

Subdivision of molecularly-classified groups by new gene signatures in breast cancer patients

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Abstract. Gene expression patterns as well as gene interactions are under investigation for their involvement in tumour heterogeneity. The molecular classification of breast cancer based on hormone receptor expression, grade and HER2 receptor levels, is indicative but not adequate enough to complete the prognostic data. The objectives of this study were to validate the prognostic value of 19 genes, solely, and as parts of classifiers (sets of genes), in breast cancer patients and to determine whether the expression of these genes and classifiers is correlated with breast cancer molecular classification. Gene expression was examined in the blood of 88 breast cancer patients and 50 healthy controls using multiplex quantitative real-time PCR. Patients with a second primary malignancy showed a statistically significant difference when compared with: i) patients with a single breast cancer, for an 8-gene classifier ($P < 0.02$); and ii) healthy individuals (classifier FBX033, FLJ339115) ($P < 0.01$), with respect to gene expression. The classifier ENY2, USP38 was associated with the development of primary breast cancer. A newly established classifier (ENY2, USP38, RPS7, Osbpl-1 and ETF1) indicated a statistically significant association with HER2 subtype patients, compared to patients with a different molecular classification ($P < 0.04$). The gene FLJ33915 was differentially expressed in a subgroup of HER2-positive patients with infiltrated axillary lymph nodes ($P < 0.028$). We validated the prognostic value of 4 classifiers for primary and second primary malignancy. Evidence of a classifier predicting the HER2 subtype and the

gene FLJ33915 which subdivides HER2 subtype patients is also presented.

Introduction

Breast cancer is one of the most common malignancies affecting women, with a lifetime risk of about 1 in 10. Breast cancer is considered both genetically and histopathologically heterogeneous (1). The mechanisms underlying breast cancer progression remain undetermined and are under investigation. The major prognostic characteristics for this disease have been based on conventional prognostic indicators, such as lymph node status, oestrogen receptor status, c-erb2 gene, tumour size and histological grade. However, it is still difficult to determine an accurate patient prognosis. Genetic expression (gene signatures) provides the basis for improving the molecular classification of breast cancer (2). Recently, an effort has been made to correlate the tumour characteristics of the patient with certain gene signatures. A classification scheme provides a very important framework for the study of breast cancer. The new form of classification represents four molecular subtypes with clinically distinct behaviour, that perhaps arise from different precursor cells in the breast. The true prognostic value of the various molecular classes is necessary because there is a strong correlation between molecular class and conventional histopathological variables. The subtypes of molecular classification are 4: Luminal A (positive hormone receptors, low grade), Luminal B (positive hormone receptors, high grade), HER2 positive subtype and Basal cell type (the latter of which includes triple negative patients) (3-5).

In order to explore the molecular basis of breast carcinogenesis aiming towards a more accurate prognosis and more effective therapeutic intervention, studies tend to focus on the microarray analysis of the whole transcriptome and proteome of the tumour, from the patients' blood (1,6,7).

Profiles of transcription and translation have shown specific changes among different types of cancer as a result of sequential mutation and signal amplification, distinguishing cancers from normal tissues. Moreover, the different gene expression profiles are likely to reflect distinct tumour subtypes involving different phenotypes and clinical features (8-11). Changes in the expression level of cancer-related genes occur much earlier than morphological changes, and they lead to a different degree

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of cellular differentiation (12). A characteristic expression profile in the blood may contribute to cancer prognosis. New molecular tumour markers can potentially be used for more accurate classification and drug targets for effective personalized therapy (13-19). The predictive power of these approaches is much greater than that of the currently used approaches based on the tumour characteristics, but this remains to be validated in prospective clinical studies.

The objective of the present study was to evaluate the predictive power of five sets of genes previously found to be correlated with primary breast cancer and second primary malignancies in breast cancer patients (17) and to determine whether the deregulation of these genes is correlated with breast cancer molecular classification.

Materials and methods

Patients. Blood was collected from 88 breast cancer patients with a 3-10 year follow-up after primary tumour excision. Blood was also collected from 50 age-matched healthy volunteers. The protocol was approved by the Ethics Committee of the Errikos Dynant Hospital and informed consent was signed by all the patients and healthy individuals participating in the study.

