Drug metabolism-related genes as potential biomarkers: analysis of expression in normal and tumour breast tissue

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Abstract The complex role of drug metabolism-related enzymes and their possible influence in cancer development, treatment and outcome has not yet been completely elucidated. There is evidence that these enzymes can activate certain environmental procarcinogens to more toxic derivatives and thus a role has been proposed for them in carcinogenesis. The fact that they can also inactivate a number of chemotherapeutic drugs has raised the possibility of these enzymes influencing the sensitivity of tumour cells to anticancer agents. In this report, we analyse the expression of drug metabolism-related genes within a whole genome microarray study of 104 breast cancer and 17 normal breast specimens. Kaplan–Meier survival curves, Chi-squared, and Cox Regression analyses were used to identify associations between expression of gene transcripts and patients' clinicopathological and survival data. Our results show that several of these genes are significantly expressed in both normal and tumour tissue; in many cases, expression is altered in the tumour specimens as compared to normal breast tissue. Moreover, expression of ARNT2 and GST A1 was correlated with prognosis. Kaplan–Meier analysis showed expression of ARNT2 mRNA to correlate significantly with favourable disease outcome for patients, in terms of

both their disease-free survival ($P = 0.0094$) and overall survival ($P = 0.0018$) times from diagnosis, while detection of GST A1 mRNA correlated with shortened disease-free survival ($P = 0.0131$) and overall survival ($P = 0.0028$). Multivariate analysis indicated GST A1 expression to be an independent prognostic factor for overall survival ($P = 0.045$). Our results suggest a possible use of ARNT2 and GST A1 as prognostic breast cancer biomarkers.

Keywords Biomarkers · Breast · Drug metabolism · Microarrays · Prognostic factors

Introduction

Drug metabolism-related enzymes protect the body from the toxic effects of endogenous and exogenous compounds. Their ability to metabolise carcinogens and anticancer drugs lends them the potential to influence tumour development and sensitivity to chemotherapy and therefore tumour progression and treatment outcome [1–5].

Cytochromes P450 are a family of enzymes implicated in the biotransformation of both xenobiotics and endogenous compounds [6]. Their primary functions are the synthesis of steroids and bile acids and the detoxification of many substances, such as drugs and environmental agents. Cytochromes P450 are considered the most important enzymes carrying out Phase I reactions, where substrates are prepared for conjugation and excretion, while several studies have proposed a role for these enzymes in carcinogenesis, cancer progression and treatment outcome [7–10]. Glutathione (GSH) is the major soluble antioxidant present in mammalian cells and can also be conjugated to endogenous compounds or xenobiotics as means of detoxifying them. It has also been reported as a mediator in signalling pathways

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that modulate cell proliferation, differentiation and apoptosis [2, 11, 12].

Expression of enzymes involved in drug metabolism has been detected in every different type of tissue studied to date, both normal and tumour; techniques employed for detection include RT-PCR, Western blot, immunocytochemistry and immunohistochemistry. Enzymatic activity measurements have also been performed in tissue homogenates and/or isolated microsomes (cell fractions mainly composed of endoplasmic reticulum membranes). Although the presence of metabolic enzymes in both normal and tumour tissue has been demonstrated, reports attempting to compare the relative expression of these enzymes in malignant versus normal tissue have generated contradictory results [13–16].

Microarray technology constitutes a more recent approach for the detection and quantification of gene expression in a particular type of tissue. This technique allows for direct analysis of the expression of thousands of different genes at the same time; it is therefore a powerful tool for the study of differential patterns of expression in normal and tumour tissue.

This study involved the analysis of 17 normal specimens and 104 breast cancer biopsies. Whole genome expression microarrays were used in an attempt to identify differences in the expression profiles of normal and malignant breast tissue specimens.

