1.52)	0.0001328	1533
ZNF367	195828	1.08 (0.8274 - 1.41)	0.571	295

Based on the mechanistic data underpinning the OncoMasTR panel, the applicants also believe the predictive power of the panel will have a capacity in predicting response to CDK4/6 inhibitors such as palbociclib. Palbociclib is an orally active, highly selective inhibitor of the cyclin-dependent kinases CDK4/6, which was initially assessed as a combination therapy with letrozole in advanced ER+ Her2+ breast cancer, in the PALOMA-1 trial (Richard S. Finn, 2014). Results from this trial have shown that the addition of palbociclib to a standard regimen extends survival by 10 months, which is a very promising result in these late-stage patients. Based on the mechanistic data underpinning OncoMasTR, the Applicant believes that it is likely to have predictive utility in terms of response to this novel therapy.

Calculating the MTR10+CDKN2A signature score in pablociclib treated cell lines

Pablociclib is an inhibitor of cyclin D kinases and its effects on human breast cancer cell lines were examined previously by Finn *et al.* Briefly, 47 human cell lines, representing the molecular subtypes of breast cancer, were treated with pablociclib and their gene expression profiles, along their IC50 values, were calculated. The gene expression data was downloaded from the Gene Expression Omnibus for the 47 cell lines, along with the accompanying IC50 data (accession number GSE18496). The gene expression data for the 10 MTRs described here was split on a gene by gene basis using median expression across all cell lines as a cut-off. Those cell lines with greater or lower than median expression of a gene were given a value of 2 or 1 for that gene, respectively. This was repeated for each of the ten genes. The expression of CDKN2A across the cell lines was split equally in three, those cell lines with high or low expression were given a value of 2 and those with an intermediate expression level were given a value of 1. A score was then calculated for each cell line by summing the individual gene scores. Figure 13 shows a plot of IC50 values versus the signature score (correlation co-efficient=0.319, p-value=0.03). The significant p-value from the *in vitro* data suggests that the MTRs can provide predictive value in respect to patients receiving CDK4/6 inhibitors to treat cancer.

In the specification the terms "comprise, comprises, comprised and comprising" or any variation thereof and the terms “include, includes, included and including" or any variation thereof are considered to be totally interchangeable and they should all be afforded the widest possible interpretation and vice versa.

The invention is not limited to the embodiments hereinbefore described but may be varied in both construction and detail.

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Claims

1. A method of predicting risk of recurrence of cancer in an individual with cancer, the method comprising a step of assaying a cancer sample from the individual for positive expression of at least four genes, or proteins encoded by said genes, selected from FOXM1, UHRF1, PTTG1, E2F1, MYBL2, HMGB2, ATAD2, E2F8, ZNF367 and TCF19, wherein positive expression of the at least four genes, or proteins encoded by said genes, correlates with increased risk of recurrence of cancer compared with an individual with cancer who does not exhibit positive expression of the at least four genes or proteins encoded by those genes.

2. A method of predicting risk of recurrence of cancer in an individual with cancer following treatment with CDK4/6 inhibitors, the method comprising a step of assaying a cancer sample from the individual for positive expression of at least four genes, or proteins encoded by said genes, selected from FOXM1, UHRF1, PTTG1, E2F1, MYBL2, HMGB2, ATAD2, E2F8, ZNF367 and TCF19, wherein positive expression of the at least four genes correlates with increased risk of recurrence of cancer in an individual with cancer following treatment with CDK4/6 inhibitors compared with an individual with cancer who does not exhibit positive expression of the at least four genes or proteins encoded by those genes.

3. A method according to Claim 1 or Claim 2, wherein the at least four genes selected are FOXM1, PTTG1, UHRF1 and HMGB2.

4. A method according to any one of Claims 1 to 3, wherein the cancer is selected from the group comprising node-negative, ER-positive breast cancer; early stage, node positive breast cancer; multiple myeloma, prostate cancer, glioblastoma, lymphoma, fibrosarcoma; myxosarcoma; liposarcoma; chondrosarcoma; osteogenic sarcoma; chordoma; angiosarcoma; endotheliosarcoma; lymphangiosarcoma; lymphangioendotheliosarcoma; synovioma; mesothelioma; Ewing's tumour; leiomyosarcoma; rhabdomyosarcoma; colon carcinoma; pancreatic cancer; breast cancer; ovarian cancer; squamous cell carcinoma; basal cell carcinoma; adenocarcinoma; sweat gland carcinoma; sebaceous gland carcinoma; papillary carcinoma; papillary adenocarcinomas; cystadenocarcinoma; medullary carcinoma; bronchogenic carcinoma; renal cell carcinoma; hepatoma; bile duct carcinoma; choriocarcinoma; seminoma; embryonal carcinoma; Wilms' tumour; cervical cancer; uterine cancer; testicular tumour; lung carcinoma; small cell lung carcinoma; bladder carcinoma; epithelial carcinoma;

glioma; astrocytoma; medulloblastoma; craniopharyngioma; ependymoma; pinealoma; hemangioblastoma; acoustic neuroma; oligodendroglioma; meningioma; melanoma; retinoblastoma; and leukemias.

5. A method according to Claim 4, wherein the cancer is breast cancer.

6. A method according to Claim 5, wherein the breast cancer is early stage, node-negative breast cancer, and optionally wherein the breast cancer is early stage, node-negative or early stage, node positive, ER positive breast cancer.

7. A method of predicting risk of recurrence of breast cancer in an early stage, node negative breast cancer patient, the method comprising a step of assaying a cancer tumour sample from the patient for positive expression of at least four genes, or proteins encoded by those genes, selected from FOXM1, UHRF1, PTTG1, E2F1, MYBL2, HMGB2, ATAD2, E2F8, ZNF367 and TCF19, wherein positive expression of the at least four genes, or proteins encoded by those genes, correlates with increased risk of recurrence of cancer compared with a patient with cancer who does not exhibit positive expression of the at least four genes or proteins encoded by those genes.

8. A method of determining a 5-year survival rate or a 10-year survival rate of an individual diagnosed with breast cancer, the method comprising a step of assaying a cancer tumour sample from the individual for positive expression of at least four genes, or proteins encoded by those genes, selected from FOXM1, UHRF1, PTTG1, E2F1, MYBL2, HMGB2, ATAD2, E2F8, ZNF367 and TCF19, wherein positive expression of the at least four genes, or proteins encoded by those genes, correlates with decreased chance of a 5-year survival rate or a 10-year survival rate compared with an individual with cancer who does not exhibit positive expression of the at least four genes or proteins encoded by those genes.

9. A method according to Claim 1, Claim 2, Claim 7 or Claim 8, the method further comprising the step of assaying for the expression of $p16^{INK4A}$ gene or a protein encoded by said gene, wherein dysregulated expression of $p16^{INK4A}$, in combination with positive expression of the at least four genes or proteins encoded by those genes, correlates with increased risk of recurrence of cancer, or a decreased chance of a 5-year survival rate or a 10-year survival rate, compared with an individual with cancer who does not exhibit dysregulated expression of $p16^{INK4A}$ and positive expression of the at least four genes or proteins encoded by those genes.

10. A method of identifying a cancer patient that is suitable for treatment with a therapy for preventing recurrence or progression of the cancer, the method comprising a step of assaying a cancer sample from the cancer patient for positive expression of at least four genes selected from FOXM1, UHRF1, PTTG1, E2F1, MYBL2, HMGB2, ATAD2, E2F8, ZNF367 and TCF19, wherein positive expression of the at least four genes or proteins encoded by those genes compared with an individual with cancer who does not exhibit positive expression of the at least four genes or proteins encoded by those genes, is indicative that the cancer patient is suitable for treatment with a therapy for preventing recurrence or progression of the cancer.

11. A method according to Claim 10, the method further comprising the step of assaying for the expression of $p16^{INK4A}$ gene or a protein encoded by said gene, wherein dysregulated expression of $p16^{INK4A}$, in combination with positive expression of the at least four genes or proteins encoded by those genes, when compared with an individual with cancer who does not exhibit dysregulated expression of $p16^{INK4A}$ and positive expression of the at least four genes or proteins encoded by those genes, is indicative that the cancer patient is suitable for treatment with an adjuvant therapy for preventing recurrence or progression of the cancer.

12. A method according to Claim 10 or Claim 11, wherein the therapy is a neoadjuvant or an adjuvant therapy, or a combination of both.

13. A method according to Claim 12, wherein the neoadjuvant therapy and adjuvant therapy is an agent selected from trastuzumab, lapatinib, neratinib, afatinib, pertuzumab, CDK4/6 inhibitors, cyclophosphamide, methotrexate, 5-fluorouracil, gemcitabine, adriamycin (doxorubicin), epirubucin, docetaxel, paclitaxel, capecitabine, and tamoxifen.

14. A system for obtaining data from at least one test sample obtained from at least one individual, the system comprising:

a determination module configured to receive at least one test sample and perform at least one test analysis on the test sample to assay for expression of at least four genes or proteins encoded by those genes selected from FOXM1, UHRF1, PTTG1, E2F1, MYBL2, HMGB2, ATAD2, E2F8, ZNF367 and TCF19;

optionally, a storage system for storing expression data generated by the determination module; and

a display module for displaying a content based in part on the data output from said determination module, wherein the content comprises a signal indicative of the expression of at the least two genes or proteins encoded by those genes.

15. The system according to Claim 14, wherein the at least four genes, or proteins encoded by said genes, are FOXM1, PTTG1, UHRF1 and HMGB2.

16. A method for monitoring the effectiveness of treatment of cancer in an individual with cancer, the method comprising a step of assaying a cancer sample from the individual with cancer for expression of at least four genes or proteins encoded by said genes selected from FOXM1, UHRF1, PTTG1, E2F1, MYBL2 and HMGB2, wherein higher expression of at least four genes selected from FOXM1, UHRF1, PTTG1, E2F1, MYBL2, HMGB2, ATAD2, E2F8, ZNF367 and TCF19 correlates with ineffective treatment and poor outcome compared with an individual with cancer who has lower expression of the at least four genes or proteins encoded by those genes.

17. The method according to Claim 16, further comprising the step of assaying the cancer sample for expression of the $p16^{INK4A}$ gene, or a protein encoded by said gene, in combination with assaying the at least four genes or proteins encoded by said genes, whereby dysregulated expression of $p16^{INK4A}$ correlates with ineffective treatment and poor outcome compared with an individual with cancer who has moderate expression of $p16^{INK4A}$.

18. The method according to Claim 16 or Claim 17, wherein the at least four genes, or proteins encoded by said genes, are FOXM1, PTTG1, UHFR1 and HMGB2.

19. A method according to Claim 16 to 18, wherein the treatment is a neoadjuvant or an adjuvant therapy, or a combination of both.

20. A method according to Claim 19, wherein the neoadjuvant therapy and adjuvant therapy is an agent selected from trastuzumab, lapatinib, neratinib, afatinib, pertuzumab, CDK4/6 inhibitors, cyclophosphamide, methotrexate, 5-fluorouracil, gemcitabine, adriamycin (doxorubicin), epirubucin, docetaxel, paclitaxel, capecitabine, and tamoxifen.

21. A method of predicting risk of recurrence or progression of breast cancer in a patient, and treating the patient with a therapy for preventing recurrence of the cancer, the method comprising a step of assaying a cancer sample from the patient for positive expression of

at least four genes selected from FOXM1, UHRF1, PTTG1, E2F1, MYBL2, HMGB2, ATAD2, E2F8, ZNF367 and TCF19, wherein positive expression of the at least four genes, or proteins encoded by those genes, correlates with increased risk of recurrence or progression of cancer compared with a patient with cancer who does not exhibit positive expression of the at least four genes, or proteins encoded by those genes; and administering a neoadjuvant or an adjuvant therapy, or a combination of both, to the patient to prevent recurrence or progression of the cancer.

22. A method according to Claim 21, wherein the neoadjuvant therapy and adjuvant therapy is an agent selected from trastuzumab, lapatinib, neratinib, afatinib, pertuzumab, CDK4/6 inhibitors, cyclophosphamide, methotrexate, 5-fluorouracil, gemcitabine, adriamycin (doxorubicin), epirubucin, docetaxel, paclitaxel, capecitabine, and tamoxifen.

23. A method according to Claim 21 or Claim 22, the method further comprising the step of assaying for the expression of $p16^{INK4A}$ gene or a protein encoded by said gene, wherein dysregulated expression of $p16^{INK4A}$, in combination with positive expression of the at least four genes or proteins encoded by those genes, correlates with increased risk of recurrence or progression of cancer compared with a patient with cancer who does not exhibit dysregulated expression of $p16^{INK4A}$ and positive expression of the at least four genes, or proteins encoded by those genes.

Figure 1A

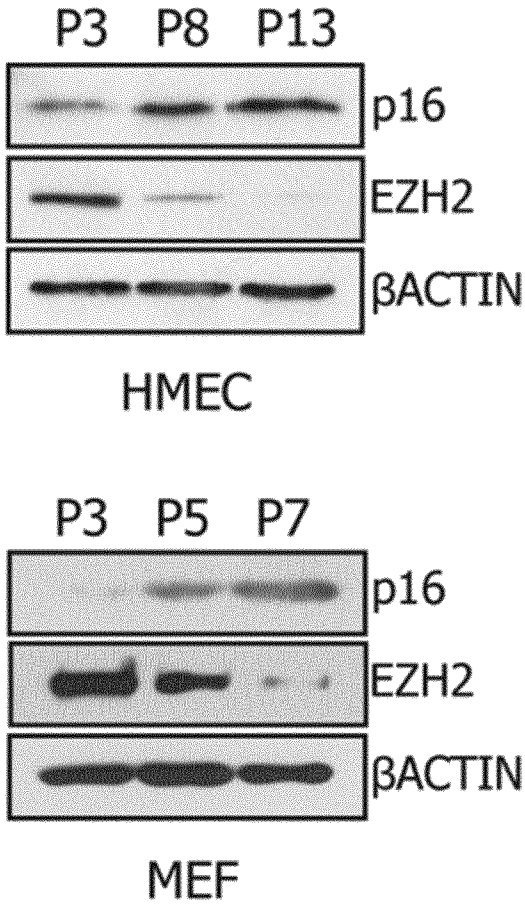


Figure 1B

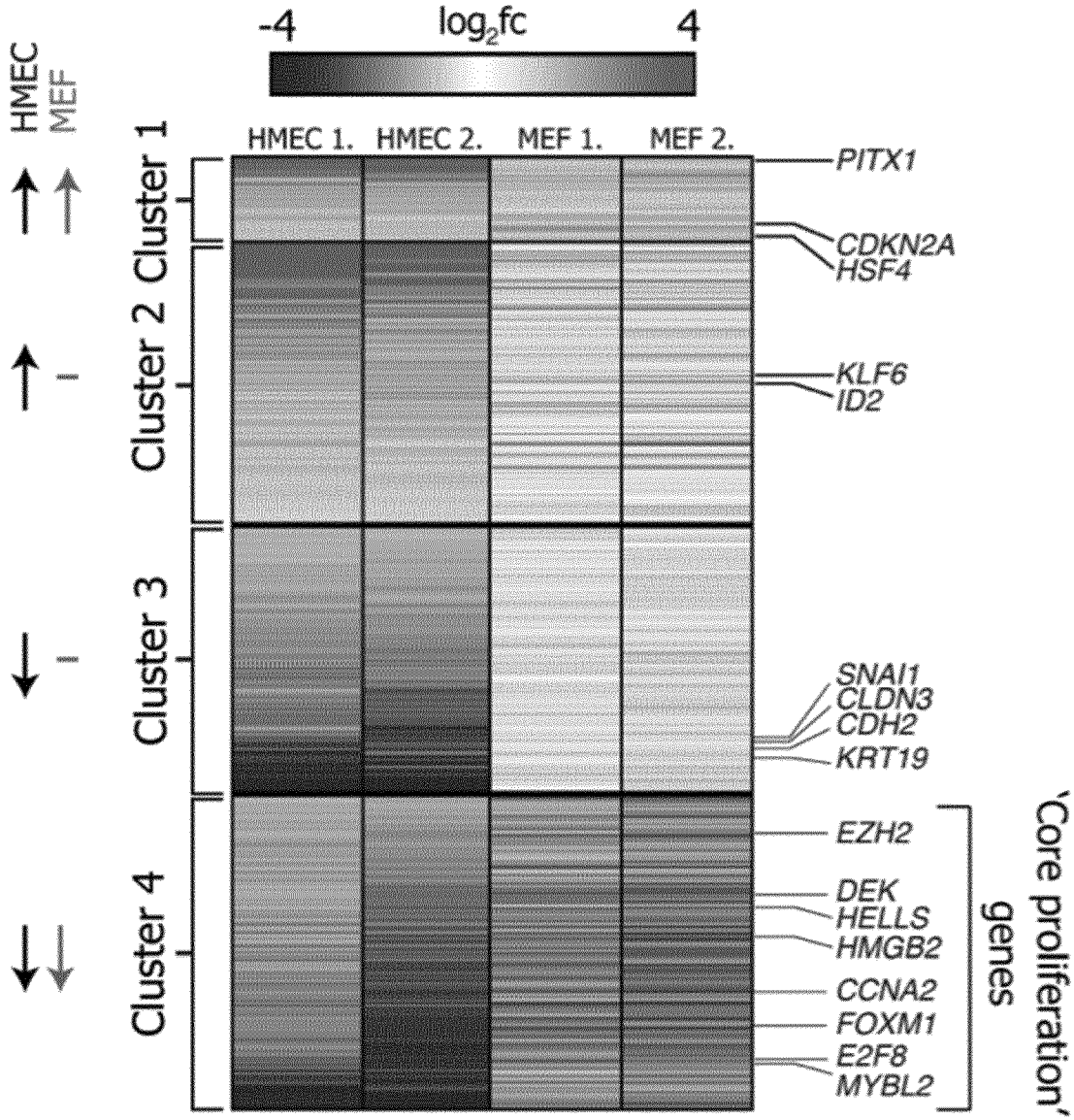

-4
log2fc
4
HMEC
MEF
HMEC 1.
HMEC 2.
MEF 1.
MEF 2.
Cluster 1
Cluster 2
Cluster 3
Cluster 4
PITX1
CDKN2A
HSF4
KLF6
ID2
SNAI1
CLDN3
CDH2
KRT19
EZH2
DEK
HELLS
HMGB2
CCNA2
FOXM1
E2F8
MYBL2
'Core proliferation' genes