Eligibility for the study required histologically-confirmed breast cancer, including patients of all stages with a World Health Organization (WHO) performance status of 0-2. All patients had been treated with surgery, chemotherapy and/or endocrine treatment and/or radiotherapy. Before enrollment in the study the patients were clinically evaluated.

Staging was determined by chest and abdominal CT scans, bone scans and occasionally, MRIs. All patients had normal liver and renal function tests. The patients were divided on the basis of their histopathological characteristics and the molecular classification subgroups. All of the clinicopathological characteristics (age, stage, histological grade, tumour size, metastasis and lymph node involvement) are shown in Table I.

Gene selection. The 19 genes investigated were selected on the basis of their association with primary breast cancer and with the development of second primary tumours in breast cancer patients (17). These genes were part of 4 classifiers genes: i) FLJ38663, LOC34563, MTRF1L, COMMD1, C10ORF22, STARD7, BAG3 and SNX26; ii) RPS7, OSBPL1, ETF1; iii) FBX033, FLJ339115; and iv) ENY2, USP38 (Table II). In addition, the genes HNRPC, SET, HSPE1 and HCG2040681 were tested although they were not categorised as a classifier. The downregulation of these genes was statistically significantly correlated with single and second primary cancer development ($P < 0.00001$) (Table II). Two endogenous housekeeping genes (18S and β -actin) were included and were used to normalize the expression levels of the other genes.

RNA isolation. Total RNA was isolated from freshly collected blood after discarding the first 3 ml beforehand, in order to avoid epithelial cell contamination. RNA concentration and quality were examined spectrophotometrically (BioSpec Nano, Shimantzu, Japan) and by agarose gel electrophoresis. RNA extraction was obtained using TRI-Reagent (MRC) according to the manufacturer's instructions.

Table I. Clinicopathological data of patients.

Characteristics	Patients (n=88)	Healthy individuals (n=50)
Age (years), median (range)	57.2 40-74	55.7 38-65
Tumour size (cm)		
<2	40	
≥ 2 - <5	40	
≥ 5	8	
Histological grade		
I	15	
II	38	
III	35	
Stage		
I	12	
II	33	
III	26	
IV	17	
Metastasis	17	
ER status positive	66	
PR status positive	43	
HER2 status positive	25	
Lymph node status	38	
Molecular classification		
Luminal A	27	
Luminal B	26	
HER2 subtype	25	
Basal-like tumours	10	
Second primary development	14	

qRT-PCR. The examination of the expression levels of the 19 genes was obtained by multiplex quantitative-real time PCR (qRT-PCR): primer set, probes and PCR conditions used in each case were selected using the software Beacon Designer 7.0 (Premier Biosoft International). The primers were further examined using the FastPCR software and by carrying out NCBI blast (Table III). Prior to the multiplex qRT-PCR analysis, these 19 genes were separated into 4 sets that were designed in such a way that led to the compatibility amongst the primers, probes and fluorophores in each reaction. The primer, probe and fluorophore compatibility for each multiplex set was examined and approved by Bio-Rad Laboratories (Table III). Each RT reaction was carried out using 1 μ g of RNA with an iScript cDNA synthesis kit (Bio-Rad Laboratories) according to the manufacturer's instructions. The obtained cDNA was amplified by multiplex qRT-PCR. Prior to the original experiment, each RT-PCR product was examined by enzyme digest and sequencing. Each reaction was obtained in 25 μ l using 12.5 μ l IQ Multiplex Powermix (Bio-Rad Laboratories), 2 μ l cDNA, 0.3 μ M of each primer and 0.2 μ M of each probe. In each case 18S and β -actin were used as internal controls. Each reaction was performed in duplicate for each patient. The validation of the product

Table II. Multiplex qRT-PCR analysis.