Materials and methods

Patient selection

Tissue specimens from 104 cases of primary breast cancer (prior to any treatment with tamoxifen or chemotherapeutic agents), on which clinical follow-up and pathologic material, including snap-frozen tissue, were available for analysis from the 1993–1997 files of St Vincent's University Hospital Pathology Department, Dublin, Ireland, were included in this study. The patients involved underwent potentially curative resection at the hospital. Unfixed fresh breast resection specimens were received in Pathology Laboratory within maximum 30 min of resection. The tissue was examined macroscopically and samples of tumour snap frozen in liquid nitrogen and subsequently stored at $-70^{\circ}\text{C}/-80^{\circ}\text{C}$ prior to RNA isolation. A number of clinical and pathological parameters were abstracted from patients' charts including details on age, post-operative treatment and follow-up, tumour stage, and hormonal analysis. Pathologic material was examined on each case by SK. Tumours were typed [17] and graded [18] as previously described. Staging was performed according to the TNM system of the UICC [19]. Seventeen non-cancerous breast biopsies were also included in these studies to represent normal breast tissue.

RNA extraction

For RNA analyses, dissected tumours that had been snap-frozen in liquid nitrogen and then stored at $-70^{\circ}\text{C}/-80^{\circ}\text{C}$ until required were homogenised, on ice, in 1 ml TriReagent (Sigma; Poole, UK) and total RNA was subsequently isolated according to the manufacturer's instructions. RNA quantity and purity were assessed at 260 nm and 280 nm using a Nanodrop (ND-1000; Labtech. International); an Agilent bioanalyser (Agilent 2100; Agilent Technologies) was used to assess RNA qualitatively after isolation and, subsequently, following biotin-labelling and after fragmentation.

Microarray analysis

About 100 ng of each specimen was amplified and labelled using the Affymetrix GeneChip Eukaryotic 2 Cycle Labelling Assays for Expression Analysis, (Affymetrix; 900494) according to the manufacturer's instructions (www.affymetrix.com/products/reagents/specific/cdna2.affx). Gene expression was examined using whole genome microarrays (Affymetrix; U133 Plus 2.0; 900470). Microarray hybridisation, normalisation and filtering were performed as we have previously described [20].

Statistical analysis

Statistical (univariate and multivariate) analyses of the results were performed using the SPSS 10.1 software package. Descriptive statistics were used to summarise patient characteristics and statistical analysis of the results was performed using Pearson's χ^2 test to investigate relationships between mRNA expression and clinicopathological and histopathologic findings. Kaplan–Meier survival curves were established and were subsequently checked using the log-rank, Breslow and Tarone-ware tests (P -values represent log-rank, unless otherwise indicated) to assess the prognostic significance of expression of analysed mRNAs in tumour cells. Multivariate survival analyses were performed using the Cox regression backward stepwise likelihood ratio. The data was censored at 5 years for multivariate analysis. A value of $P < 0.05$ was considered statistically significant.

Results

Patient characteristics

This study involved analysis of 104 breast cancer biopsies (removed prior to any treatment with tamoxifen or chemotherapeutic agents) from patients aged between 31 years

and 89 years at the time of diagnosis (mean age = 58 years). Twenty-seven were less than 50 years and seventy-seven women were 50 years, or older, at diagnosis.

The size of the tumours ranged between 0.6 cm and 8.0 cm (mean = 2.79 cm). Eighteen tumours were T1 (<2 cm) in maximal dimension; 83 were T2 (2–5 cm) and 3 tumours were T3 (>5 cm). Eighty-two were invasive ductal carcinoma, 17 were invasive lobular and five were tumours of special type (two tubular and three mucinous). Eleven tumours were grade 1; 40 were grade 2; and 53 were grade 3. Sixty-seven tumours were oestrogen receptor (ER) positive and 34 were ER negative (ER status was determined by Enzyme Immuno-Assay (EIA); a positive result was defined as more than 200 fmol/g protein). ER status was not available for 3 patients. Forty-five tumours had no axillary metastases and 59 tumours had metastasised to axillary lymph nodes.

Sixty-nine women were treated with post-operative tamoxifen; 26 did not receive tamoxifen. Fifty patients were treated with adjuvant systemic chemotherapy (CMF +/- adriamycin); 45 patients did not receive chemotherapy. Details regarding tamoxifen and systemic chemotherapy were not available for 9 patients. Maximal follow-up was 3,026 days with a mean follow-up of 1,887 days.

