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Expression levels of HER2/*neu* and those of collocated genes at 17q12-21, in breast cancer

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Abstract. HER2/*neu* is associated with poorer clinical outcome in breast cancer. Expression patterns of co-localised cancer-associated genes at 17q12-21 were examined using RT-PCR. The study group consisted of a 96-patient cohort. Relative quantity of mRNA expression was calculated using the comparative cycle threshold method and Qbase software. Results were analysed to detect expression patterns among the genes, and to identify associations between expression levels and clinical data. Levels of HER2/*neu* correlated with those of *GRB7* (r=0.551, p<0.001), *RARA* (r=0.391, p<0.001), *RPL19* (r=0.549, p<0.001) and *LASP1* (r=0.399, p<0.001). *GRB7* was significantly inversely associated with improved DFS at 60 months (p=0.036). *RARA* levels were greater in HER2/*neu*-positive as opposed to HER2/*neu*-negative patients (p=0.021); levels were significantly higher in ER-positive patients, relative to those who were ER-negative (p=0.003). Levels of *RPL19* were significantly higher in the HER2/*neu*-overexpressing (p=0.010) and luminal B subtypes (p=0.007). *LASP1* levels were higher in those patients who had been classified clinically as HER2/*neu*-positive (p=0.004). This study reaffirms the correlation between HER2/*neu* and the co-localised *LASP1* and *GRB7*; the latter target may hold additional significance in addition to being a surrogate marker for HER2/*neu* expression. The relationship identified between *RARA* and ER-positivity may herald an avenue for targeted therapy of these tumours.

Introduction

The molecular taxonomy of breast cancer, as first described by Perou *et al* (1), has revolutionised how clinicians approach and manage breast cancer. Our treatment options have not, as yet, evolved to the point where each subtype can be considered entirely separate, however. In particular, numerous questions remain in relation to HER2/*neu* function, its influence on tumourigenesis and cancer progression and, most importantly from a clinical standpoint, the underlying mechanisms through which it impacts on our treatment strategies. One approach to answering these questions has been to focus on the area of chromosome 17 in which HER2/*neu* is located, 17q12-21 (the HER2/*neu* amplicon), in order to identify other genes in the vicinity.

Growth factor receptor-bound protein 7 (GRB7) forms part of the GRB7 family of proteins, along with GRB10 and GRB14, and it acts as an adaptor molecule. Numerous binding partners have been identified for GRB7, including HER2/*neu* (2). *GRB7* has been demonstrated to be coamplified with HER2/*neu* (2,3) in breast tumour biopsies. In addition, it is included on Oncotype DX, the commercially available multigene molecular assay which can provide individualised risk estimates for patients with breast cancer, based on the expression levels of 16 cancer-related genes in reference to 5 invariant genes (4).

Other co-located targets of interest include retinoic acid receptor α (*RARA*), an estrogen-regulated gene with potential as a target for cancer prevention and therapy because treatment with retinoids leads to cell growth arrest and apoptosis in ER-positive breast cancer cells (5,6), and ribosomal protein 19 (*RPL19*) a member of a family comprising >70 different proteins that form the large and small ribosomal subunits. Whilst protein expression of RPL19 was first demonstrated to correlate with that of HER2/*neu* in 1993 (7), and in 2003 it was shown to be co-expressed with HER2/*neu* on a cDNA microarray (8), its expression has not yet been examined by RT-PCR (real-time polymerase chain reaction), and its association with clinicopathological variables or outcome in breast cancer remains to be elucidated.

Given that many of the chemotherapeutic agents currently in use target the microtubule, including the microtubule-stabilising agents, paclitaxel docetaxel and the epithilones A and B, and the microtubule-destabilising agents, vincristine and vinblastine, γ-tubulin is potentially a target in cancer chemotherapy, γ-tubulin 1 (*TUBG1*) was the first γ-tubulin gene to be discovered in 1991 (9), and its upregulation has been postulated to facilitate rapid division of tumour cells (10). Interestingly, in some preclinical studies, taxane resistance has been reversed with the use of trastuzumab in HER2/*neu*-positive tumours (11), and the relationship between HER2/*neu* and *TUBG1* certainly warrants further consideration.