Multiplex set	Gene	Classifier	PCR conditions (1st cycle at 95°C for 3 min)
1	C10ORF22	1	95°C for 15 sec
	COMMD1	1	58°C for 1 min, x40 cycles
	MTRF1L	1	
	STARD7	1	
2	BAG3	1	
	SNX26	1	56°C for 1 min, x40 cycles
	LOC345630	1	
3	FLJ38663	1	
	FBX033	2	58°C for 1 min, x40 cycles
	FLJ33915	2	
4	HNRPC		
	SET		57°C for 1 min, x40 cycles
	HSPE1		
	HCG2040681		
5	RPS7	3	
	OSBPL1	3	57°C for 1 min, x40 cycles
	ETF1	3	
6	ENY2	4	
	USP38	4	58°C for 1 min, x40 cycles
	ACTB		
	18S		

Six gene sets were obtained, each one composed from 2 to 4 genes; here the PCR conditions used for the analysis of each gene set are also described.

identity and expression was obtained by the melting curve. We used a two-step amplification reaction according to the manufacturer's instructions (Table II) using the IQ5 thermal cycler (Bio-Rad Laboratories).

We analysed the data with the LightCycler software. Briefly, three serial 10-fold dilutions of cDNA from 50 normal individuals were amplified in duplicates to construct standard curves and to set the baseline. Standard curves generated by the software were used for extrapolation of the expression level for the unknown samples based on their threshold cycle (Ct) values. For each reaction, melting curves and agarose gel electrophoresis of PCR products, enzyme digests and sequencing, were used to verify the identity of the amplification products. All experiments were performed with at least two independent PCR reactions.

Statistical analysis. In our study, the gene expression of the 50 healthy individuals was set as the normal baseline. A gene was considered to be significantly differentially expressed (over- or underexpressed), if the ratio of the expression level in the cancer sample to the expression level in the blood of healthy individuals was higher than 4.0, which indicated a 4-fold increase in expression, or if the ratio was lower than 0.3. The results obtained by real time RT-PCR for each gene and patients' clinicopathological data, molecular staging and

second primary tumour development were analysed using Multivariate Analysis of Variance (MANOVA). When significant differences were observed, discriminant function analysis was used to assess the relative contribution of each dependent variable. Each group of genes was considered the independent variable, while the patients' clinicopathological data, molecular staging and development of a second primary tumour were the dependent variables. All statistical analyses were performed using SPSS software for Windows (SPSS Inc., Chicago, IL) and a P-value of <0.05 was considered to be statistically significant.

Results

The molecular classification subgroups of patients (Luminal A, B, HER2 subtype and Basal) were compared for gene deregulation levels of the classifiers and of each gene solely. MANOVA for the five-gene classifier ENY2, USP38, RPS7, OSBPL1 and ETF1 revealed a statistically significant difference between HER2 subtype patients and the rest of the molecular subgroups (Wilks' lambda: 0.85, F=2.42, P<0.04). Discriminant analysis indicated that ETF1 is the most important gene predictor, separating the HER2 subtype from the rest of the subtypes, (Wilks' lambda: 0.93, F=5.32, P<0.02). Furthermore, FLJ33915 gene expression was found to differ

Table III. Primers, probes and fluorophores for each gene, used in multiplex qRT-PCR.