Expression of drug-metabolism related genes

The presence of mRNAs from several different drug metabolism-related enzymes was detected in both normal and tumour tissue. As shown in Table 1, drug metabolism-related genes appear to be frequently expressed in both normal and tumour tissue. Some of these genes also show differential regulation, their expression levels significantly changed in malignant as compared to normal tissue. Amongst the cytochromes P450, CYP2C8, CYP2D6, CYP2E1 and CYP2J2 show increased frequency of expression in tumour as compared to normal tissue, while CYP1A1, CYP2C18, CYP2W1 and CYP3A5 are more commonly found in normal than in tumour tissue. As for the (GSH)-related genes, only two appear to be differentially regulated in tumour and normal tissue. Expression of GSH transferase (GST) M4 mRNA is less frequent in tumour than in normal specimens, while that of GSH peroxidase 2 is more common in tumour tissue. The transcript for aryl hydrocarbon receptor nuclear translocator protein 2 (ARNT2) is also considerably more frequent in tumour than in normal specimens, suggesting that it could be used as a biomarker of disease.

Alterations in expression can be seen in a different aspect in Table 2, which shows significant changes in the expression of genes involved in drug metabolism as detected in normal and tumour tissue. In Table 2, changes

in expression are stated as fold change between normal and tumour sample values; these values are related to the intensity of microarray probe binding and are expressed in arbitrary units. For every gene of interest, the microarray platform used has 11 different probes; Table 2 refers to the results obtained with specific probes, hence the existence of more than one entry for a single gene.

As can be seen in Table 2, frequency of expression does not necessarily correlate with changes in the expression levels of a particular gene; many of the genes listed in Table 1 are not differentially expressed in malignant as compared to normal tissue. The cytochromes P450 in this list are upregulated in tumour tissue, in accordance with the general tendency observed in P450 expression studies. The GSH-related genes, on the other hand, appear to be preferentially downregulated in tumour tissue, with the exception of GSH reductase, GST omega 2, GSH synthetase and one of the probes of GST A4.

For those genes that were commonly expressed (i.e., present in 15–85% of specimens), the possibility of a correlation between level of expression and overall survival, relapse-free survival, relevance as a positive (good) or negative (bad) prognostic factor, age at diagnosis, ER status, lymph node spread, tumour type, tumour size and tumour grade was analysed. The results shown in Table 3 indicate that expression of ARNT2 positively correlates with patient survival, while GST A1 is associated with poorer prognosis.

Prognostic analysis of ARNT2 and GST A1 for disease-free survival and overall survival

To further understand the implications of our findings, patient disease-free survival was plotted relative to the presence or absence of GST A1 and ARNT2 (Fig. 1). The graphs shown in Fig. 1 present censored data: this means that the data have been corrected by taking into account those patients for whom complete follow-up information was not available. This is a common event in this type of study: some patients move to a different place, or no longer attend follow-up clinics. Their data are therefore included in the study, but only up to the last time that information was available on their status. After that, they're no longer considered for the statistics calculations.

In relation to relapse-/disease-free survival (RFS), Kaplan–Meier analysis indicated ARNT2 expression to be a favourable factor associated with extended RFS (Fig. 1(i); $P = 0.0094$) and overall survival (OS) (Fig. 1(ii); $P = 0.0018$) from time of diagnosis. This was supported by χ^2 analysis (Table 4), indicating ARNT2 presence to be significantly associated with smaller tumour sizes ($P = 0.006$) and with relapse-free survival ($P = 0.027$) and

Table 1 Expression of members of the cytochrome P450 superfamily, glutathione-related genes and drug metabolism-related transcription factors in breast tissue samples expressed as percentage of samples positive for each particular gene