Figure 1C

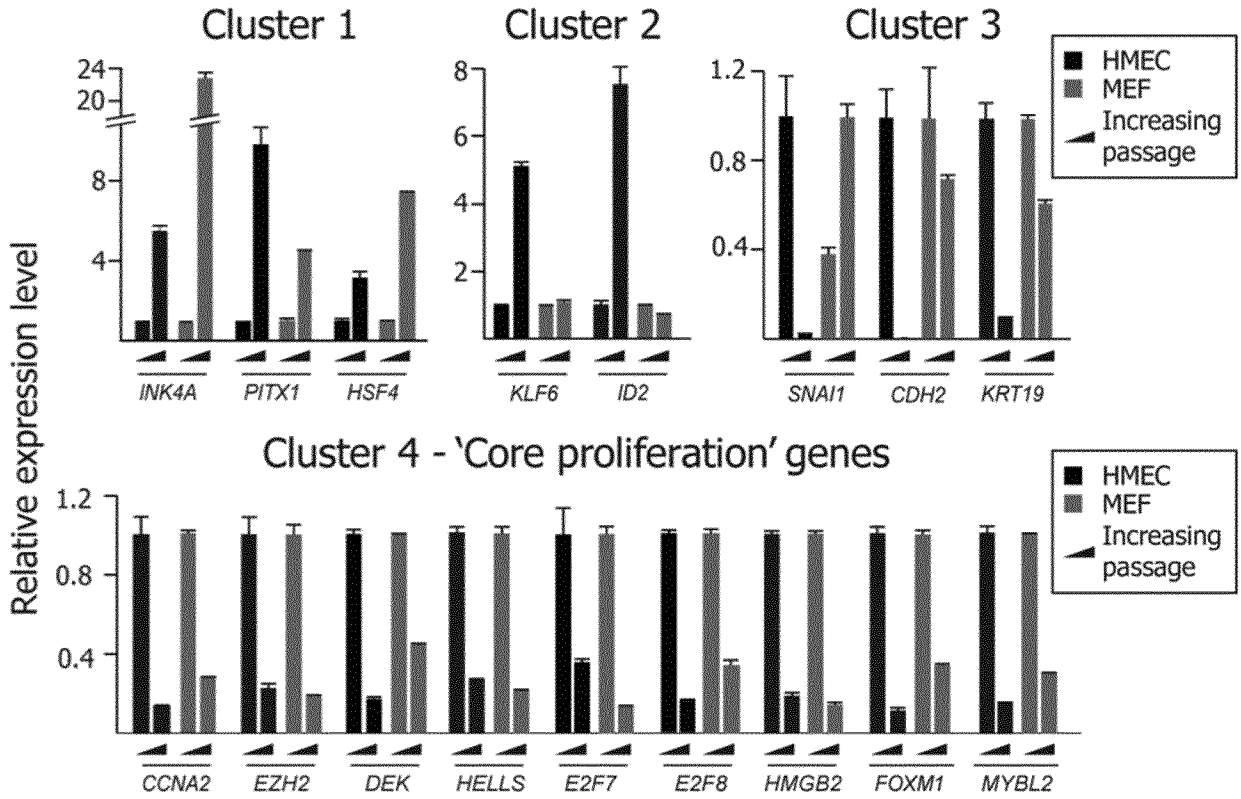

Cluster 1
Cluster 2
Cluster 3
HMEC
MEF
Increasing passage
Relative expression level
24
20
8
4
8
6
4
2
1.2
0.8
0.4
INK4A
PITX1
HSF4
KLF6
ID2
SNAI1
CDH2
KRT19
Cluster 4 - 'Core proliferation' genes
HMEC
MEF
Increasing passage
1.2
0.8
0.4
CCNA2
EZH2
DEK
HELLS
E2F7
E2F8
HMGB2
FOXM1
MYBL2

Figure 1D

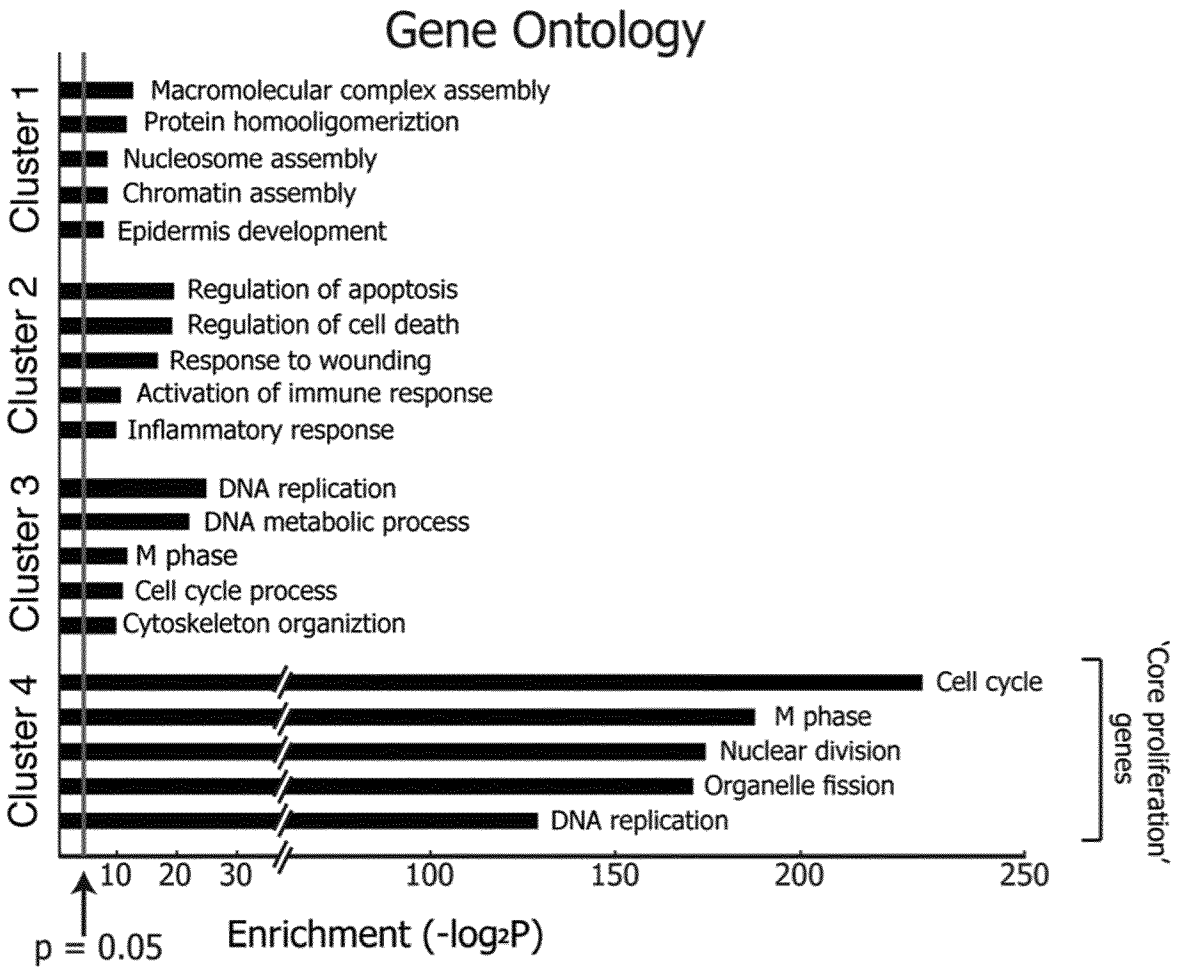

Gene Ontology
Cluster 1
Macromolecular complex assembly
Protein homooligomeriztion
Nucleosome assembly
Chromatin assembly
Epidermis development
Cluster 2
Regulation of apoptosis
Regulation of cell death
Response to wounding
Activation of immune response
Inflammatory response
Cluster 3
DNA replication
DNA metabolic process
M phase
Cell cycle process
Cytoskeleton organiztion
Cluster 4
Cell cycle
M phase
Nuclear division
Organelle fission
DNA replication
'Core proliferation' genes
10 20 30 100 150 200 250
p = 0.05
Enrichment (-log2P)

Figure 1E

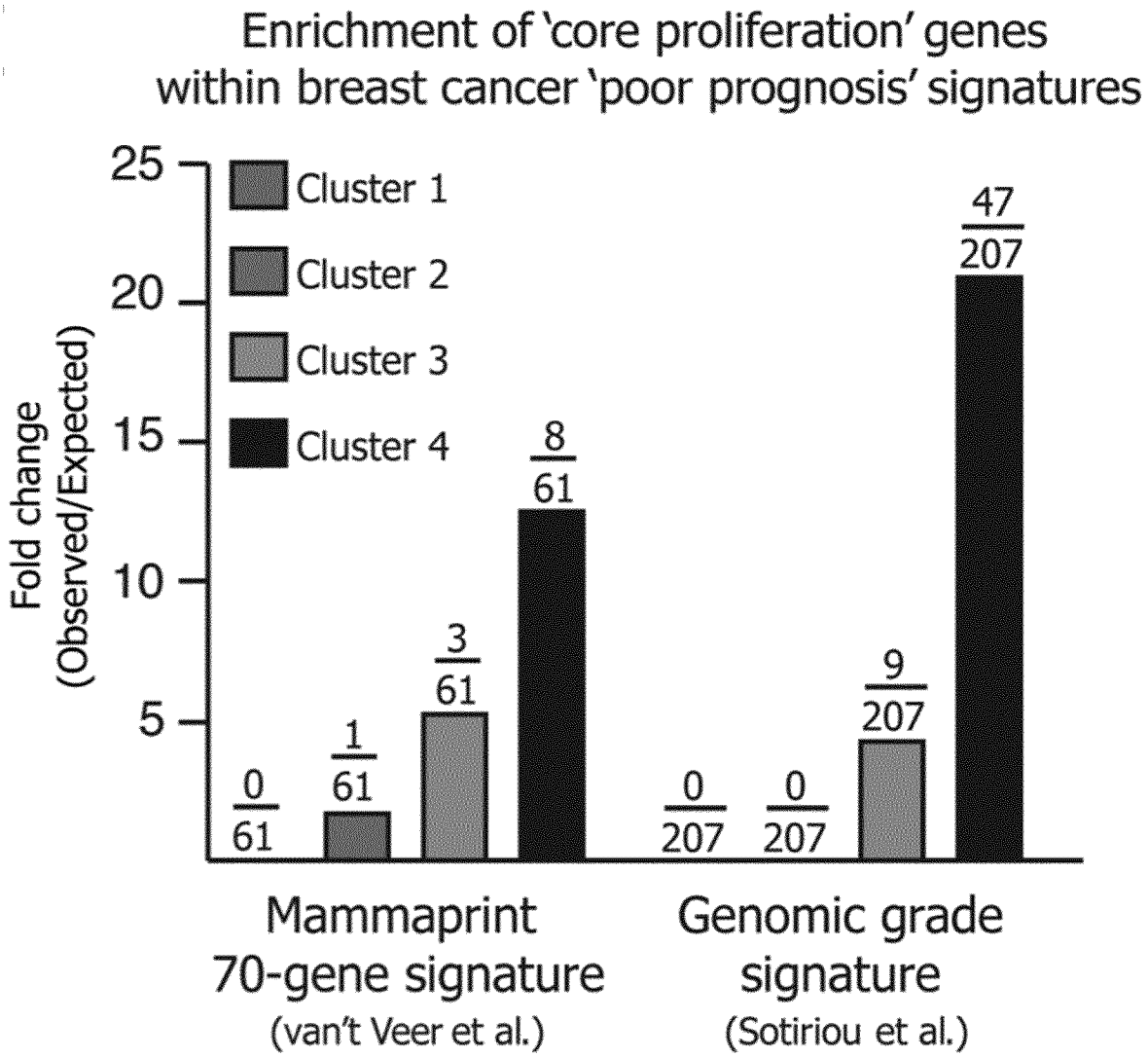

Enrichment of 'core proliferation' genes
within breast cancer 'poor prognosis' signatures
Fold change
(Observed/Expected)
25
20
15
10
5
Cluster 1
Cluster 2
Cluster 3
Cluster 4
0/61
1/61
3/61
8/61
0/207
0/207
9/207
47/207
Mammaprint
70-gene signature
(van't Veer et al.)
Genomic grade
signature
(Sotiriou et al.)

Figure 2A

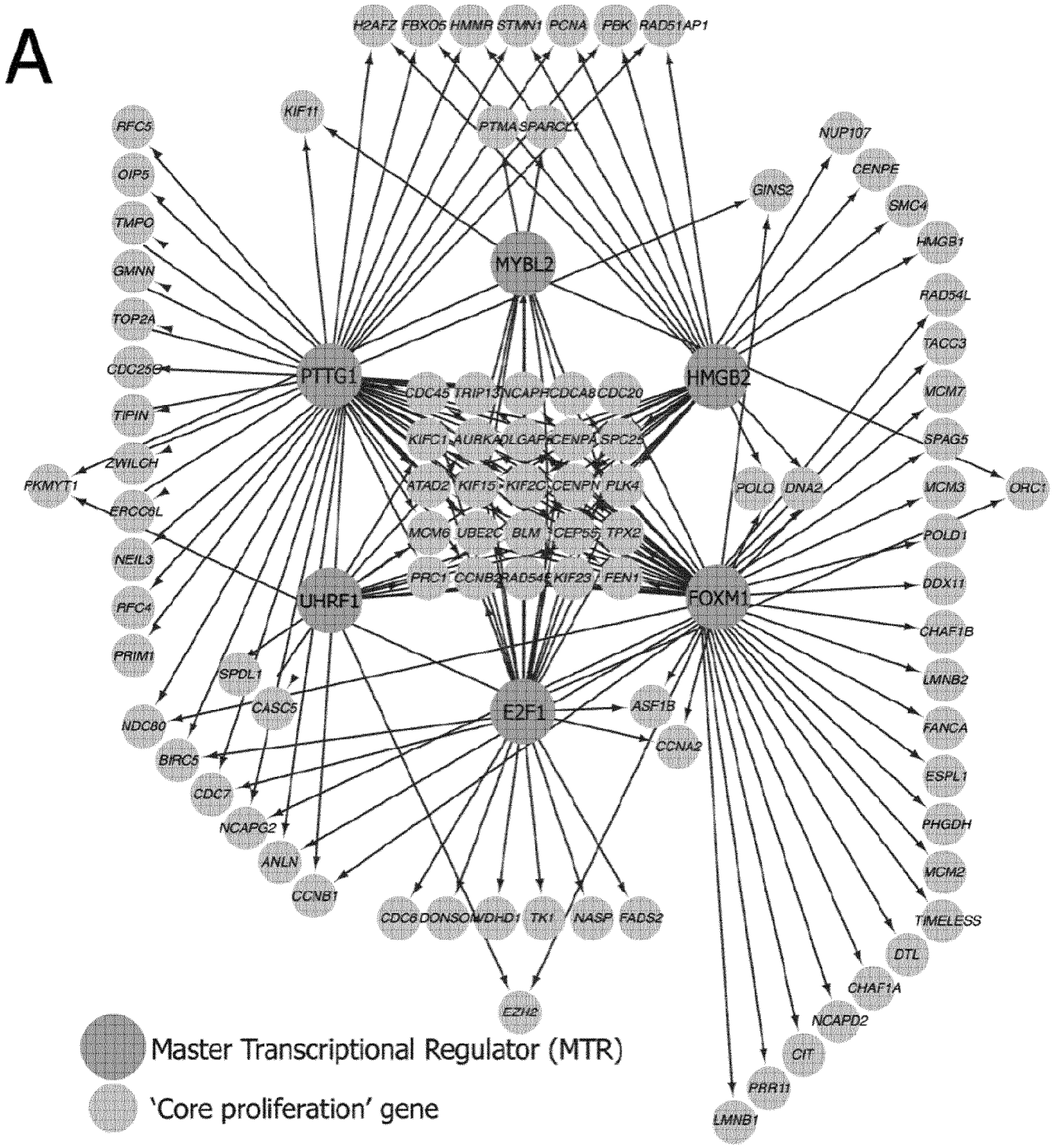

A
H2AFZ
FBXO5
HMMR
STMN1
PCNA
PBK
RAD51AP1
KIF11
PTMA
SPARCL1
RFC5
OIP5
TMPO
GMNN
TOP2A
CDC25C
TIPIN
ZWILCH
PKMYT1
ERCC6L
NEIL3
RFC4
PRIM1
MYBL2
PTTG1
HMGB2
UHRF1
FOXM1
E2F1
CDC45
TRIP13
NCAPH
CDCA8
CDC20
KIFC1
AURKA
DLGAP5
CENPA
SPC25
ATAD2
KIF15
KIF2C
CENPN
PLK4
MCM6
UBE2C
BLM
CEP55
TPX2
PRC1
CCNB2
RAD54B
KIF23
FEN1
NUP107
CENPE
GINS2
SMC4
HMGB1
RAD54L
TACC3
MCM7
SPAG5
POLQ
DNA2
MCM3
ORC1
POLD1
DDX11
CHAF1B
LMNB2
FANCA
ESPL1
PHGDH
MCM2
TIMELESS
DTL
CHAF1A
NCAPD2
CIT
PRR11
LMNB1
SPDL1
CASC5
NDC80
BIRC5
CDC7
NCAPG2
ANLN
CCNB1
ASF1B
CCNA2
CDC6
DONSON
RRM2
TK1
NASP
FADS2
EZH2
Master Transcriptional Regulator (MTR)
'Core proliferation' gene