Gene	Primer	Probe	Fluorophore
C10ORF22	F: GCCGGGACTGCCACTATTAC R: AGACCTTGGGACCTGGATAGG	TCCCTCGCACCAGAAGTCATCGGC	5'Texas Red 3'BHQ2
STARD7	F: ATCCAATGTACTCACGGGATTATG R: ATATGATCTGACCCTGACGAATTC	CACTCGGATGCTCCACAGCACGC	5'Cy5 3'BHQ3
MTRF1L	F: GGCTCATTAATCAGTTATGGTTCC R: CAGCACTGTCCGTGGTATTTAC	CCCAGCTCCACTGGCTCGCTTAGT	5'FAM 3'BHQ1
COMMD1	F: ACATCTGACCAAGCTGCTGTC R: GCTGAGTGCCTTGACTGAGAC	ATCAACTCTCCAGCTCAGGCCCCG	5'HEX 3'BHQ1
BAG3	F: CTCAGAGGTCCCAGTCACC R: GAGGAGGATGAGGATGAGCAG	CATGCCAGAAACCACTCAGCCAGA	5'FAM 3'BHQ1
SNX26	F: TGGTGGTGGAGTTTCTGCTC R: CTTCCTCCAGCGTCAGCAG	CCTGTTCCAGCGACACCTTCACCTC	5'HEX 3'BHQ1
FLJ38663	F: CATGGGGACTCCGGCTTTG R: CTTCGAGTTCAATTCTCATCCAAGG	AGGGTAGTCCTTCTTGCTGCCAT	5'Texas Red 3'BHQ2
LOC345630	F: GCCACTTTCTCATCTCCATCAAG R: TCATAGGGCTCCAGGGTCAG	TCCACCGCATCCGCCGAGG	5'Cy5 3'BHQ3
FBX033	F: GGGACTGGAGGGGAGGAAG R: AACTTCTGAAGGTTCTGTTGTTTC	ACCAGCACGCAAAGCACCAGC	5'HEX 3'BHQ1
FLJ33915	F: GCCCAGGCGAGGTGGAAGG R: GACCAGGGACGCTCGATTTTC	ACGTCTGCCTCAGCCTGCTCG	5'FAM 3'BHQ1
HNRPC	F: GGCTTCAATTCTAAGAGTGGACAG R: TCCAGGTTTTCCAGGAGAGAATC	TGGGTCAGCTCCTTCTTAATGGCCT	5'FAM 3'BHQ1
SET	F: GAAATATAACAAACTCCGCCAACC R: AATTCTGTCACTTCAACTCTGGTC	CAGTGCCTCTTCATCTTCCTCCCA	5'HEX 3'BHQ1
HSPE1	F: TTGAAAGGAGTGCTGCTGAAAC R: CACGCTAACTGGTTGAATCTCTC	AGAACCCGATCCAACAGCGACTACT	5'Texas Red 3'BHQ2
HCG2040681	F: TGCAGGAGTTTAAAACGAGAGTG R: CCCATCCAGTGACTTTGCTTTAG	TCCTTCCCTTTGCCTGTGGTGTCA	5'Cy5 3'BHQ3
RPS7	F: TCTTCTGGAGCTGGAGATGAAC R: TGAGGAACGGGAACAAAGATTATG	AGCTTTCCGACCACCACCAACTTC	5'FAM 3'BHQ1
OSBPL1	F: GAGTGGGGAGAAGCTGAAGG R: CTGCCATTTCCGACTGTGTATC	CCACCCTCTTCCGCATCACATCC	5'HEX 3'BHQ1
ETF1	F: AGGAGGAGGCGAGAAGATGG R: GAAATCTGGTCTTTGGGAGGAATG	ACGACCCAGTGCTGCCGAC	5'Texas Red 3'BHQ2
ENY2	F: GTAACGGTCTCAGCGCAAG R: AACTTTTGGTTAATCGCTGCTCTC	CATCTTGCTAACCACCATCACCGCG	5'FAM 3'BHQ1
USP38	F: CAGCATCTTTTTGCCTTTCTGG R: CTGAGGTATTCAGAACAGTCTTGC	ACACAGAGGGAAGCATAACGCACCT	5'HEX 3'BHQ1
ACTB	F: GCACAGAGCCTCGCCTTTG R: CATGCCGGAGCCGTTGTC	CCGCCGCCGTTCCACACC	5'Texas Red 3'BHQ2
18S	F: GGCTCATTAATCAGTTATGGTTCC R: CGGGTTGGTTTTGATCTGATAAATG	TGGTCGCTCGCTCCTCTCCTACT	5'Cy5 3'BHQ3

For each reaction, compatible fluorophores were used. BHQ, Black Hole Quencher.

significantly in lymph node positive HER2 subtype patients vs. lymph node negative HER2 subtype patients (0.35 ± 0.08 and 0.63 ± 0.08 , respectively, $P < 0.028$) (Table IV). None of the

other genes or classifiers of genes examined in this study were statistically significantly correlated with any molecular classification subtype.