<i>Gene ID</i>	<i>Gene name</i>	<i>Normal (%)</i>	<i>Tumour (%)</i>
NM_000499	CYP1A1	23.5	6.7
NM_000761	CYP1A2	88	96
NM_000104	CYP1B1	100	100
NM_000762	CYP2A6	35.3	30.5
NM_000767	CYP2B6	0	4.8
NM_000770	CYP2C8	11.8	4.8
NM_030878	CYP2C8	5.9	31.4
NM_000771	CYP2C9	17.6	5.7
NM_000772	CYP2C18	41.2	15.2
NM_000106	CYP2D6	11.8	52.4
AF182276	CYP2E1	47	77
NM_000775	CYP2J2	35.3	79
NM_024514	CYP2R1	11.8	33.3
BC012027	CYP2U1	0	1
NM_017781	CYP2W1	47	33.3
NM_017460	CYP3A4	58.8	61.9
NM_022820	CYP3A43	11.8	9.5
NM_000777	CYP3A5	58.8	13.3
NM_000765	CYP3A7	23.5	30.5
NM_000941	NADPH cytochrome P450 oxidoreductase	82	95
NM_000846	Glutathione transferase A1	23.5	18.1
NM_000847	Glutathione transferase A3	5.9	10.5
NM_001512	Glutathione transferase A4	100	100
NM_015917	Glutathione transferase K1	100	100
NM_000561	Glutathione transferase M1	94.1	76.2
NM_000848	Glutathione transferase M2	94.1	95.2
A1459140	Glutathione transferase M3	94	81
NM_000850	Glutathione transferase M4	52.9	21.9
NM_000851	Glutathione transferase M5	100	92.4
NM_004832	Glutathione transferase O1	100	100
NM_000852	Glutathione transferase P1	100	100
NM_000853	Glutathione transferase T1	76.5	80
NM_000854	Glutathione transferase T2	23.5	39
NM_001498	Gamma glutamyl cysteine synthetase, heavy subunit	100	98.1
NM_002061	Gamma glutamyl cysteine synthetase, light subunit	100	98.1
NM_005265	Gamma glutamyl transpeptidase	88.2	97.1
NM_000581	Glutathione peroxidase 1	100	100
NM_002083	Glutathione peroxidase 2	23.5	50.5
NM_002084	Glutathione peroxidase 3	94.1	98.1
NM_002085	Glutathione peroxidase 4	100	100
NM_003996	Glutathione peroxidase 5	5.9	1.9
NM_000637	NADPH Glutathione reductase	47.1	61
NM_001621	Aryl hydrocarbon receptor	100	98.1
NM_014862	Aryl hydrocarbon receptor nuclear translocator protein 2	17.6	75.2

Table 2 List of drug metabolism-related genes with significant changes in expression levels in tumour relative to normal breast tissue

<i>Probe set</i>	<i>Gene name</i>	<i>Normals</i>	<i>Tumours</i>	<i>Fold change</i>	<i>P value</i>
202435_s_at	CYP1B1	649	973	1.5	0.018
214320_x_at	CYP2A6	37	472	12.8	0.008
1494_f_at	CYP2A6	37	394	10.7	0.023
200736_s_at	Glutathione peroxidase 1	4,152	1,867	−2.2	<0.001
214091_s_at	Glutathione peroxidase 3	2,159	169	−12.8	<0.001
201348_at	Glutathione peroxidase 3	3,189	210	−15.2	<0.001
201106_at	Glutathione peroxidase 4	5,192	2,084	−2.50	<0.001
225609_at	Glutathione reductase	259	494	1.9	<0.001
235405_at	Glutathione S-transferase A4	58	173	3.0	<0.001
202967_at	Glutathione S-transferase A4	314	204	−1.5	0.005
217751_at	Glutathione S-transferase kappa 1	1,294	953	−1.4	0.027
227163_at	Glutathione S-transferase omega 2	60	323	5.3	<0.001
203815_at	Glutathione S-transferase theta 1	550	242	−2.3	0.019
201415_at	Glutathione synthetase	273	491	1.8	<0.001

Fold change is calculated as intensity values of normal/intensity values of tumours

overall survival ($P = 0.002$) 5 years after breast cancer diagnosis.