Figure 2B

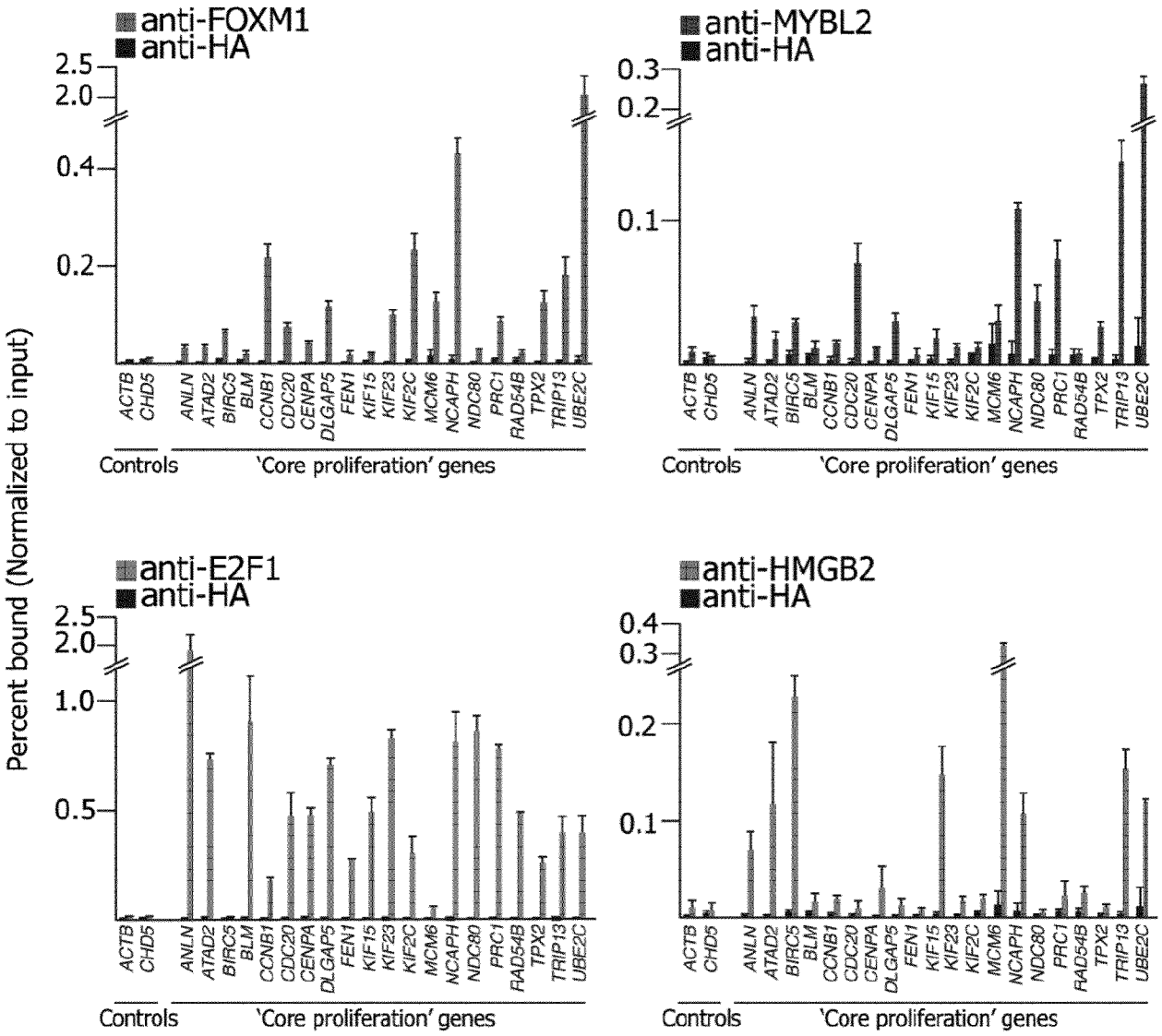

anti-FOXM1
anti-HA
anti-MYBL2
anti-HA
anti-E2F1
anti-HA
anti-HMGB2
anti-HA
Percent bound (Normalized to input)
Controls
'Core proliferation' genes
ACTB
CHD5
ANLN
ATAD2
BIRC5
BLM
CCNB1
CDC20
CENPA
DLGAP5
FEN1
KIF15
KIF23
KIF2C
MCM6
NCAPH
NDC80
PRC1
RAD54B
TPX2
TRIP13
UBE2C

Figure 2C

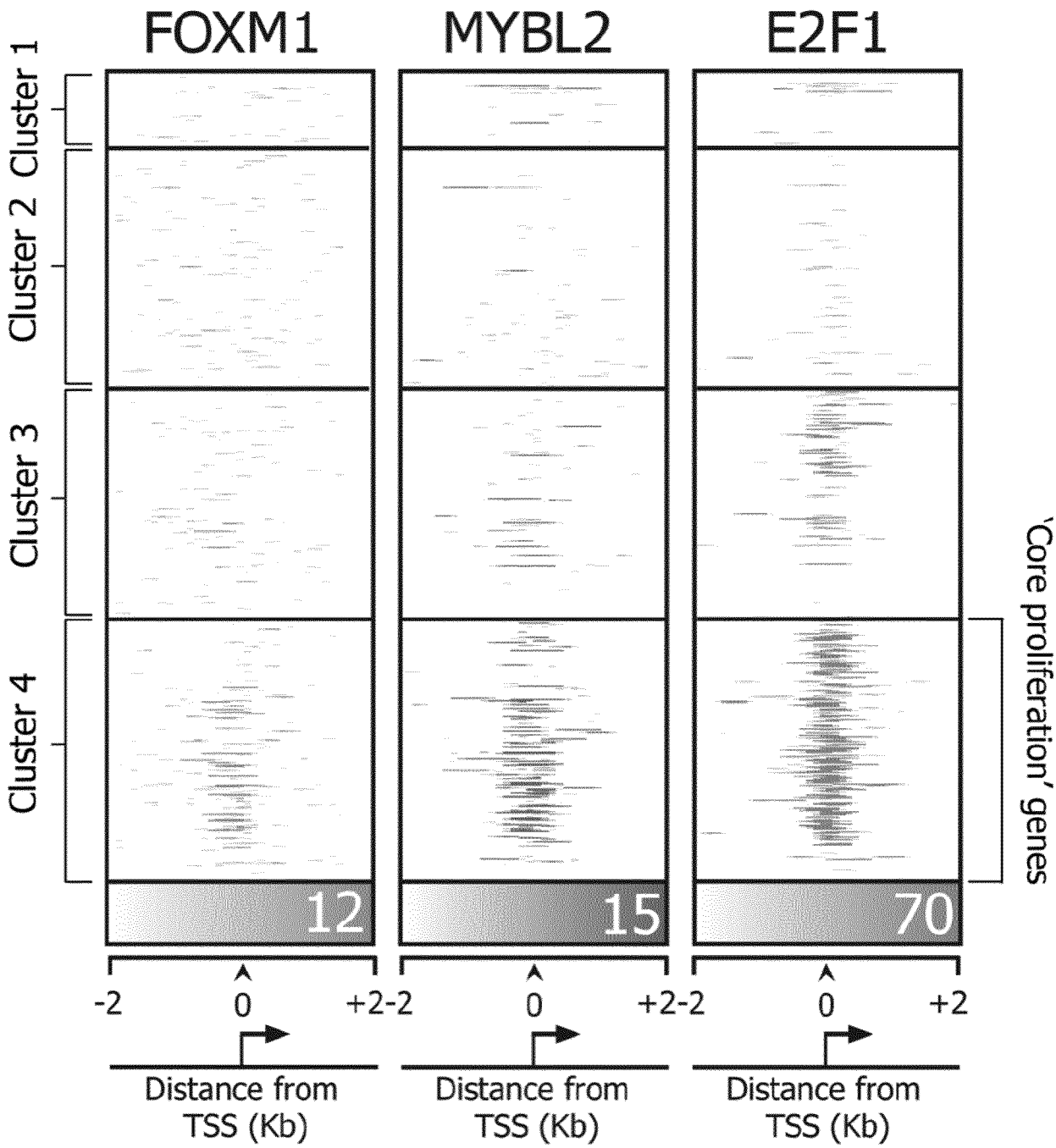

FOXM1
MYBL2
E2F1
Cluster 1
Cluster 2
Cluster 3
Cluster 4
'Core proliferation' genes
12
15
70
-2
0
+2
Distance from TSS (Kb)

Figure 2D

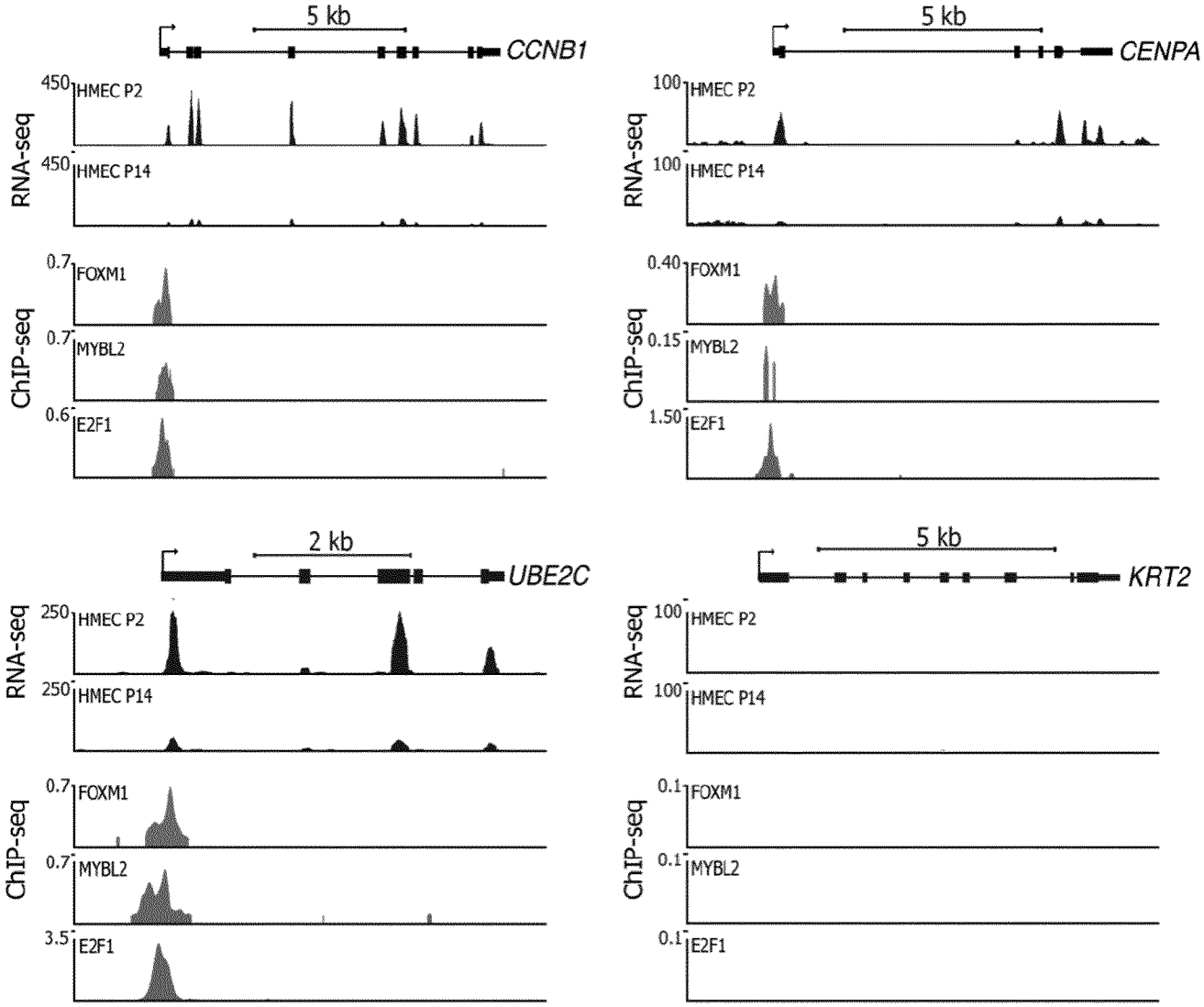

5 kb
CCNB1
RNA-seq
450 HMEC P2
450 HMEC P14
ChIP-seq
0.7 FOXM1
0.7 MYBL2
0.6 E2F1
5 kb
CENPA
RNA-seq
100 HMEC P2
100 HMEC P14
ChIP-seq
0.40 FOXM1
0.15 MYBL2
1.50 E2F1
2 kb
UBE2C
RNA-seq
250 HMEC P2
250 HMEC P14
ChIP-seq
0.7 FOXM1
0.7 MYBL2
3.5 E2F1
5 kb
KRT2
RNA-seq
100 HMEC P2
100 HMEC P14
ChIP-seq
0.1 FOXM1
0.1 MYBL2
0.1 E2F1

Figure 3A

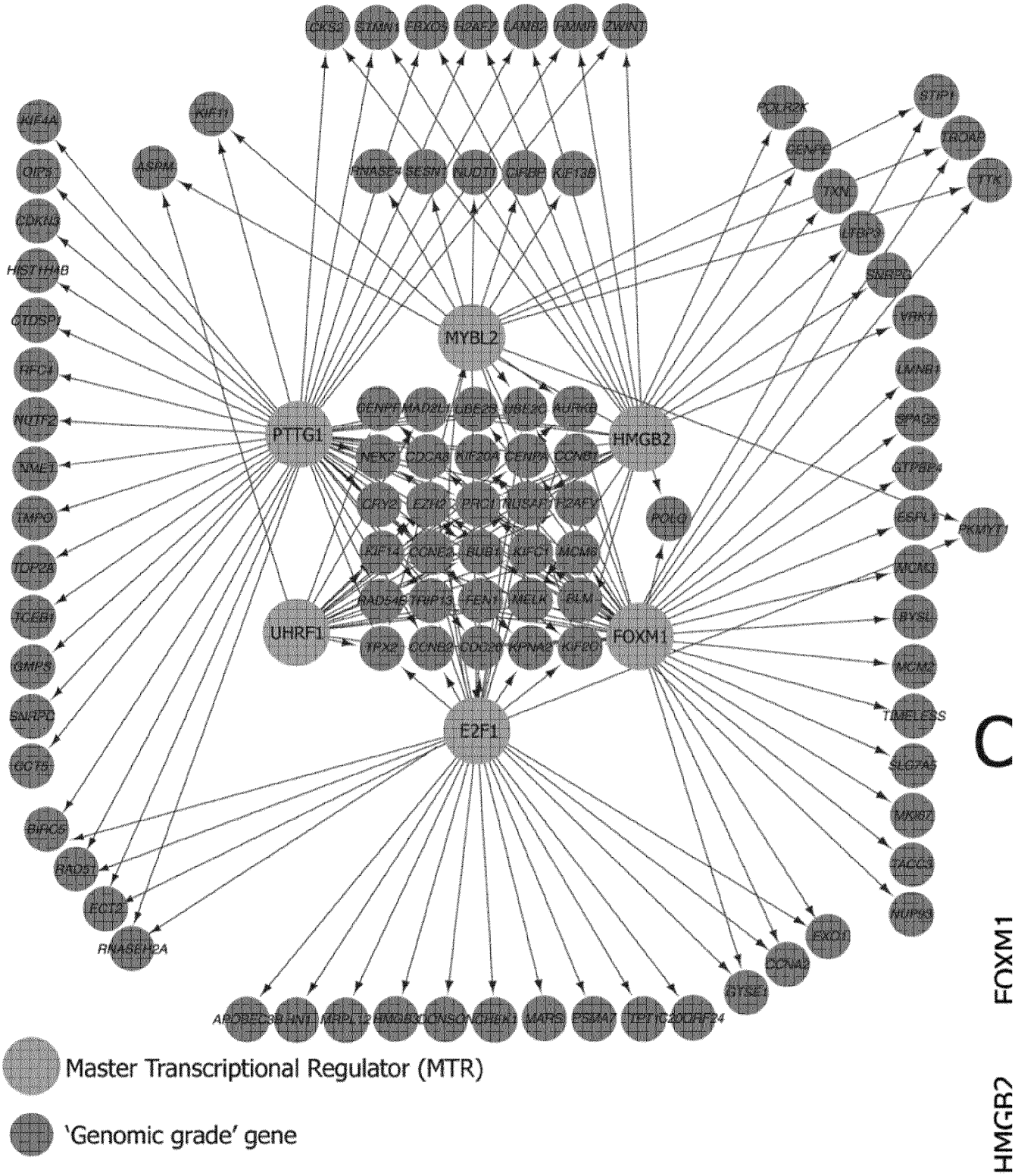

CKS2
STMN1
FBXO5
H2AFZ
LAMB2
PRMMR
ZWINT
KIF11
ASPM
RNASET2
SESN1
NUDT1
CIRBP
KIF13B
MYBL2
PTTG1
HMGB2
UHRF1
FOXM1
E2F1
Master Transcriptional Regulator (MTR)
'Genomic grade' gene
C
FOXM1
HMGB2