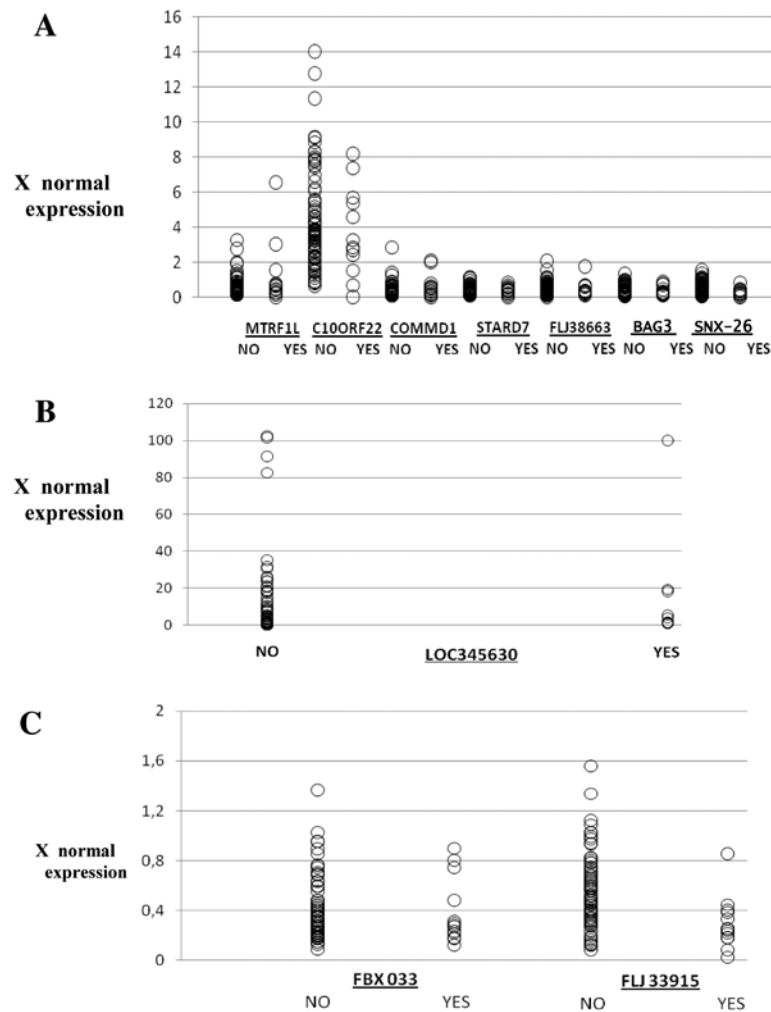


Figure 1. (A) The mRNA levels (times to normal expression) of MTRF1L, C10ORF22, COMMD1, STARD7, FLJ38663, BAG3, SNX26 and LOC345630, in patients negative (NO) or positive (YES) to the development of second primary malignancies using real-time RT-PCR. Each circle represents a patient. (B) Although we changed the scale for LOC345630 expression, three patients with very high values have not been included in this representation. Furthermore, it should be noted that although there is little difference in the expression of each of these genes between individuals with a single cancer and individuals with a second primary tumour, together as a classifier, they represent a very useful tool for the determination of single breast cancer individuals susceptible to developing a second primary tumour. (C) The mRNA levels (times to normal expression) of FBX033 and FLJ33915 in patients negative (NO) or positive (YES) to the development of second primary malignancies using real-time RT-PCR.

Table IV. Association of genes and classifiers with a second primary malignancy and HER2 subgrouping.

Classifier	Genes	Function	Statistical significance (P-value)
First	FLJ38663, LOC34563, MTRF1L, COMMD1, C10ORF22, STARD7, BAG3, SNX26	Second primary tumour predictor (single tumour vs. second primary)	<0.02
Third	FBX033, FLJ339115	Second primary tumour predictor (single tumour vs. normal)	<0.01
Fifth	ENY2, USP38, RPS7, OSBPL1, ETF1, FLJ33915	HER2 subtype predictor HER2 subtype subgrouping (with and without lymph node infiltration)	0.02 <0.028

The two-gene classifiers ENY2 and USP38 were observed to be differentially expressed in breast cancer patients when compared to healthy individuals. Using descriptive statistics we found that 85/88 patients (95.6%) presented ENY2 expres-

sion levels lower than 0.4 of the normal expression levels and 70/88 patients presented USP38 expression at levels lower than 0.4 of the normal expression. In addition, we also observed that 72/88 patients (81.8%) presented C10ORF >3, 86/88 (97.7%)

Table V. Description of the genes included in our study and their association with cancer.