In contrast to ARNT2, Kaplan–Meier analysis indicated GST A1 presence in tumours to be significantly associated with shortened RFS (Fig. 1(iii); $P = 0.0131$) and OS (Fig. 1(iv); $P = 0.0028$) times from breast cancer diagnosis. As summarised in Table 4, this is in agreement with X^2 analysis indicating GST A1 to be associated with spread of cancer to lymph nodes ($P = 0.031$) and with relapse ($P = 0.006$) and death ($P = 0.001$) from breast cancer within 5 years of diagnosis. Furthermore, multivariate analysis (Table 5) indicated GST A1 expression ($P = 0.045$), as well as lymph node status ($P < 0.0005$), tumour grade ($P = 0.003$), and ER status ($P = 0.0026$) to be independent prognostic factors for OS.

Discussion

A number of studies have clearly demonstrated that cytochromes P450 and other drug-metabolism related enzymes are commonly expressed in both normal and tumour tissue. Literature also suggests a differential regulation of these enzymes in malignant cells, although the nature of these differences is still a matter of controversy.

Analysis of a large number of breast tumour specimens was performed in our laboratory using whole genome gene expression microarrays. A large proportion of the specimens studied expressed a variety of drug metabolism-related genes; in many cases, significant alterations in gene expression were found in tumour as compared to normal specimens.

It is known that the expression of CYP2J2 increases cell proliferation and inhibits apoptosis [10], so it is not unexpected that CYP2J2 mRNA was more often expressed in tumours than in normal tissue. CYP2W1, however, has recently been hailed as a tumour-specific P450 [21], with no or very low levels of CYP2W1 mRNA and protein found in normal adult tissues, so it was unexpected to find that mRNA expression of this enzyme in normal tissue was more frequent than in tumour specimens. The low prevalence of CYP1A1 mRNA expression found in this analysis is in agreement with previous results reporting lower frequency of CYP1A1 expression in tumour as compared to normal tissue [22]. Very low levels of this enzyme were also found in both breast tumour specimens and their surrounding normal tissue by RT-PCR analysis [14]. However, the same authors found decreased expression of CYP2E1 in tumour specimens by immunoblotting, which is in contradiction with the observed mRNA expression frequencies in our results. These inconsistencies could be attributed to posttranscriptional events, since mRNA levels do not always correlate with protein levels.

GSH transferase M4 (GST M4) mRNA expression was found to be more common in normal than tumour tissue. The role played by this enzyme in cancer remains obscure, but a recent study suggested an association between GST M4 polymorphisms and lung cancer risk, implying that the activity of this enzyme might be involved in tumour development [23]. GSH peroxidase 2 mRNA was also found to be more frequently expressed in tumour as compared to normal breast tissue; this enzyme can protect cells from free radicals and also regulate proliferation and apoptosis through redox signalling [24] and thus its

Table 3 Correlation between mRNA expression and overall survival (OS), relapse-free survival (RFS), age at diagnosis (RFS), age at diagnosis, oestrogen receptor (ER) status, lymph node spread, tumour type, tumour size and tumour grade

mRNA	OS	G/B	RFS	G/B	Age at diagnosis	ER status	Lymph node spread	Tumour type	Tumour size	Tumour grade
CYP2A6	N	N/A	N	N/A	N	N	N	N	N	N
CYP2C8	N	N/A	N	N/A	N	N	N	N	N	N
CYP2C18	N	N/A	N	N/A	N	N	N	N	N	N
CYP2D6	N	N/A	N	N/A	N	Y (ER+)	N	N	N (but $P = 0.052$, $P \propto$ smaller)	N
CYP2E1	N	N/A	N	N/A	N	N	N	N	N	N
CYP2J2	N	N/A	N	N/A	N	N	N	N	–	N
CYP2R1	N (but $P = 0.081$)	$P \sim$ good outcome	N	N/A	N	N	N	N	N	N
CYP2W1	N	N/A	N	N/A	N	N	N	N	N	N
CYP3A4	N	N/A	N	N/A	N	N	N	N	N	N
CYP3A7	N	N/A	N	N/A	N	N	N	N	N	N
ARNT2	Y ($P = 0.002$)	G	Y ($P = 0.009$)	G	N	N (but $P = 0.052$, $P \propto$ ER+)	N	N	–	$P \propto$ lower grade
GST A1	Y ($P = 0.003$)	B	Y ($P = 0.013$)	B	N	N	–	N	N	N
GST M1	N	N/A	N	N/A	N	N	N	N	N	N
GST M3	N	N/A	N	N/A	N	N	N	N	N	N
GST M4	N	N/A	N	N/A	Y ($P = 0.030$, $P \propto$ older age)	N	–	N	N	N
GST T1	N	N/A	N	N/A	N	N	N (but $P = 0.054$)	N	N	N
GST T2	N	N/A	N	N/A	Y ($P \propto$ older patients)	N	N	N	N	N
Glutathione peroxidase 2	N	N/A	N	N/A	–	N	N	N	N	N
NADPH Glutathione reductase	N	N/A	N	N/A	N	N	N	N	N	N