Figure 3B

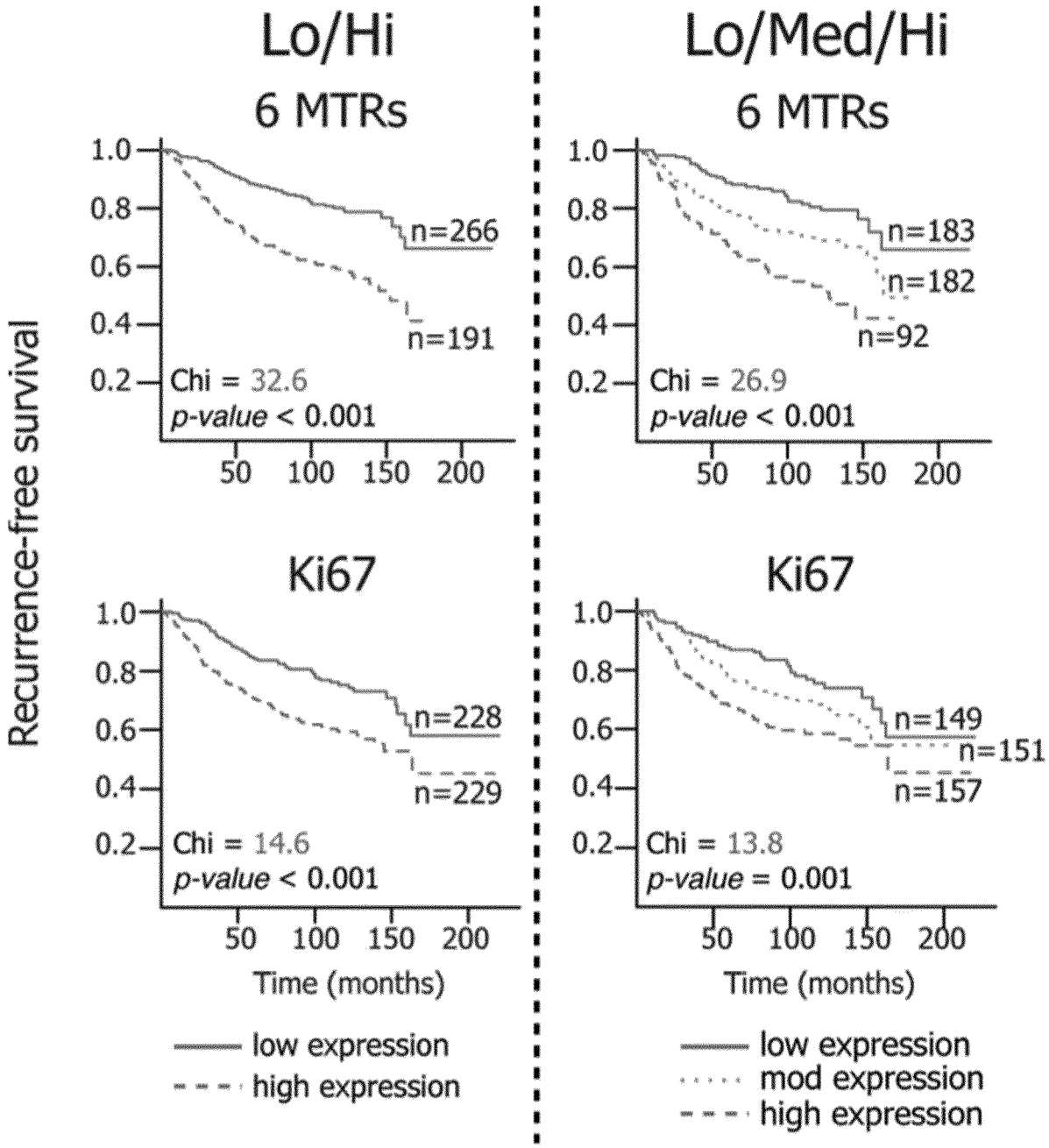

Lo/Hi
6 MTRs
Lo/Med/Hi
6 MTRs
Recurrence-free survival
1.0
0.8
0.6
0.4
0.2
n=266
n=191
Chi = 32.6
p-value < 0.001
n=183
n=182
n=92
Chi = 26.9
p-value < 0.001
50 100 150 200
Ki67
Ki67
n=228
n=229
Chi = 14.6
p-value < 0.001
n=149
n=151
n=157
Chi = 13.8
p-value = 0.001
Time (months)
low expression
high expression
mod expression

Figure 3C

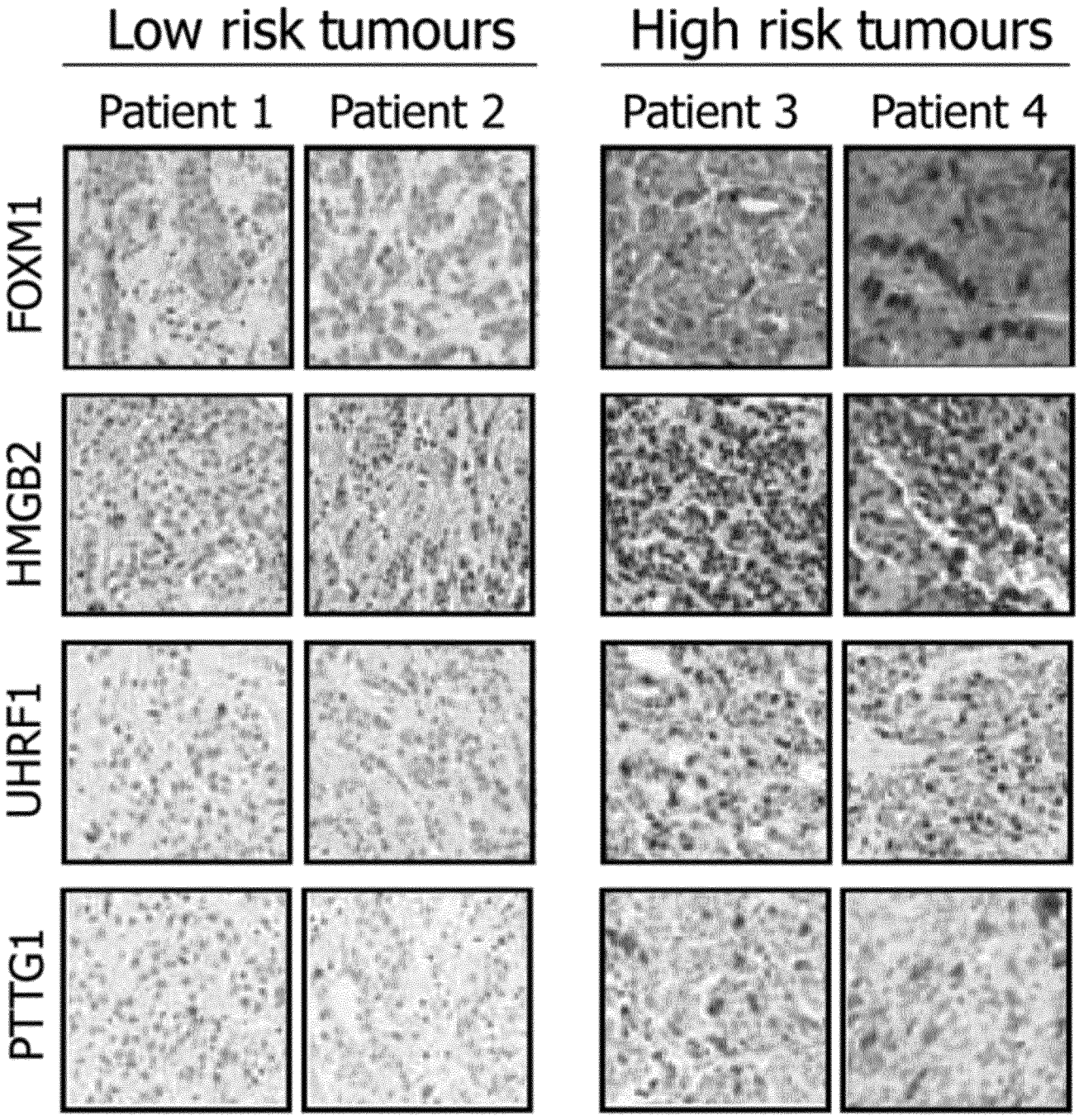

Low risk tumours
High risk tumours
Patient 1
Patient 2
Patient 3
Patient 4
FOXM1
HMGB2
UHRF1
PTTG1

Figure 3D

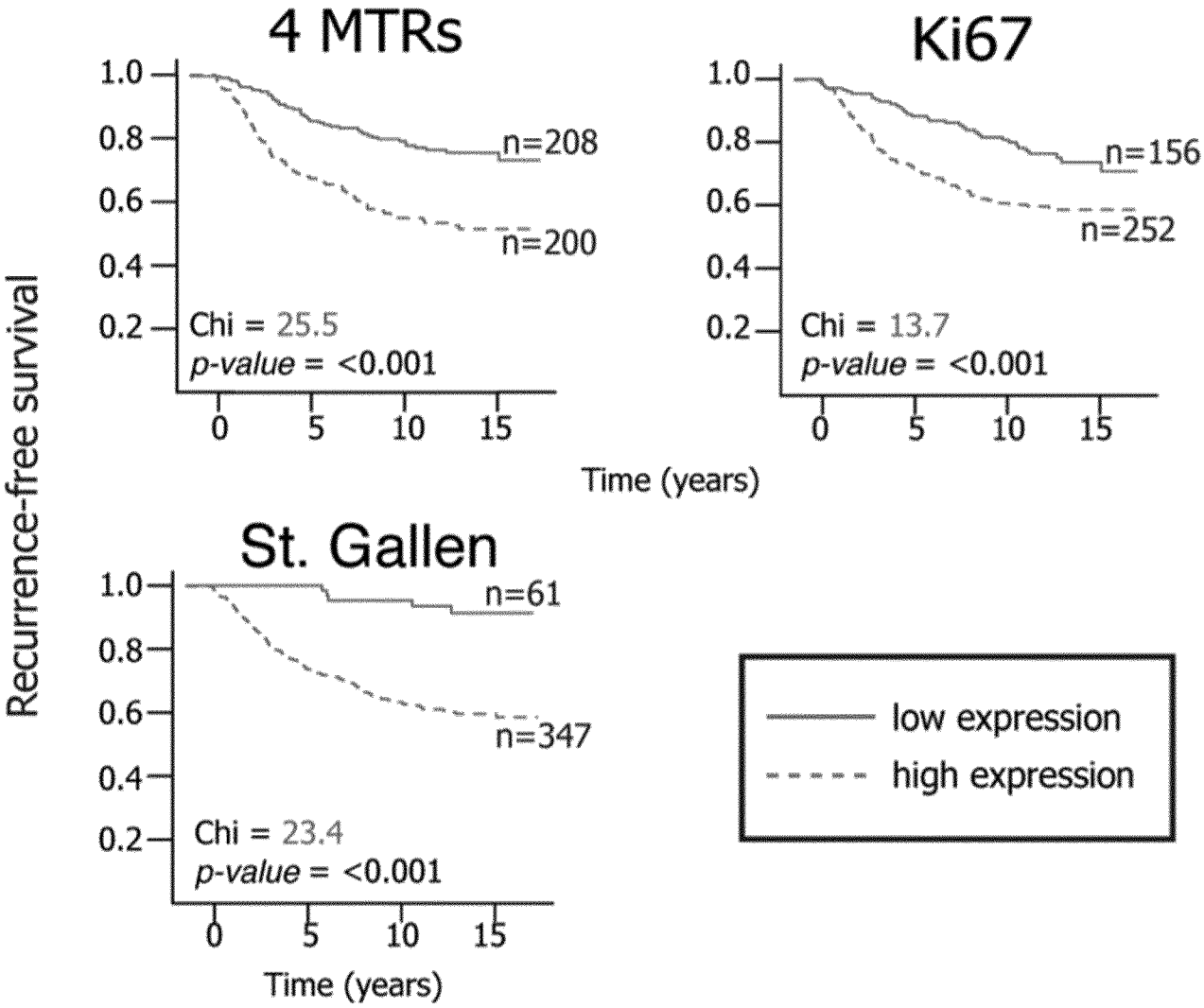

4 MTRs
Ki67
St. Gallen
Recurrence-free survival
1.0
0.8
0.6
0.4
0.2
n=208
n=200
Chi = 25.5
p-value = <0.001
n=156
n=252
Chi = 13.7
p-value = <0.001
n=61
n=347
Chi = 23.4
p-value = <0.001
0
5
10
15
Time (years)
low expression
high expression

Figure 3E

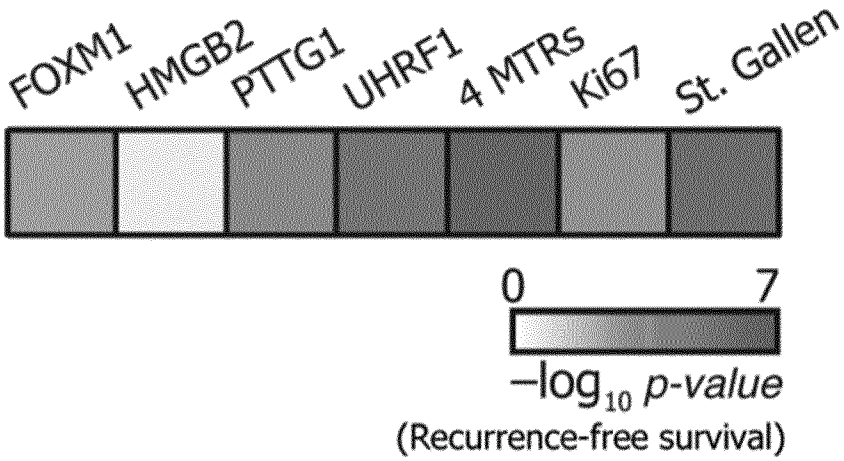

FOXM1
HMGB2
PTTG1
UHRF1
4 MTRs
Ki67
St. Gallen
0
7
–log10 p-value
(Recurrence-free survival)