Gene	Function	Cancer involvement
HCG2040681	Unclassified gene	
FBXO33	Protein-ubiquitin ligase; F-box proteins interact with SKP1 through the F box, and they interact with ubiquitination targets through other protein interaction domains and mediate the ubiquitination and subsequent proteasomal degradation of target proteins (UPP) (20)	
FLJ38663	Nuclear gene encodes a mitochondrial matrix protein that appears to contribute to peptide chain termination in the mitochondrial translation machinery; two different 1-bp deletions (resulting in the same premature stop codon) result in decreased mitochondrial translation, decreased levels of oxidative phosphorylation complexes and encephalomyopathy; alternative splicing results in multiple transcript variants (21)	
ENY2	Component of the transcription regulatory histone acetylation (HAT) complex SAGA, a multiprotein complex that activates transcription by remodeling chromatin and mediating histone acetylation and deubiquitination; it may also participate in mRNA export and accurate chromatin positioning in the nucleus by tethering genes to the nuclear periphery; USP 38 ubiquitin specific peptidase which is involved in ubiquitin catabolism (22)	
HNRPC	Essential for mitochondrial protein biogenesis, together with CPN60; it binds to CPN60 in the presence of Mg-ATP and suppresses the ATPase activity of the latter (23)	Breast cancer
SET	Highly conserved nuclear phosphoprotein that is ubiquitously expressed (24,25); SET has been suggested to regulate G2/M and in transition by modulating cyclin B-CDK1 activity	Overexpressed in solid tumours of the breast, stomach, uterus and rectum and in leukaemia (26)
HSPE1	Proangiogenic growth factor functions together with VEGF	Involved in the metastatic biology of ovarian cancer (27)
OSBPL1	Membrane-bound protein that binds oxysterols and may inhibit their cytotoxicity and, there are alternative transcriptional splice variants that have not have not yet been fully characterised (28)	
RPS7	Ribosomal protein is a component of the 40S subunit; this protein belongs to the S7E family of ribosomal proteins; it is located in the cytoplasm; as is typical for genes encoding ribosomal proteins, there are multiple processed pseudogenes of this gene dispersed through the genome; in our case the RPS7 mRNA was induced up to 33 times more than the normal levels and it is believed to be a p53 MDM2 interaction modulator (29)	
ETF1	Functions as an omnipotent translation termination factor, decoding all 3 stop codons (30)	
BAG3	Promotes substrate release after binding to the Hsc70/Hsp70 ATPase domain and inhibits its chaperone activity (31)	
C10ORF	Human thiol dioxygenases include cysteine dioxygenase (CDO; MIM 603943) and cysteamine (2-aminoethanethiol) dioxygenase (ADO; EC 1.13.11.19); CDO adds 2 oxygen atoms to free cysteine, whereas ADO adds 2 oxygen atoms to free cysteamine to form hypotaurine (32)	

Table V. Continued.

Gene	Function	Cancer involvement
COMMD1	Associated with copper homeostasis, NF- κ B signalling, and sodium transport and in HIF-1 signalling; hypoxia-inducible factors (HIFs) also regulate oxygen homeostasis, which control angiogenesis, erythropoiesis, glycolysis and cell survival/proliferation under normal and pathological conditions (33)	
STARD7	Unknown function; its existence is supported by mRNA and EST data; the predicted gene product contains a region similar to the STAR-related lipid transfer (START) domain, which is often present in proteins involved in the cell signalling mediated by lipid binding; some transcripts occur only in cancer cell lines (34)	
SNX26	This gene encodes a member of the sorting nexin family; members of this family contain a phox (PX) domain, which is a phosphoinositide-binding domain, and they are involved in intracellular trafficking; alternative splice variants encoding different isoforms have been identified in this gene (35)	
USP38	Ubiquitin specific peptidase is involved in ubiquitin catabolism (36)	
LOC345630	Unclassified gene	
MTRF1L	Mitochondrial peptide chain release factor directs the termination of translation in response to the peptide chain termination codons UAA and UAG (37)	
FLJ33915	Hypothetical protein	

and RPS7 <0.21 and 62/88 patients (70.5%) presented FBX033 expression <0.3. In all of the cases, 1 was considered as the normal expression level.