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One final target of interest is LIM and SH3 domain protein 1 (*LASP1*). Originally identified in a cDNA library of metastatic breast cancer (12), the cellular function of *LASP1* remains unknown. Knockdown of *LASP1* in the breast cancer cell lines BT-20 and MCF-7, and the ovarian cancer cell line SKOV-3 has however been demonstrated to result in strong inhibition of proliferation and migration (13,14). In 2006, *LASP1* was identified as one of 5 genes down-regulated in poor prognosis, lymph-node-positive breast cancer (15). *LASP1* has also been identified as a potential p53 (a tumour suppressor) target in hepatocellular carcinoma, with further work suggesting that p53 may play a role in influencing tumour metastasis through *LASP1* (16).

The primary aim of this work was to further characterise, using RT-PCR, the relationship between HER2/*neu* and the aforementioned candidate genes (*GRB7, RARA, RPL19, TUBG1* and *LASP1*) located at the HER2/*neu* amplicon on chromosome 17, thus identifying targets with prognostic or predictive significance in the clinical management of breast cancer.

Materials and methods

The study group for RT-PCR consisted of a 96-patient cohort (Table I); mean patient age at diagnosis of breast cancer was 57.6 years (SD 13.3, range 28-92), and the median period of follow-up was 49 months (range 1-224) (Table I). HER2/*neu* status had been previously determined by scoring membranous staining according to the HercepTest™ (Dako) protocol. Similarly, routine clinical determination of ER and PR status had been performed at Galway University Hospital using the Allred scoring method (17). Patients were classified according to tumour characteristics as follows: luminal A (estrogen receptor (ER)-positive and/or progesterone receptor (PR)-positive and not HER2/*neu*-positive); luminal B (ER-positive and/or PR-positive and HER2/*neu*-positive); HER2/*neu* overexpressing (HER2/*neu*-positive, ER-negative, PR-negative); and basal-like or triple-negative (ER-negative, PR-negative, HER2/*neu*-negative). According to this classification, 35 (36.5%) patients in the study group had luminal A breast cancer, 31 (32.3%) had luminal B, 17 (17.7%) were HER2/*neu* overexpresssing, and 13 (13.5%) were basal-like. Fifty-seven patients (59.4%) were ER-positive and 55 (57.3%) were PR-positive; the PR status for 1 patient was unknown. Forty-eight patients (50.0%) were HER2/*neu*-positive, and 48 (50.0%) were HER2/*neu*-negative.

RT-PCR. Following informed, signed consent, tissue specimens were snap-frozen in liquid nitrogen immediately following surgical excision, and subsequently stored at -80˚C. Ethical approval from the Galway University Hospital's Research Ethics Committee was granted to store and use this material for translational research programmes. This tissue was later homogenised, and total RNA was extracted. RNA concentration and purity was determined using a NanoDrop spectrophotometer. Aliquots of RNA equivalent to 1 μg were reverse transcribed using SuperScript™ III (Invitrogen). RT-PCR reactions were then carried out in final volumes of 10 μl using a 7900HT sequence detection system [Applied Biosystems (AB)], using TaqMan probes, optical 96-well fast plates and sequence detection system (SDS) software (AB). RT-PCR reaction volumes consisted of cDNA (1 μl), mastermix (5 μl), TaqMan probe mix (0.5 μl), and nuclease-free water (3.5 μl).

Table I. Study group.

	No.
Grade	
0	5
1	5
2	28
3	58
T	
1	18
2	48
3	23
4	6
Unknown	1
Nodal status	
0	50
1	16
2	15
3	13
Unknown	2
M	
0	77
1	16
Unknown	3
NPI	
1	6
2	8
3	39
4	39
Unknown	4
Stage	
1	17
2	37
3	23
4	16
Unknown	3
ER status	
ER^+	57
ER^-	39
Unknown	0
PR status	
PR^+	55
PR^-	40
Unknown	1
HER2/neu status	
HER2/*neu*$^+$	48
HER2/*neu*$^-$	48
Unknown	0

cDNA, synthesised from commercially available breast cancer cell line RNA, was included on each 96-well plate as an interassay control. All reactions were performed in triplicate, and the threshold standard deviation for intra- and inter-assay replicates was 0.3.

Relative quantity of mRNA expression was calculated using the comparative cycle threshold (ΔΔCt) method and Qbase software. The geometric mean of the cycle threshold value of the endogenous control genes *PPIA* and *MRPL19* was used to normalize the data, and the lowest expressed sample was used as a calibrator. Relative quantities were logarithmized (log10), and distribution of the data set was assessed using the Kolmogorov-Smirnov test in SPSS version 15.0. Logarithmized values did not vary significantly from a normal distribution. Statistical tests employed to assess the data included Pearson's correlation coefficient, Student's t-test and one-way ANOVA for between groups analysis. Kaplan-Meier curves and the log-rank test were employed to investigate for significant relationships between gene amplification and disease-free and overall survival.