Figure 4A

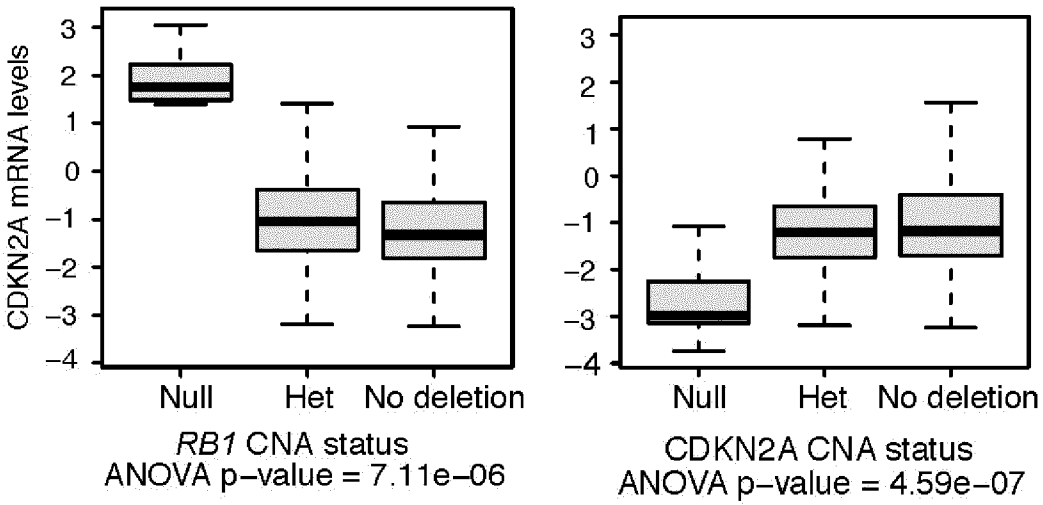


Figure 4B

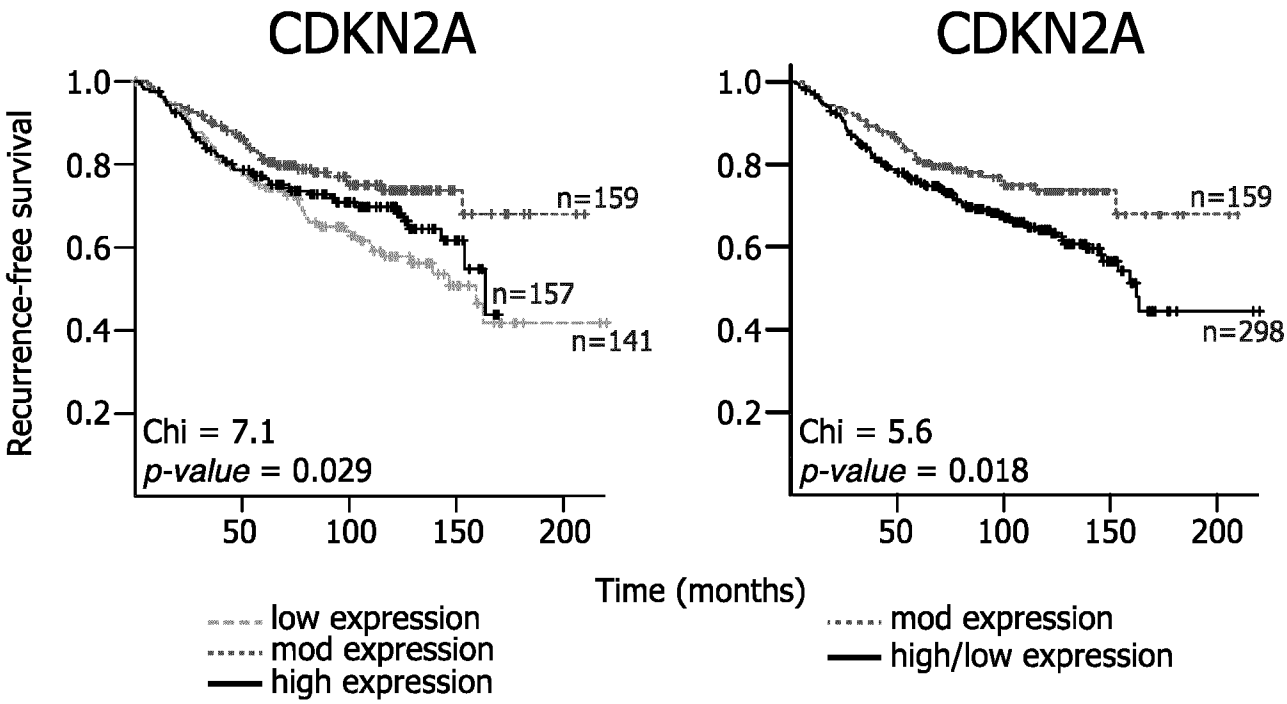


Figure 4C

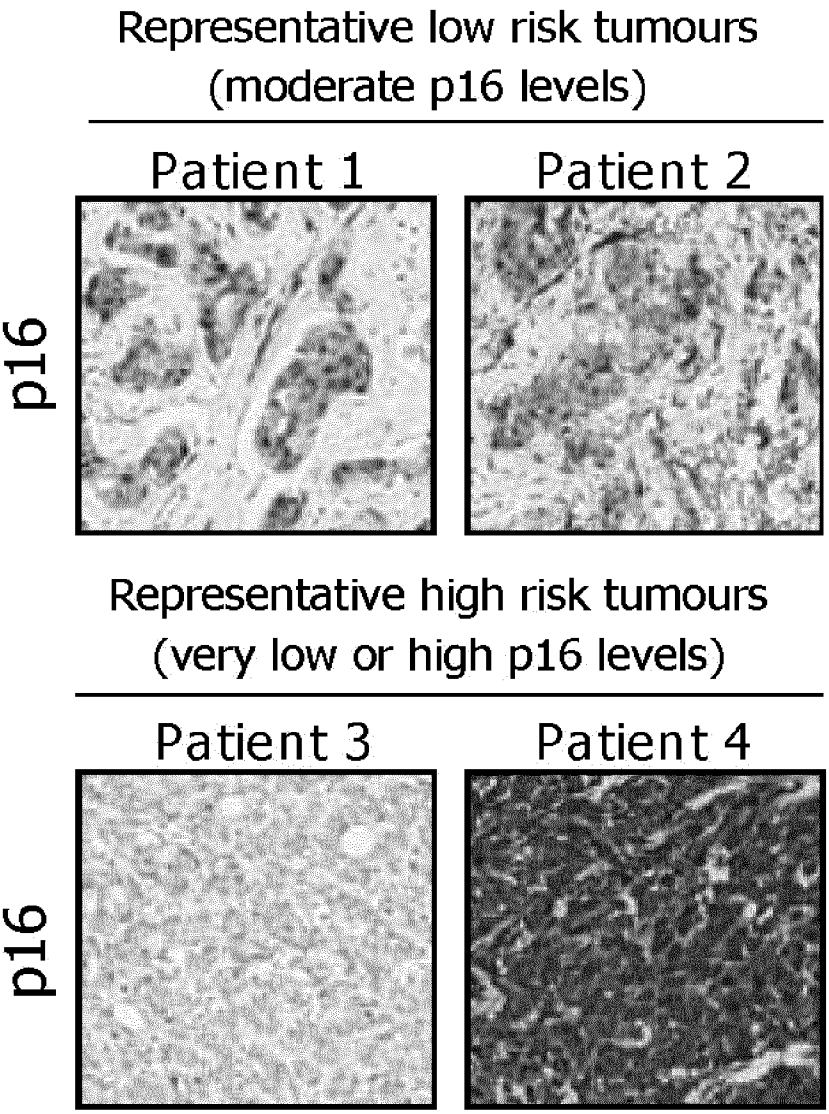

Representative low risk tumours
(moderate p16 levels)
Patient 1
Patient 2
p16
Representative high risk tumours
(very low or high p16 levels)
Patient 3
Patient 4
p16

Figure 4D

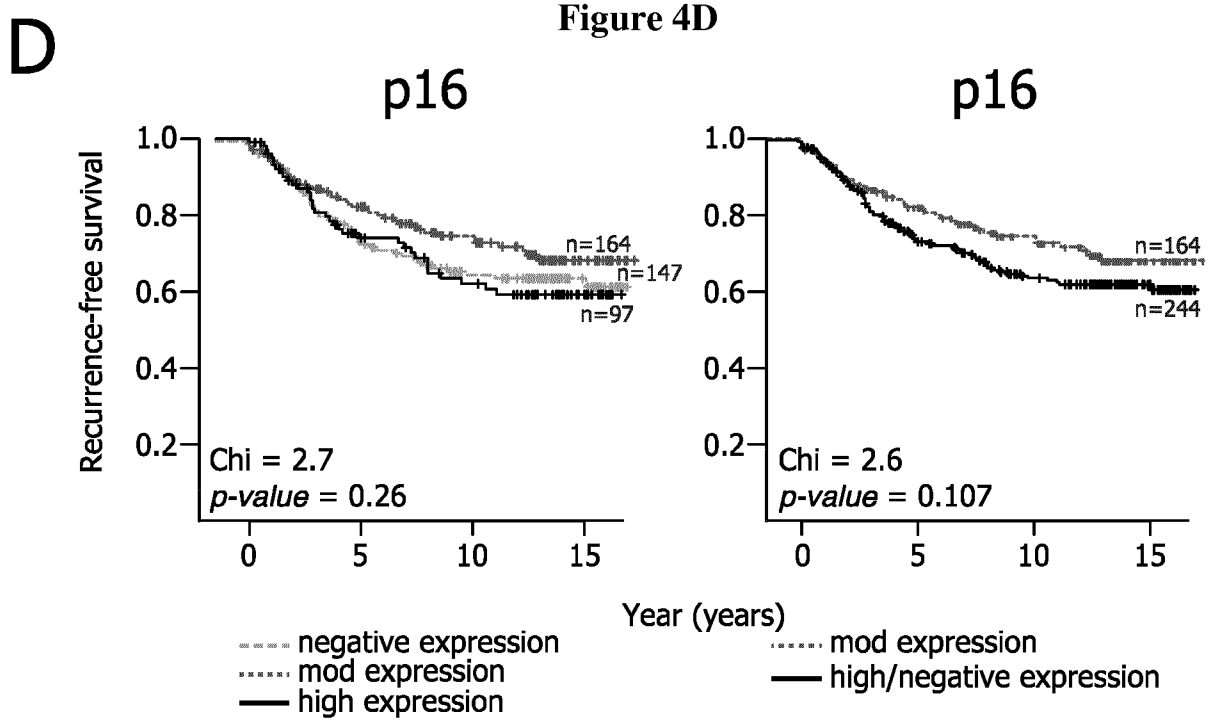


Figure 4E

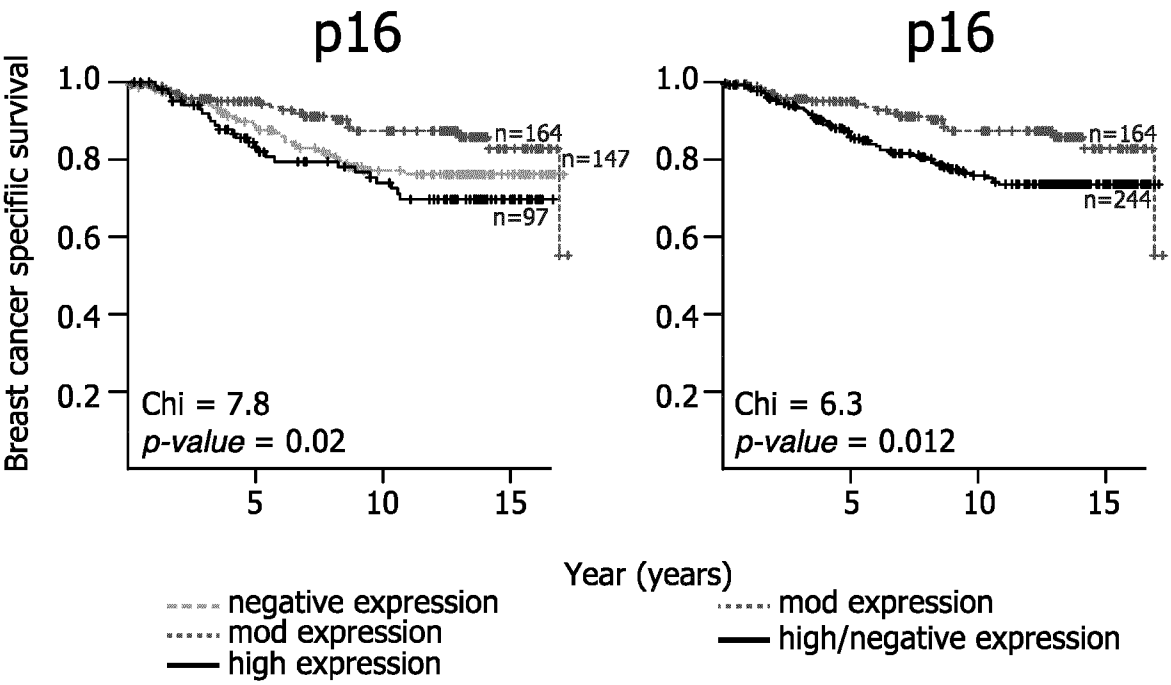


Figure 5A

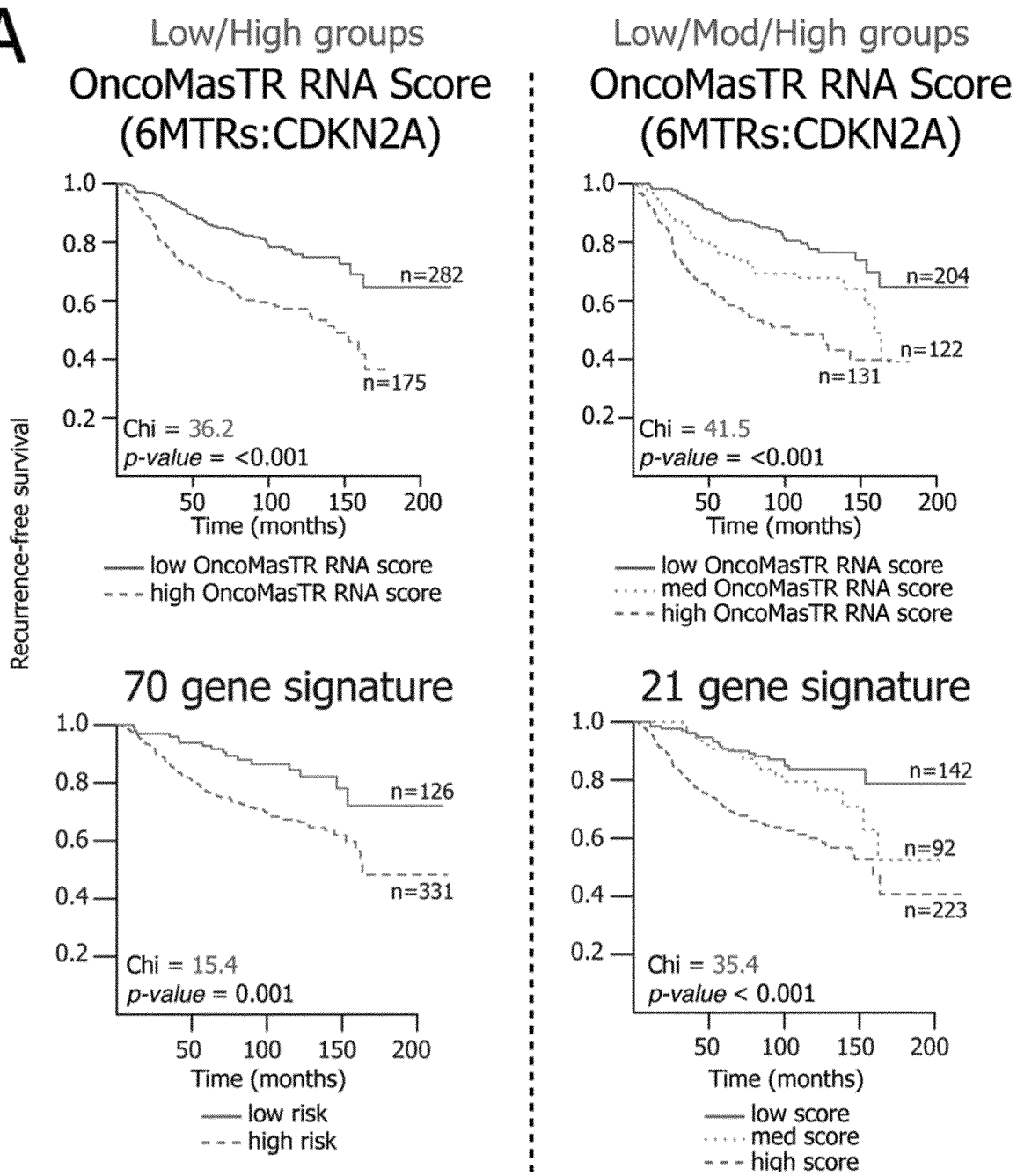

A
Low/High groups
OncoMasTR RNA Score
(6MTRs:CDKN2A)
Recurrence-free survival
1.0
0.8
0.6
0.4
0.2
n=282
n=175
Chi = 36.2
p-value = <0.001
50 100 150 200
Time (months)
low OncoMasTR RNA score
high OncoMasTR RNA score
Low/Mod/High groups
OncoMasTR RNA Score
(6MTRs:CDKN2A)
n=204
n=122
n=131
Chi = 41.5
p-value = <0.001
Time (months)
low OncoMasTR RNA score
med OncoMasTR RNA score
high OncoMasTR RNA score
70 gene signature
n=126
n=331
Chi = 15.4
p-value = 0.001
Time (months)
low risk
high risk
21 gene signature
n=142
n=92
n=223
Chi = 35.4
p-value < 0.001
Time (months)
low score
med score
high score

Figure 5B

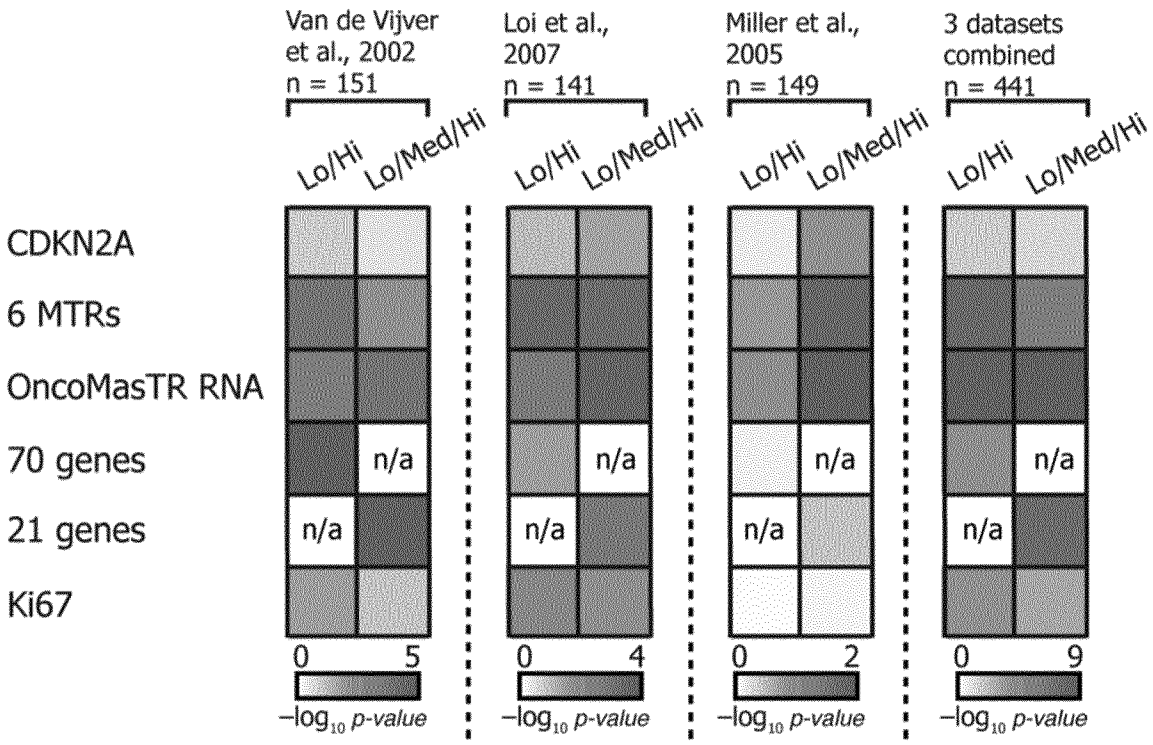

Van de Vijver et al., 2002 n = 151
Loi et al., 2007 n = 141
Miller et al., 2005 n = 149
3 datasets combined n = 441
Lo/Hi
Lo/Med/Hi
CDKN2A
6 MTRs
OncoMasTR RNA
70 genes
21 genes
Ki67
n/a
0 5
0 4
0 2
0 9
−log10 p-value