G good prognostic factor, B bad prognostic factor, α associated with, P probability value, Y yes, N no, N/A not applicable

Fig. 1 Kaplan–Meier survival curves for ARNT2 ((i) & (ii)) and GST A1 ((iii) & (iv)) mRNA presence or absence and their association with disease/relapse-free survival (RFS) and overall survival (OS), respectively

Table 4 Correlation between clinicopathological factors and expression of GST A1 and ARNT2 mRNA in breast carcinoma

Characteristics	No. of cases	GST A1 (%)	<i>P</i>	No. of cases	ARNT2 (%)	<i>P</i>
Age (yr)						
<50	6/27	22.2	0.537	18/27	66.7	0.245
≥50	13/77	16.9		60/77	77.9	
Tumour size						
<2.8 cm	11/56	19.6	0.695	48/56	85.7	0.006*
≥2.8 cm	8/48	16.7		30/48	62.5	
Lymph node metastasis						
Negative	4/45	8.9	0.031*	33/45	73.3	0.732
Positive	15/59	25.4		45/59	76.3	
Histology grade						
I	1/11	9.1	0.453	10/11	90.9	0.084
II	6/40	15.0		33/40	82.5	
III	12/53	22.6		35/53	66.0	
Histology type						
IDC	17/82	20.7	0.381	58/82	70.7	0.132
ILC	2/17	11.8		15/17	88.2	
Special	0/5	0.0		5/5	100.0	
ER status						
Negative	9/34	26.5	0.106	22/34	64.7	0.052
Positive	9/67	13.4		55/67	82.1	
Relapse						
No relapse at 5 yrs	5/54	9.3	0.006*	45/54	83.3	0.027*
Relapsed within 5 yrs	13/41	31.7		26/41	63.4	
Alive versus died from breast cancer						
Alive & well at 5 yrs	6/64	9.4	0.001*	54/64	84.4	0.002*
Died within 5 yrs	11/29	37.9		16/29	55.2	

P values from χ^2 analyses; * indicates significant parameter; *IDC* invasive ductal carcinoma, *ILC* invasive lobular carcinoma

Table 5 Multivariate cox regression backward stepwise (likelihood ratio)

Characteristics	Overall survival (OS), <i>P</i>	Relapse-free survival (RFS), <i>P</i>
Lymph Node (spread versus no spread)	<0.0005*	<0.0005*
Histology grade (I & II versus III)	0.003*	NS
Tumour size (<2.8 cm vs. ≥2.8 cm)	(0.060)	0.048*
ER status (positivity versus negativity)	0.026*	<0.0005*
GST A1	0.045*	NS

Parameters included in the multivariate analysis included age at diagnosis, tumour size, tumour grade, lymph node status, ER status, as well as GST A1 and ARNT2 mRNA expression. Mean size (2.8 cm) was used as cut-off; grade I & II were grouped together versus grade III. This Table summarises significant factors. (* significant; NS not significant)

increased expression may confer a survival advantage to tumour cells. Expression of GSH peroxidase 2 has been established in breast tissue [25] and increased expression of this enzyme has recently been reported in lung adenocarcinomas and in normal alveolar epithelium from smokers as compared to normal lung tissue from non-smokers [26].