The 8-gene classifier (genes FLJ38663, LOC34563, MTRF1L, COMMD1, C10ORF22, STARD7, BAG3 and SNX26) was indicative of the development of second primary tumours after comparing individuals with a second primary malignancy vs. individuals with one primary malignancy, (Wilks' lambda: 0.83, F=2.61, P<0.02) (Table IV; Fig. 1A and B). The case was similar for the 2-gene classifier (FBX033 and FLJ33915), in the comparison of individuals with a second primary malignancy vs. healthy individuals. There was a statistically significant difference in the degree of deregulation (Wilks' lambda: 0.89, F=4.92, P<0.01) (Table IV, Fig. 1C).

Discussion

The 19 genes and the gene classifiers were examined in order to find correlations with the molecular classification subgroups. These genes and classifiers were also examined to determine the relationships between primary breast cancer and second primary cancer in breast cancer patients. The results of this study concur with those previously published (17), suggesting that the proposed gene classifiers may be an attractive candidate with prognostic value of breast cancer heterogeneity.

The three-gene classifier (RPS7, OSBPL1 and ETF1) that was found to be related to breast cancer development, may be

of value as a prognostic marker. Deregulation in the expression of each of these three genes was observed only in breast cancer patients and not in the healthy individuals. The patients with second primary tumours presented downregulation of FBX033, FLJ33915 gene expression (2-gene classifier) which was not observed in healthy individuals.

The 8-gene classifier was useful for the prognosis of second primary tumours in breast cancer patients. Therefore, the prognostic value for second primary tumour development is directed at: i) the 2-gene classifier (FBX033 and FLJ33915) in healthy individuals and ii) the 8-gene classifier (genes FLJ38663, LOC34563, MTRF1L, COMMD1, C10ORF22, STARD7, BAG3 and SNX26) in breast cancer patients.

Out of the 19 genes examined here, three (HNRPC, SET and HSPE1), are known to be directly related to carcinogenesis. The remaining 16 are not directly associated with cancer development (20-24) (Table V). These genes are involved in certain pathways, such as p53 protein stabilisation, the ubiquitin proteasome pathway, angiogenesis, cell survival and proliferation, G2 to M transition and protein synthesis, where defects in each of these may lead, indirectly, to cancer development (25-37). Our data suggests that some of these genes, not solely but as parts of a classifier can be used for the prognosis of breast cancer. These results were observed after comparing gene deregulation between healthy individuals, breast cancer patients and breast cancer patients with a second primary tumour.

The 5-gene classifier (ENY2, USP38, RPS7, OSBPL1 and ETF1) deregulation presents a statistically significant

difference between the HER2 subtype versus the rest of the subgroups of molecular classification. By further analysis, the ETF1 gene was conceived as the most important factor that is deregulated in the majority of patients categorised as the HER2 subtype. The fact that not all the patients with the HER2 subtype presented with significant deregulation of ETF1 may be an indication of its usefulness as a marker for the sub-grouping of the HER2 subtype group. Moreover, FLJ33915 downregulation was associated with lymph node infiltration in HER2 subtype patients. Prior to the classification of breast cancer patients into the 4 molecular classification subtypes (Luminal A, Luminal B, HER2 and Basal), some of the factors that were traditionally considered for the prognosis of the disease were not included; lymph node infiltration is one of them. The fact that FLJ33915 gene deregulation is statistically significantly associated with lymph node infiltration in patients with the HER2 subtype, suggests its potential usefulness in subdividing this subtype. This evidence is also an indication that perhaps it is wise to reconsider the evaluation of lymph nodes at least in patients with the HER2 subtype.

In conclusion, the findings summarised above suggest that the use of the genomic tests mentioned in this study may improve our ability to identify high-risk breast cancer patients prone to develop a second primary tumour and healthy individuals who may develop breast cancer. These patients may benefit from the prognostic power of the molecular signatures based on gene expression which is driven by genes that are not directly associated with cancer development. Instead, these genes are associated with tumour development and progression. Furthermore, we present evidence of a possible sub-categorisation of HER2 subtype patients, based on the expression profile of FLJ33915.

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