Figure 5C

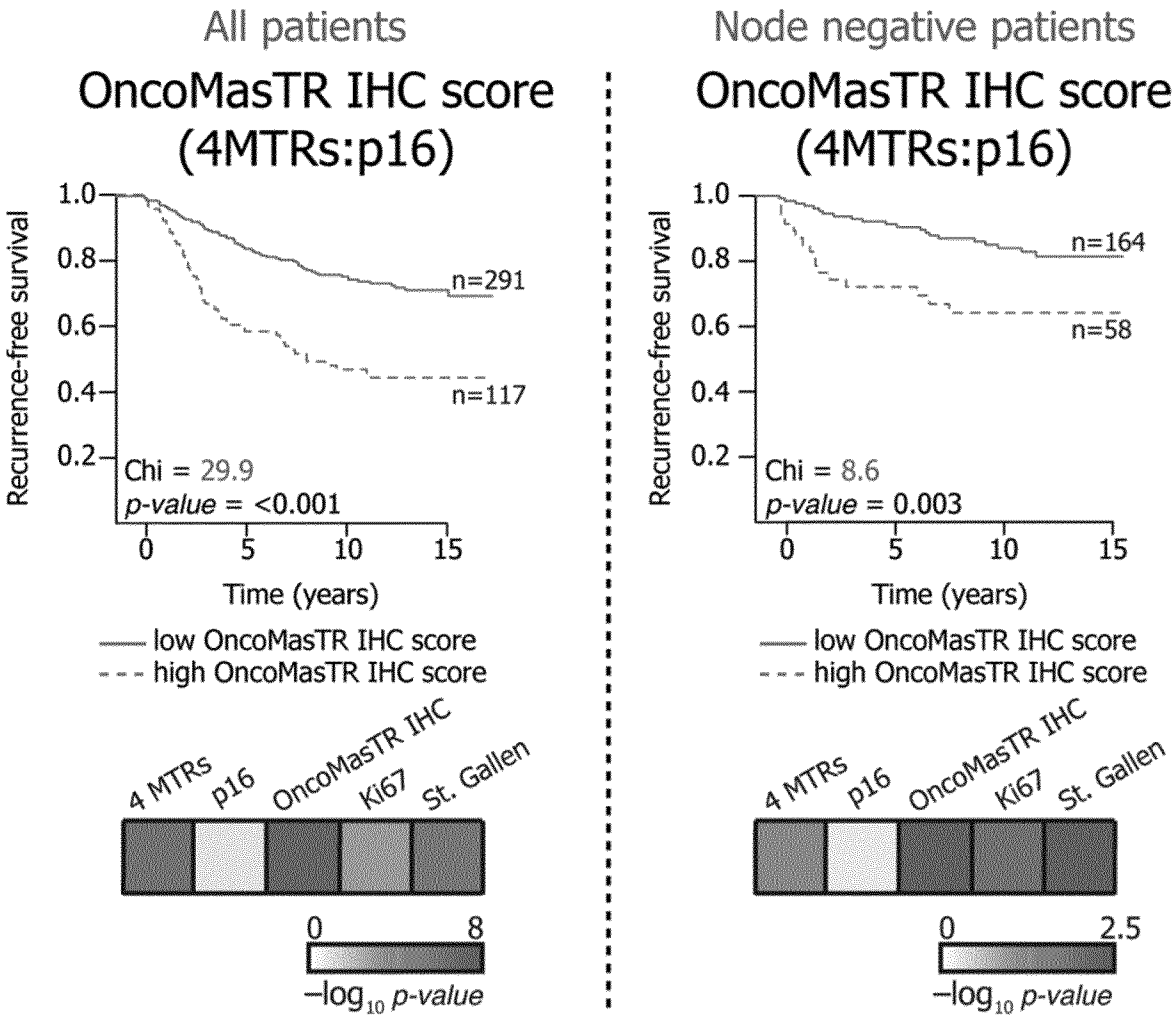

All patients
OncoMasTR IHC score (4MTRs:p16)
Recurrence-free survival
1.0
0.8
0.6
0.4
0.2
n=291
n=117
Chi = 29.9
p-value = <0.001
0
5
10
15
Time (years)
low OncoMasTR IHC score
high OncoMasTR IHC score
4 MTRs
p16
OncoMasTR IHC
Ki67
St. Gallen
0
8
−log10 p-value
Node negative patients
OncoMasTR IHC score (4MTRs:p16)
Recurrence-free survival
1.0
0.8
0.6
0.4
0.2
n=164
n=58
Chi = 8.6
p-value = 0.003
0
5
10
15
Time (years)
low OncoMasTR IHC score
high OncoMasTR IHC score
4 MTRs
p16
OncoMasTR IHC
Ki67
St. Gallen
0
2.5
−log10 p-value

Figure 5D

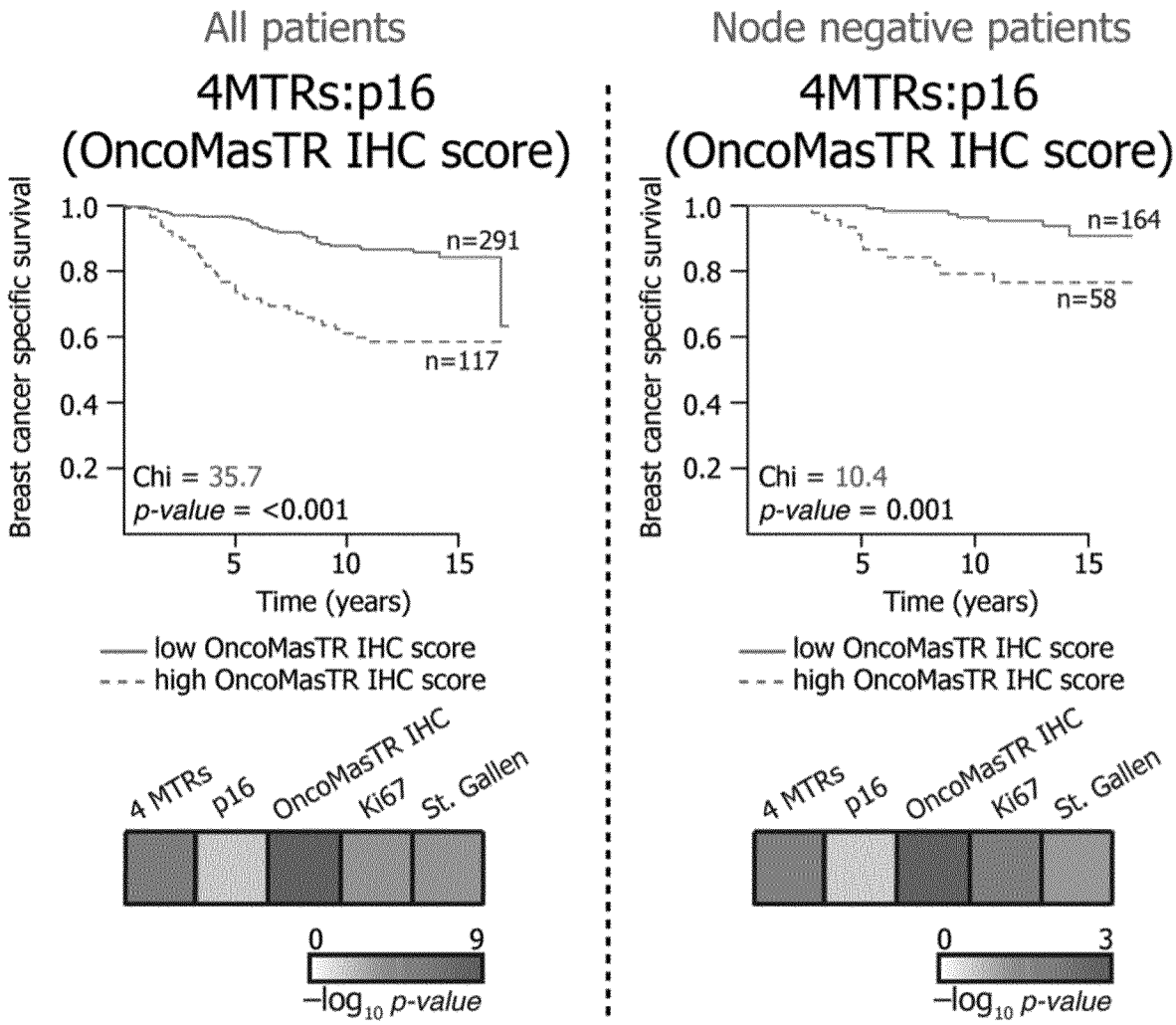

All patients
4MTRs:p16
(OncoMasTR IHC score)
Breast cancer specific survival
1.0
0.8
0.6
0.4
0.2
n=291
n=117
Chi = 35.7
p-value = <0.001
5
10
15
Time (years)
low OncoMasTR IHC score
high OncoMasTR IHC score
4 MTRs
p16
OncoMasTR IHC
Ki67
St. Gallen
0
9
−log10 p-value
Node negative patients
4MTRs:p16
(OncoMasTR IHC score)
Breast cancer specific survival
1.0
0.8
0.6
0.4
0.2
n=164
n=58
Chi = 10.4
p-value = 0.001
5
10
15
Time (years)
low OncoMasTR IHC score
high OncoMasTR IHC score
4 MTRs
p16
OncoMasTR IHC
Ki67
St. Gallen
0
3
−log10 p-value

Figure 6A

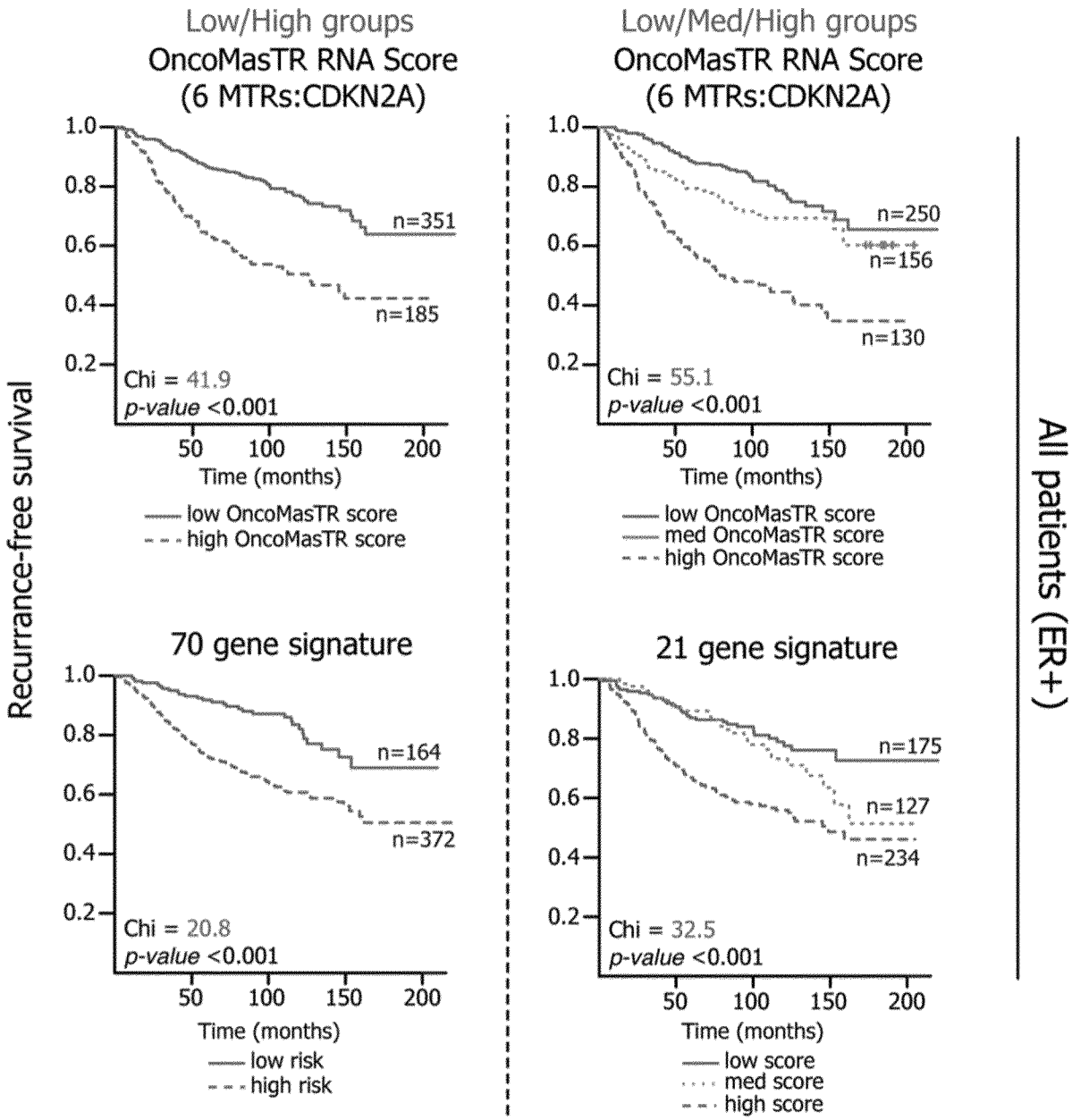
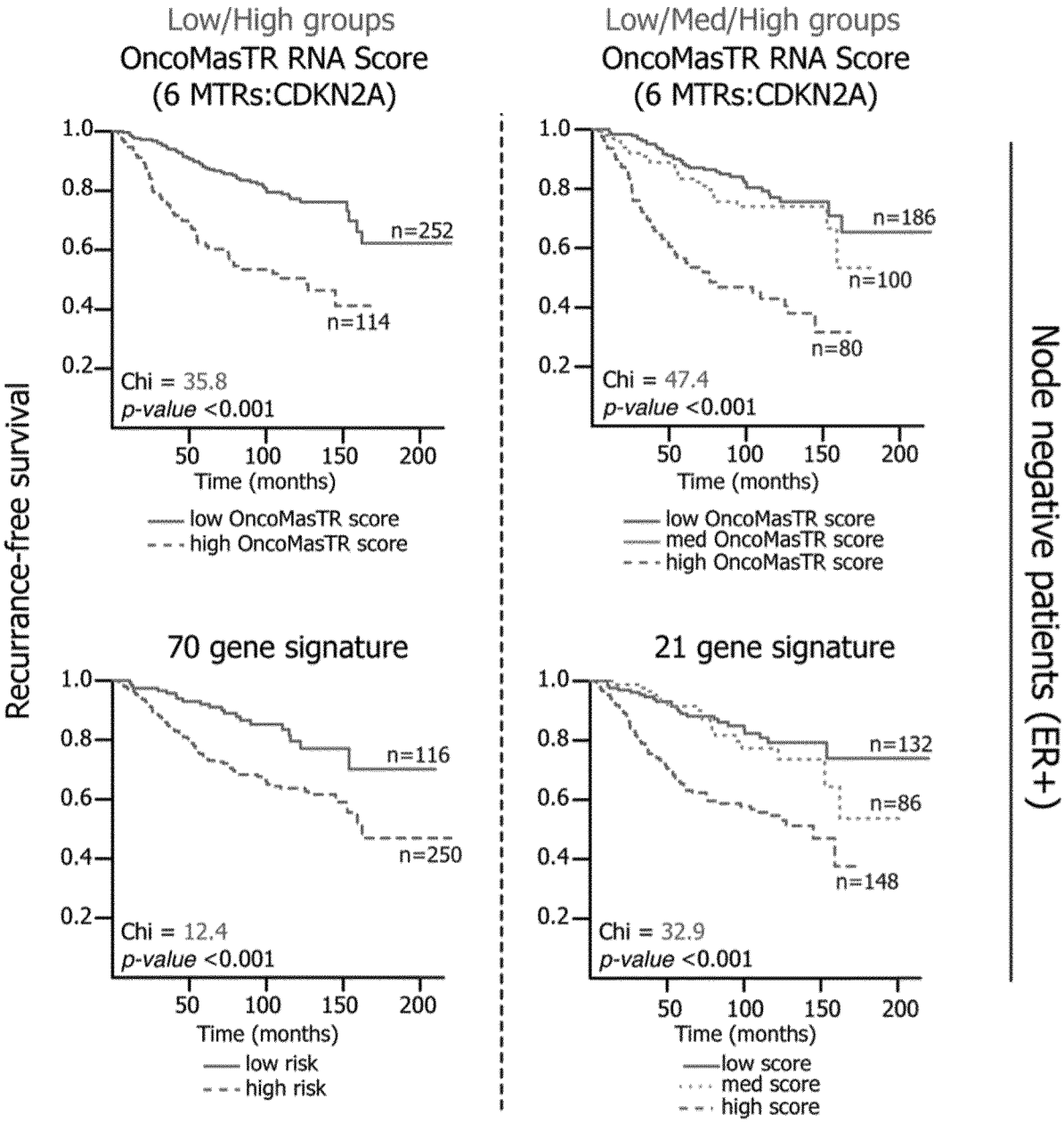

Low/High groups
OncoMasTR RNA Score
(6 MTRs:CDKN2A)
Low/Med/High groups
OncoMasTR RNA Score
(6 MTRs:CDKN2A)
Recurrance-free survival
All patients (ER+)
n=351
n=185
Chi = 41.9
p-value <0.001
n=250
n=156
n=130
Chi = 55.1
p-value <0.001
Time (months)
low OncoMasTR score
high OncoMasTR score
med OncoMasTR score
70 gene signature
21 gene signature
n=164
n=372
Chi = 20.8
p-value <0.001
n=175
n=127
n=234
Chi = 32.5
p-value <0.001
low risk
high risk
low score
med score
high score

Figure 6B


Low/High groups
OncoMasTR RNA Score
(6 MTRs:CDKN2A)
Low/Med/High groups
OncoMasTR RNA Score
(6 MTRs:CDKN2A)
Recurrance-free survival
1.0
0.8
0.6
0.4
0.2
n=252
n=114
Chi = 35.8
p-value <0.001
50 100 150 200
Time (months)
low OncoMasTR score
high OncoMasTR score
n=186
n=100
n=80
Chi = 47.4
p-value <0.001
med OncoMasTR score
70 gene signature
n=116
n=250
Chi = 12.4
low risk
high risk
21 gene signature
n=132
n=86
n=148
Chi = 32.9
low score
med score
high score
Node negative patients (ER+)