Differential mRNA expression of ARNT2 was one of the major findings in our study, with expression in tumour tissue being much more prevalent than in normal tissue specimens and significantly correlating with better prognosis. To date, no correlation has been reported in the literature between expression of this gene and cancer progression or survival. It should be noted, however, that increased mRNA expression of an AhR-related element, the aryl hydrocarbon interacting protein, was also reported in specimens from patients that had a better prognosis [27]. This points to the AhR receptor pathway as a commonly altered target in breast cancer; its deregulation, however, appears to be associated with good prognosis.

Upon binding a specific ligand, the aryl hydrocarbon receptor (AhR) dimerises with the AhR nuclear translocator protein (ARNT) and activates the transcription of its target genes. ARNT2 is a homolog of ARNT with a more restricted pattern of expression, commonly found in the central nervous system [28], although its expression has also been reported in other developing organs such as kidney [29]. The main function of ARNT2 appears to be in organ development, since knock-out or disruption of this gene in mice and zebrafish results in severe defects in organs such as brain, eyes, heart and gut [30, 31].

The AhR receptor pathway is implicated in such essential cell functions as growth, proliferation, apoptosis and epithelial-mesenchymal transition [32]. The role of the AhR in these phenomena remains undefined, as there is evidence that it can both increase and inhibit cell proliferation, as well as protect cells from apoptosis or induce cell death. One of the best characterised mechanisms of growth inhibition by the AhR involves repression of ER signalling; it is then not unreasonable to believe that ER positive tumours would display impaired growth and decreased proliferation rates. It should also be noted that

even though ER expression did not significantly correlate with ARNT2 expression, the *P* value was almost significant (*P* = 0.052). This would suggest that many of the tumours expressing ARNT2 were also ER+. AhR signalling, helped by ARNT2, might then slow down the progression of these tumours. This model would explain the better prognosis observed in patients with ARNT2-overexpressing tumours.

It should be noted that ER positive tumours are associated with better survival; since many of the tumours expressing ARNT2 are also ER positive, the better survival rates associated with ARNT2 might just be due to the fact that this protein is linked with ER positive status, which in turn is associated with better survival. Increased ARNT2 mRNA expression might thus not directly impair tumour progression but rather be associated with the better prognosis-related ER positive status. Even if this were the case, ARNT2 would still be useful in prediction of prognosis and might also be used as a marker of disease, since it is considerably upregulated in tumour as opposed to normal tissue.

Expression of GST A1 was associated with decreased overall and relapse-free survival. This might be related to therapy efficacy, since expression of GST A1 in cell lines is associated with an increase in resistance to carboplatin and cyclophosphamide [33]. Increased expression of GST A1 in tumour tissue would mean enhanced inactivation of anticancer drugs that could result in treatment failure.

Several authors have investigated whether a correlation exists between GST A1 expression in tumours and disease prognosis, sometimes generating contradictory results. Early studies carried out in breast cancer patients failed to establish a correlation between GST expression and clinicopathological features such as disease-free and overall survival [34, 35]. Confirming these results, no association was found between the expression of any of the GSTs analysed and response to treatment, overall survival and disease-free survival in ovarian cancer [36]. However, a more recent analysis found that patients homozygous for a GST A1 polymorphism that results in reduced expression of the enzyme showed a significant difference in 5-year survival after breast cancer treatment when compared to heterozygous patients [37]; indeed, low expression of GST

A1 correlated with reduced risk of death in the 5 years following diagnosis. Lower expression of GST A1 in the liver would mean that drugs such as cyclophosphamide are inactivated in a slower manner, leaving more drug available in the circulation and improving therapy efficacy.

In conclusion, gene expression microarray analysis makes possible the study of alterations in the expression of thousands of genes during cancer development and progression, providing an accurate picture of what is happening inside the cell. As shown by our results, it can also help to elucidate the connection between gene expression and cancer phenotype or therapy outcome, thereby improving our knowledge of the disease and providing biomarkers for diagnosis and individual tumour characterisation.

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