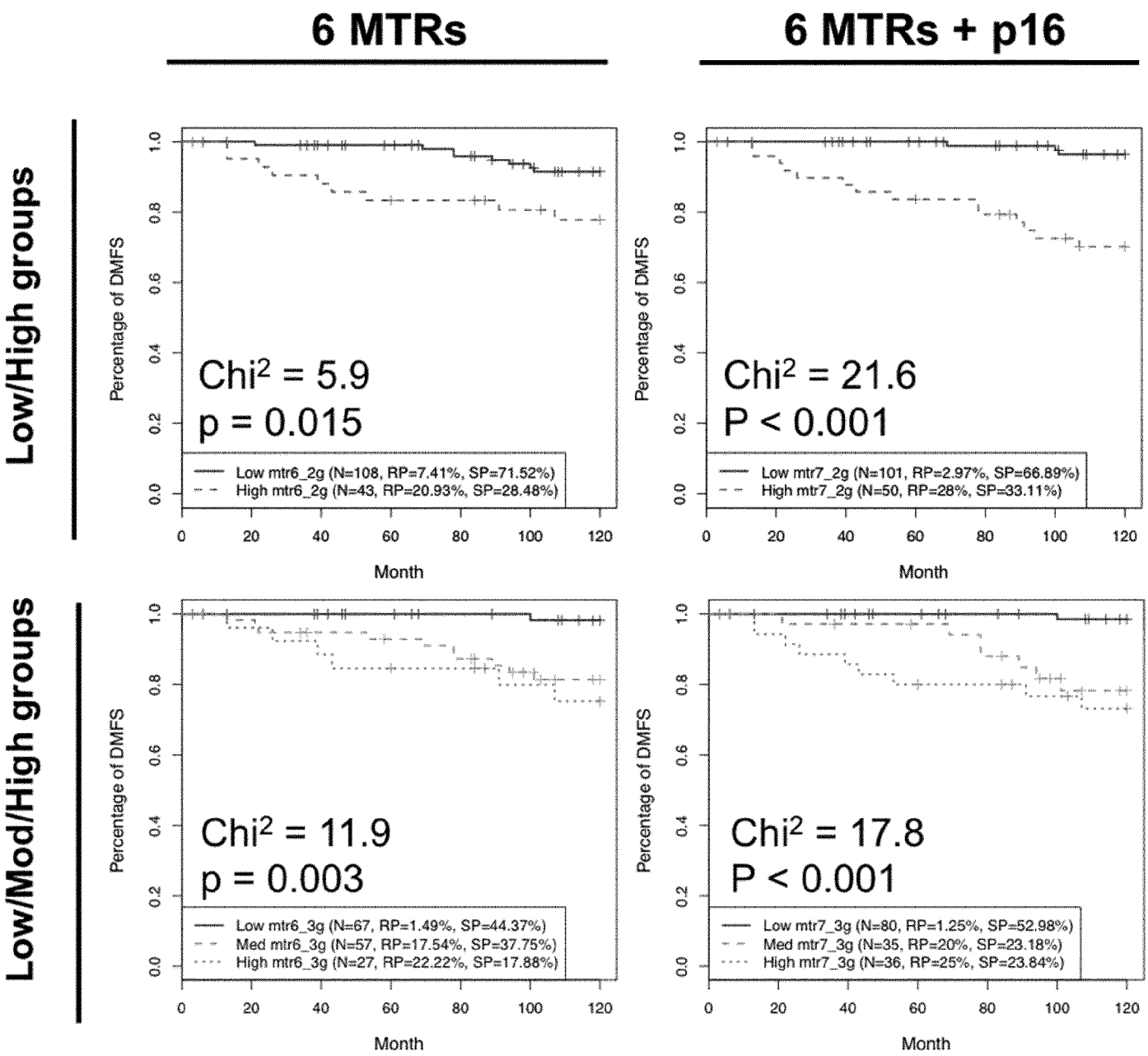

6 MTRs
6 MTRs + p16
Low/High groups
Low/Mod/High groups
Percentage of DMFS
Month
Chi² = 5.9
p = 0.015
Low mtr6_2g (N=108, RP=7.41%, SP=71.52%)
High mtr6_2g (N=43, RP=20.93%, SP=28.48%)
Chi² = 21.6
P < 0.001
Low mtr7_2g (N=101, RP=2.97%, SP=66.89%)
High mtr7_2g (N=50, RP=28%, SP=33.11%)
Chi² = 11.9
p = 0.003
Low mtr6_3g (N=67, RP=1.49%, SP=44.37%)
Med mtr6_3g (N=57, RP=17.54%, SP=37.75%)
High mtr6_3g (N=27, RP=22.22%, SP=17.88%)
Chi² = 17.8
P < 0.001
Low mtr7_3g (N=80, RP=1.25%, SP=52.98%)
Med mtr7_3g (N=35, RP=20%, SP=23.18%)
High mtr7_3g (N=36, RP=25%, SP=23.84%)

Figure 7

Figure 8

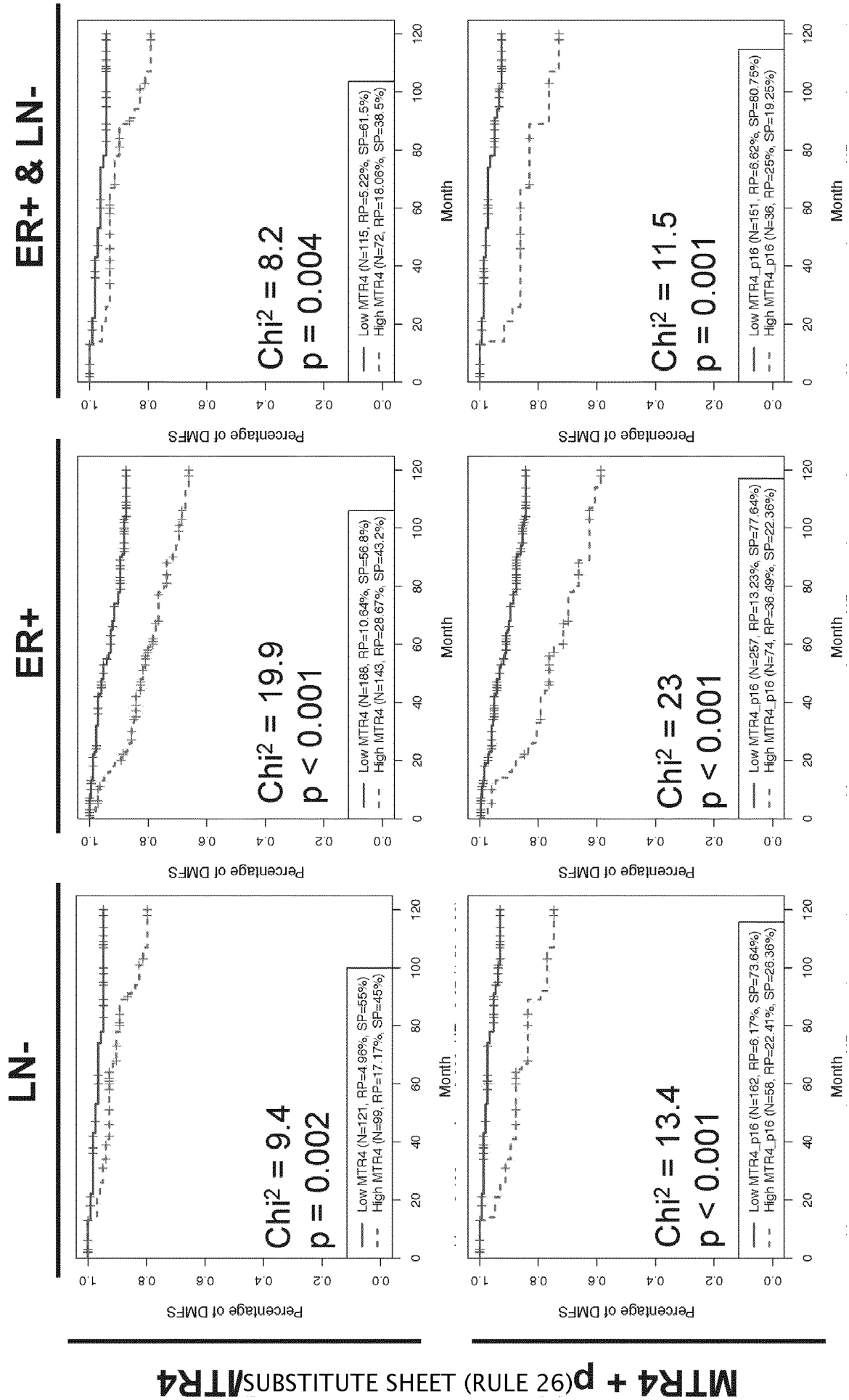


Figure 9

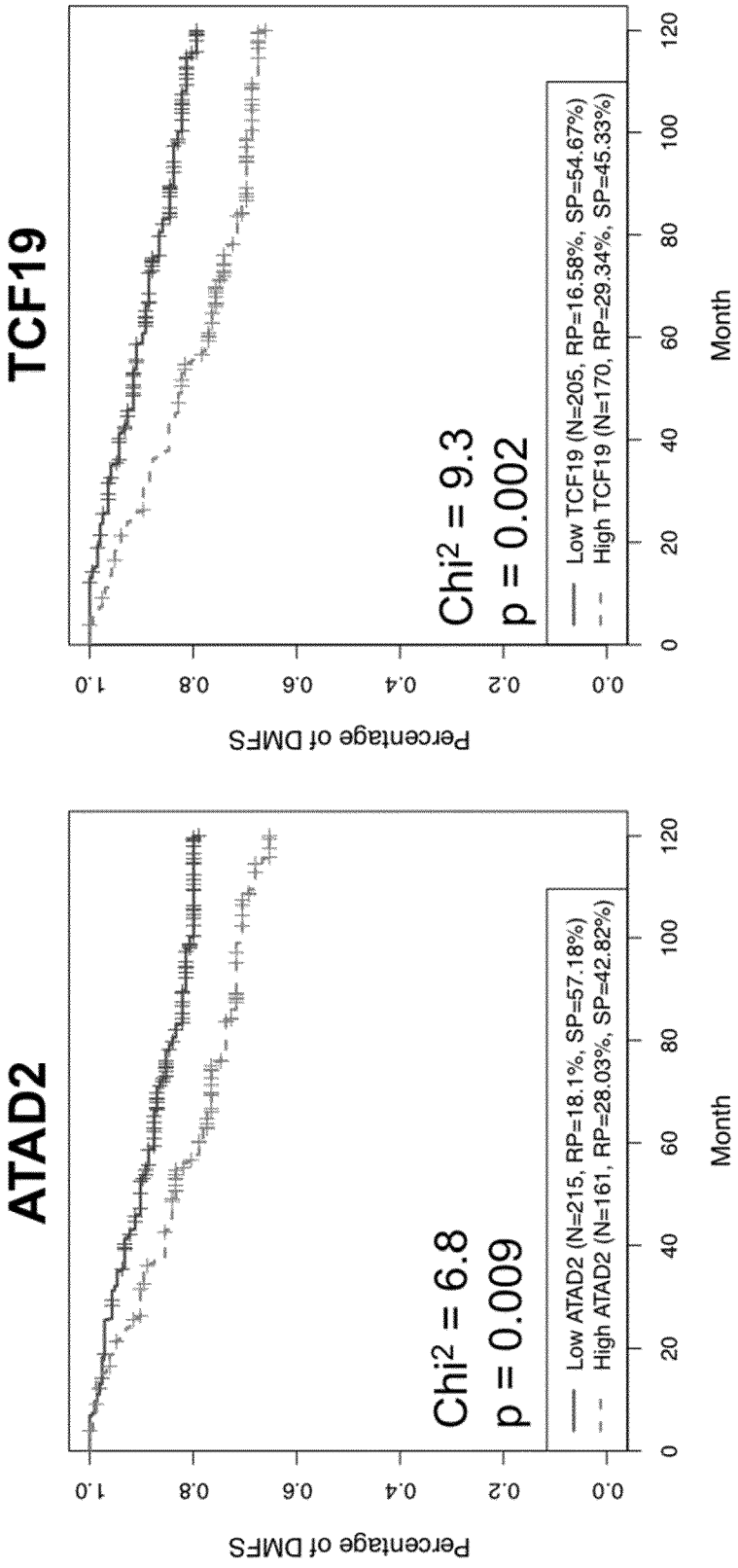
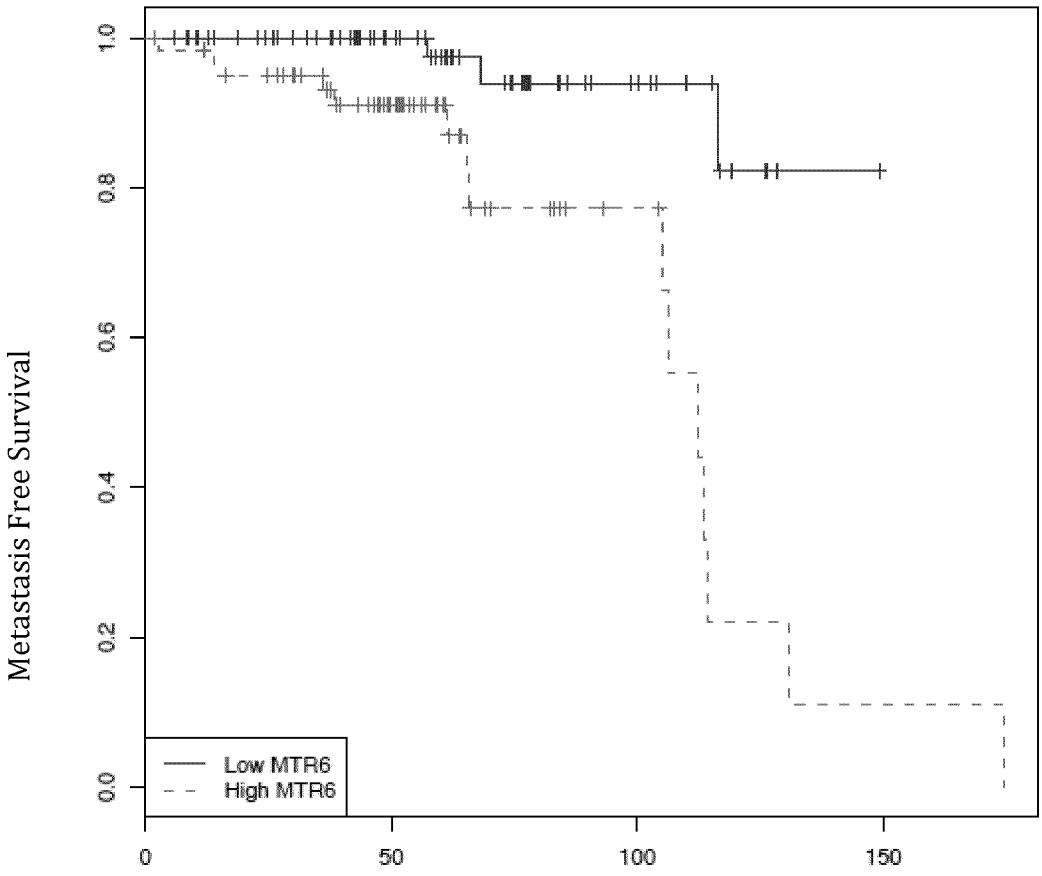
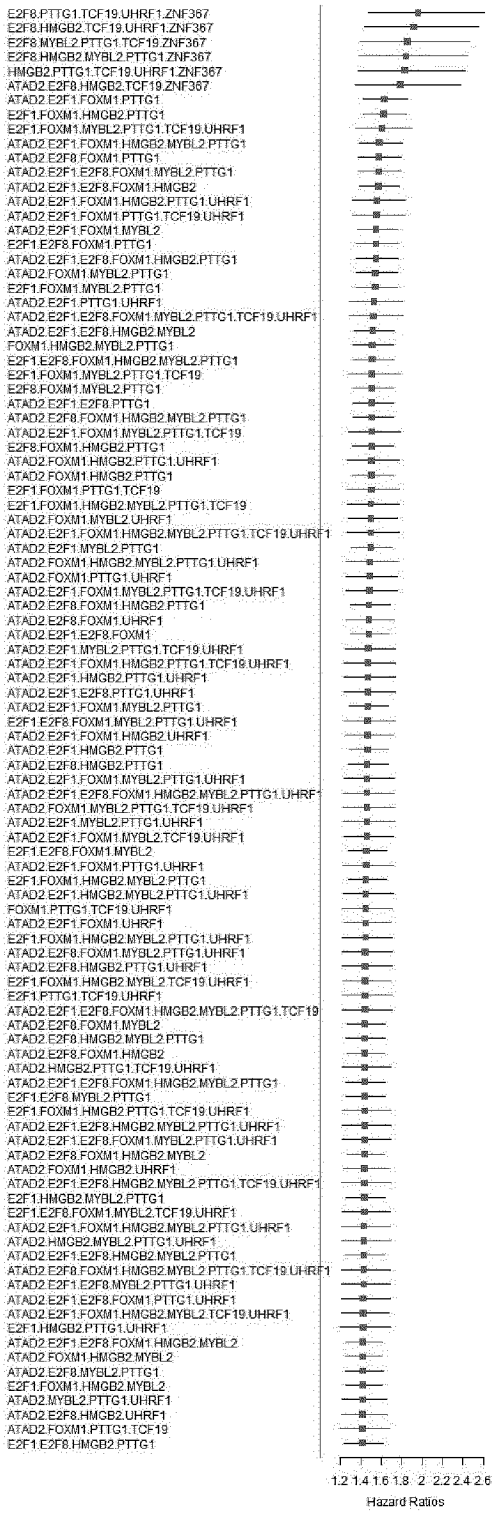

TCF19
Chi² = 9.3
p = 0.002
Low TCF19 (N=205, RP=16.58%, SP=54.67%)
High TCF19 (N=170, RP=29.34%, SP=45.33%)
Percentage of DMFS
Month
ATAD2
Chi² = 6.8
p = 0.009
Low ATAD2 (N=215, RP=18.1%, SP=57.18%)
High ATAD2 (N=161, RP=28.03%, SP=42.82%)
Percentage of DMFS
Month

Figure 10



Chisq=16.601, p-value=0, HR=7.69 (2.17-27.18)

Figure 11


E2F8.PTTG1.TCF19.UHRF1.ZNF367
E2F8.HMGB2.TCF19.UHRF1.ZNF367
E2F8.MYBL2.PTTG1.TCF19.ZNF367
E2F8.HMGB2.MYBL2.PTTG1.ZNF367
HMGB2.PTTG1.TCF19.UHRF1.ZNF367
ATAD2.E2F8.HMGB2.TCF19.ZNF367
ATAD2.E2F1.FOXM1.PTTG1
E2F1.FOXM1.HMGB2.PTTG1
E2F1.FOXM1.MYBL2.PTTG1.TCF19.UHRF1
ATAD2.E2F1.FOXM1.HMGB2.MYBL2.PTTG1
ATAD2.E2F8.FOXM1.PTTG1
ATAD2.E2F1.E2F8.FOXM1.MYBL2.PTTG1
ATAD2.E2F1.E2F8.FOXM1.HMGB2
ATAD2.E2F1.FOXM1.HMGB2.PTTG1.UHRF1
ATAD2.E2F1.FOXM1.PTTG1.TCF19.UHRF1
ATAD2.E2F1.FOXM1.MYBL2
E2F1.E2F8.FOXM1.PTTG1
ATAD2.E2F1.E2F8.FOXM1.HMGB2.PTTG1
ATAD2.FOXM1.MYBL2.PTTG1
E2F1.FOXM1.MYBL2.PTTG1
ATAD2.E2F1.PTTG1.UHRF1
ATAD2.E2F1.E2F8.FOXM1.MYBL2.PTTG1.TCF19.UHRF1
ATAD2.E2F1.E2F8.HMGB2.MYBL2
FOXM1.HMGB2.MYBL2.PTTG1
E2F1.E2F8.FOXM1.HMGB2.MYBL2.PTTG1
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E2F8.FOXM1.MYBL2.PTTG1
ATAD2.E2F1.E2F8.PTTG1
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ATAD2.E2F1.FOXM1.MYBL2.PTTG1.TCF19
E2F8.FOXM1.HMGB2.PTTG1
ATAD2.FOXM1.HMGB2.PTTG1.UHRF1
ATAD2.FOXM1.HMGB2.PTTG1
E2F1.FOXM1.PTTG1.TCF19
E2F1.FOXM1.HMGB2.MYBL2.PTTG1.TCF19
ATAD2.FOXM1.MYBL2.UHRF1
ATAD2.E2F1.FOXM1.HMGB2.MYBL2.PTTG1.TCF19.UHRF1
ATAD2.E2F1.MYBL2.PTTG1
ATAD2.FOXM1.HMGB2.MYBL2.PTTG1.UHRF1
ATAD2.FOXM1.PTTG1.UHRF1
ATAD2.E2F1.FOXM1.MYBL2.PTTG1.TCF19.UHRF1
ATAD2.E2F8.FOXM1.HMGB2.PTTG1
ATAD2.E2F8.FOXM1.UHRF1
ATAD2.E2F1.E2F8.FOXM1
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ATAD2.E2F1.FOXM1.MYBL2.PTTG1
E2F1.E2F8.FOXM1.MYBL2.PTTG1.UHRF1
ATAD2.E2F1.FOXM1.HMGB2.UHRF1
ATAD2.E2F1.HMGB2.PTTG1
ATAD2.E2F8.HMGB2.PTTG1
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ATAD2.E2F1.E2F8.FOXM1.HMGB2.MYBL2.PTTG1.UHRF1
ATAD2.FOXM1.MYBL2.PTTG1.TCF19.UHRF1
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ATAD2.E2F1.FOXM1.MYBL2.TCF19.UHRF1
E2F1.E2F8.FOXM1.MYBL2
ATAD2.E2F1.FOXM1.PTTG1.UHRF1
E2F1.FOXM1.HMGB2.MYBL2.PTTG1
ATAD2.E2F1.HMGB2.MYBL2.PTTG1.UHRF1
FOXM1.PTTG1.TCF19.UHRF1
ATAD2.E2F1.FOXM1.UHRF1
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ATAD2.E2F8.FOXM1.MYBL2.PTTG1.UHRF1
ATAD2.E2F8.HMGB2.PTTG1.UHRF1
E2F1.FOXM1.HMGB2.MYBL2.TCF19.UHRF1
E2F1.PTTG1.TCF19.UHRF1
ATAD2.E2F1.E2F8.FOXM1.HMGB2.MYBL2.PTTG1.TCF19
ATAD2.E2F8.FOXM1.MYBL2
ATAD2.E2F8.HMGB2.MYBL2.PTTG1
ATAD2.E2F8.FOXM1.HMGB2
ATAD2.HMGB2.PTTG1.TCF19.UHRF1
ATAD2.E2F1.E2F8.FOXM1.HMGB2.MYBL2.PTTG1
E2F1.E2F8.MYBL2.PTTG1
E2F1.FOXM1.HMGB2.PTTG1.TCF19.UHRF1
ATAD2.E2F1.E2F8.HMGB2.MYBL2.PTTG1.UHRF1
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ATAD2.E2F8.FOXM1.HMGB2.MYBL2
ATAD2.FOXM1.HMGB2.UHRF1
ATAD2.E2F1.E2F8.HMGB2.MYBL2.PTTG1.TCF19.UHRF1
E2F1.HMGB2.MYBL2.PTTG1
E2F1.E2F8.FOXM1.MYBL2.TCF19.UHRF1
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ATAD2.E2F1.E2F8.FOXM1.PTTG1.UHRF1
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E2F1.FOXM1.HMGB2.MYBL2
ATAD2.MYBL2.PTTG1.UHRF1
ATAD2.E2F8.HMGB2.UHRF1
ATAD2.FOXM1.PTTG1.TCF19
E2F1.E2F8.HMGB2.PTTG1
1.2 1.4 1.6 1.8 2 2.2 2.4 2.6
Hazard Ratios

Figure 12

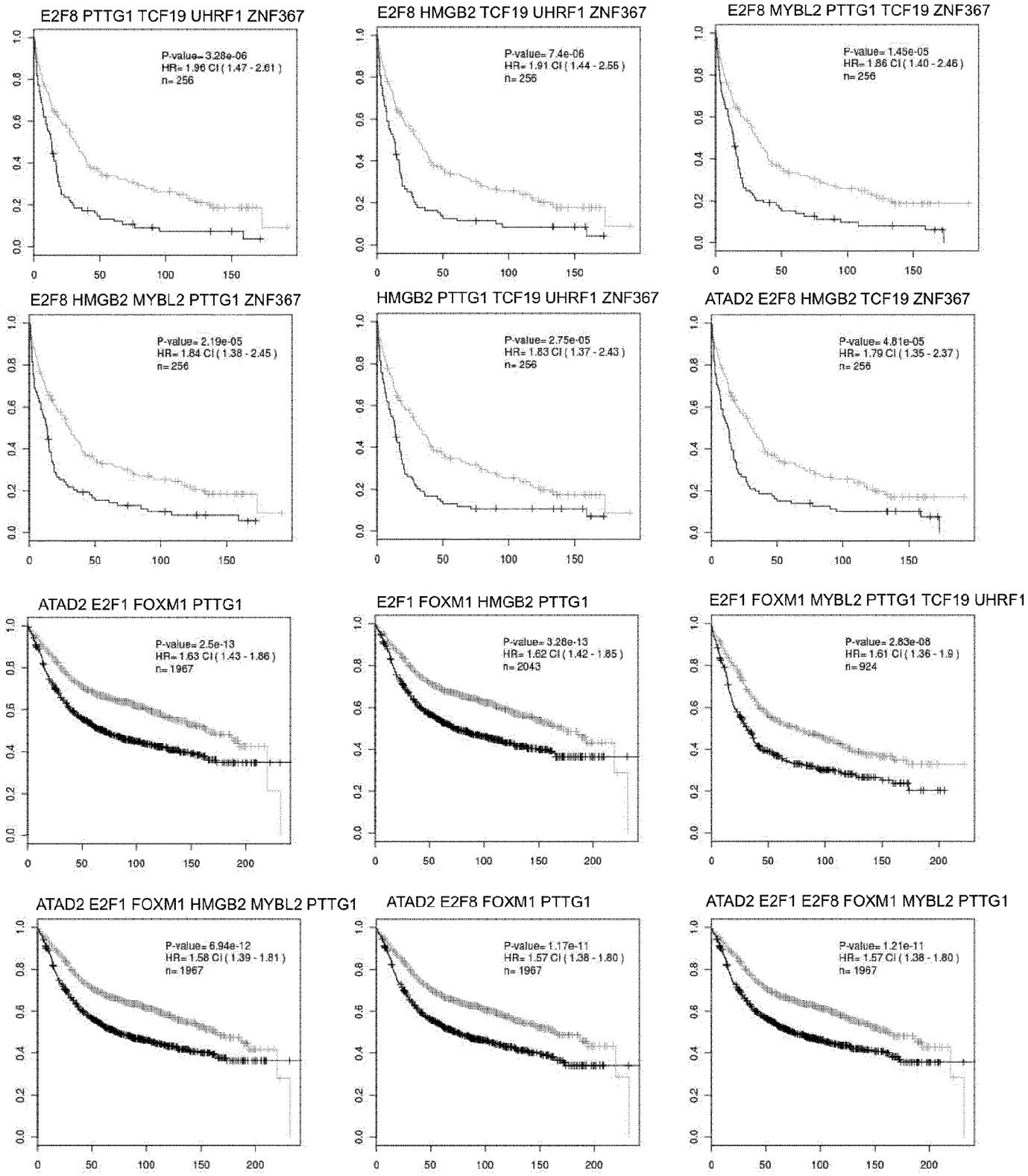

E2F8 PTTG1 TCF19 UHRF1 ZNF367
P-value= 3.28e-06
HR= 1.96 CI (1.47 - 2.61)
n= 256
E2F8 HMGB2 TCF19 UHRF1 ZNF367
P-value= 7.4e-06
HR= 1.91 CI (1.44 - 2.55)
n= 256
E2F8 MYBL2 PTTG1 TCF19 ZNF367
P-value= 1.45e-05
HR= 1.86 CI (1.40 - 2.46)
n= 256
E2F8 HMGB2 MYBL2 PTTG1 ZNF367
P-value= 2.19e-05
HR= 1.84 CI (1.38 - 2.45)
n= 256
HMGB2 PTTG1 TCF19 UHRF1 ZNF367
P-value= 2.75e-05
HR= 1.83 CI (1.37 - 2.43)
n= 256
ATAD2 E2F8 HMGB2 TCF19 ZNF367
P-value= 4.81e-05
HR= 1.79 CI (1.35 - 2.37)
n= 256
ATAD2 E2F1 FOXM1 PTTG1
P-value= 2.5e-13
HR= 1.63 CI (1.43 - 1.86)
n= 1967
E2F1 FOXM1 HMGB2 PTTG1
P-value= 3.28e-13
HR= 1.62 CI (1.42 - 1.85)
n= 2043
E2F1 FOXM1 MYBL2 PTTG1 TCF19 UHRF1
P-value= 2.83e-08
HR= 1.61 CI (1.36 - 1.9)
n= 924
ATAD2 E2F1 FOXM1 HMGB2 MYBL2 PTTG1
P-value= 6.94e-12
HR= 1.58 CI (1.39 - 1.81)
n= 1967
ATAD2 E2F8 FOXM1 PTTG1
P-value= 1.17e-11
HR= 1.57 CI (1.38 - 1.80)
n= 1967
ATAD2 E2F1 E2F8 FOXM1 MYBL2 PTTG1
P-value= 1.21e-11
HR= 1.57 CI (1.38 - 1.80)
n= 1967

Figure 12 (cont.)

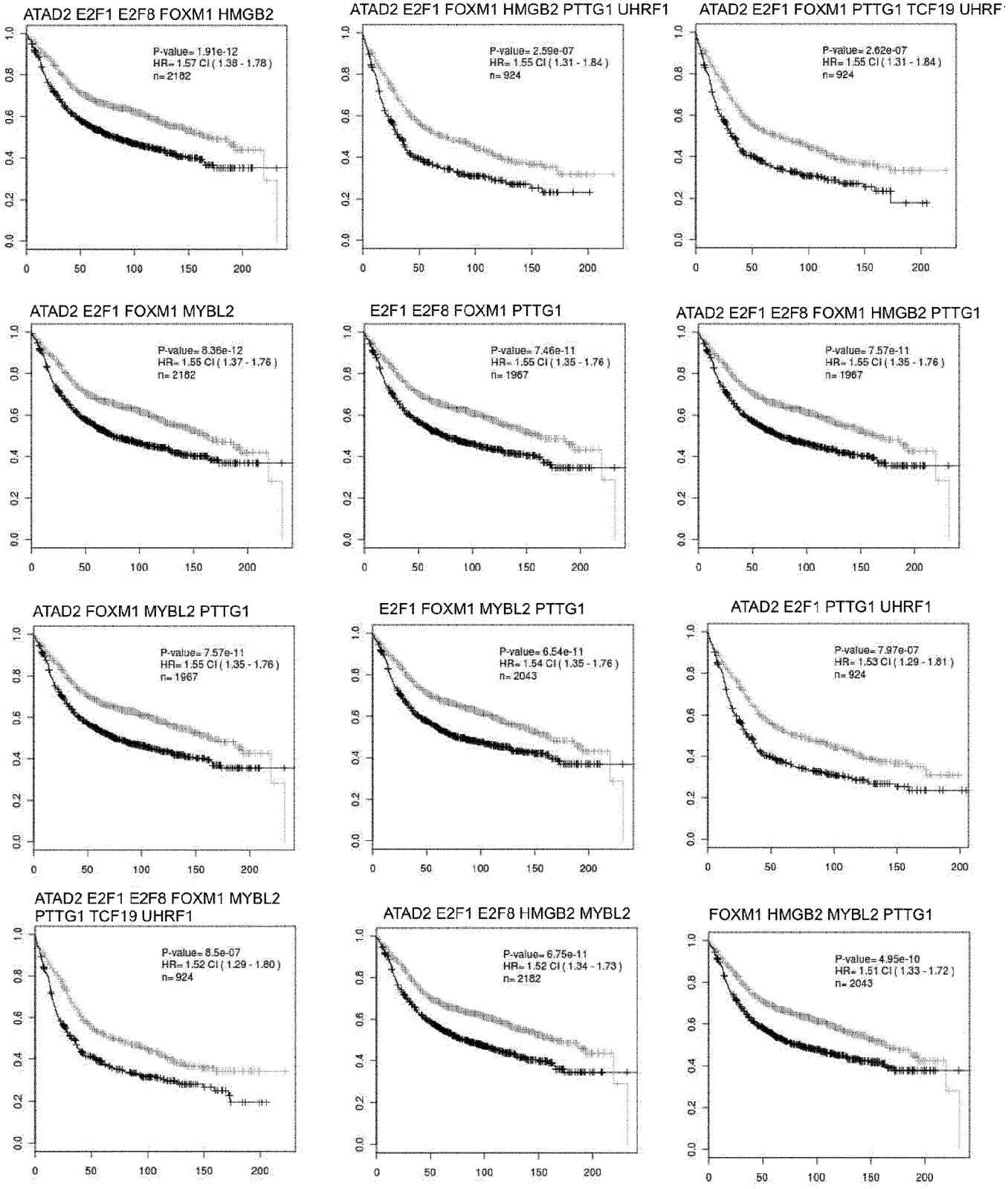
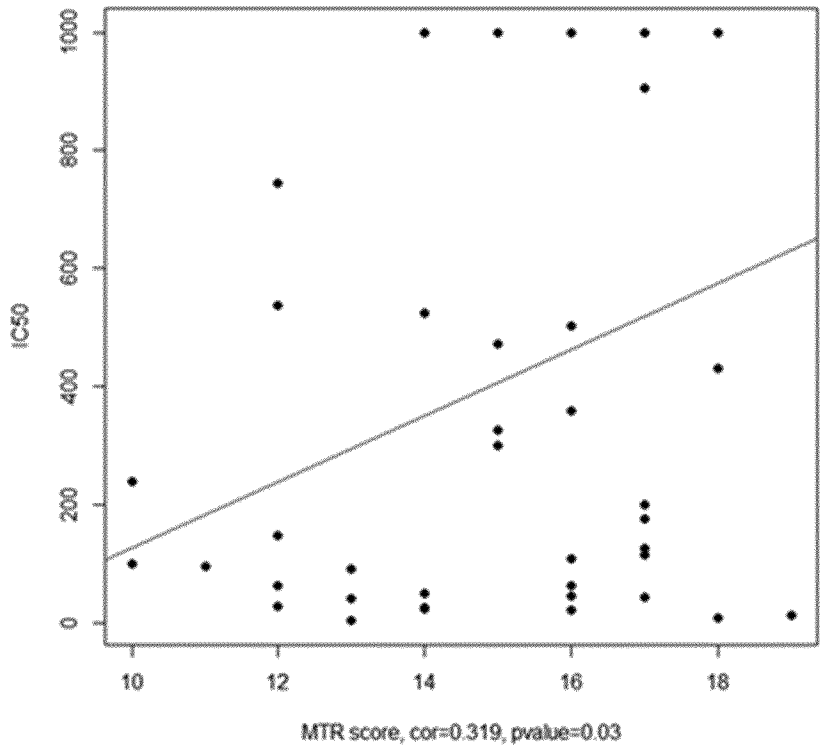


Figure 13


IC50
0
200
400
600
800
1000
10
12
14
16
18
MTR score, cor=0.319, pvalue=0.03

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/071524

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS, Sequence Search, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2014/066796 A2 (MYRIAD GENETICS INC [US]; LANCHBURY JERRY [US]; GUTIN ALEXANDER [US];) 1 May 2014 (2014-05-01)	1,2,4-8, 10, 12-14, 16,18-22
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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
14 January 2016	21/01/2016

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Gabriels, Jan

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Information on patent family members

International application No
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