Results

HER2/neu. Gene expression levels of HER2/*neu* correlated with those of *GRB7* (r=0.551, p<0.001), *RARA* (r=0.391, p<0.001), *RPL19* (r=0.549, p<0.001), and *LASP1* (r=0.399, p<0.001). HER2/*neu* expresssion did not correlate with that of *TUBG1* (r=0.173, p=0.091). Expression levels of HER2/*neu* were significantly higher in those patients who had been classified clinically as HER2/*neu*-positive using immunohistochemistry (IHC) and fluorescent *in situ* hybridisation (FISH) (p<0.001). Levels did not differ significantly between patients according to either their ER (p=0.481) or PR status (p=0.935).

Post hoc analysis using bonferroni adjustment demonstrated that levels of HER2/*neu* were significantly higher in the HER2/*neu* overexpressing and luminal B subgroups relative to levels in both the luminal A (p<0.001) and basal (p<0.001) subtypes. Gene expression levels of HER2/*neu* were significantly lower in patients who were N0 at diagnosis versus those who were N3 at diagnosis (p=0.007). No differences were seen in amplification levels of HER2/*neu* according to menopausal status (p=0.520), tumour grade (p=0.634).

Levels of HER2/*neu* were found to be significantly higher in those patients classified as NPI 4 versus those with an NPI of 3 (p=0.002). Levels were also higher in the excellent prognostic group (NPI 1), but this did not reach statistical significance. Gene expression of HER2/*neu* were found to be significantly less in stage 1 (p=0.018) and 2 (p<0.001) disease when compared to levels in patients with stage 3 disease.

Analysis of the relationship between levels of HER2/*neu* and disease-free (DFS) (p=0.651) and overall survival (OS) (p=0.505) using Kaplan-Meier curves failed to demonstrate significant relationships. No differences were noted when we looked at levels in relation to DFS (p=0.915) and OS (p=0.495) up to the 60-month time-point.

GRB7. Gene expression levels of *GRB7* were significantly higher in patients classified as HER2/*neu*-positive using IHC and FISH, versus those classified as HER2/*neu*-negative (p=0.009). Levels were significantly higher in ER-negative versus ER-positive patients (p=0.036), but no differences were noted according to PR status (p=0.469). *GRB7* levels were noted to be significantly higher in the luminal B subgroup relative to the luminal A subgroup (p=0.033); no differences were seen across the other intrinsic subtypes. Levels did not differ according to menopausal status (p=0.650), tumour grade (p=0.650), nodal status (p=0.462), stage (p=0.201), or NPI (p=0.734). In relation to survival, although no significant differences were demonstrated for our cohort overall, lower levels of *GRB7* were significantly associated with improved DFS (p=0.036) but not OS (p=0.497) up to 60 months.

RARA. Levels of *RARA* were greater in HER2/*neu*-positive as opposed to HER2/*neu*-negative patients (p=0.021). Gene expression was significantly higher in ER-positive patients, relative to those who were ER-negative (p=0.003). Levels did not differ significantly between PR-positive and PR-negative patients (p=0.403). Gene expression was significantly lower in the basal-like subtype relative to the luminal A (p=0.038), luminal B (p=0.005) and HER2/*neu* overexpressing (p=0.012) subtypes. Levels did not differ significantly between these latter 3 subgroups. No differences were seen for expression levels relative to clinicopathological variables or in relation to either DFS or OS.

LASP1. *LASP1* expression levels were significantly higher in those patients who had been classified clinically as HER2/*neu*-positive (p=0.004). Levels did not differ significantly according to ER (p=0.428) or PR (p=0.109) status. Gene expression levels were significantly higher in the HER2/*neu* overexpressing subtype relative to the luminal A group of patients (p=0.045). Levels did not differ according to menopausal status (p=0.076), grade (p=0.325), nodal status (p=0.248), NPI (p=0.887), or stage of disease (p=0.053). Whilst no significant differences were noted for *LASP1* relative to DFS or OS, a trend towards improved DFS, for those with lower levels of *LASP1*, at 60 months (p=0.069) was noted.

RPL19. RPL19 expression levels did not differ significantly according to HER2/*neu* (p=0.377), ER (p=0.167) or PR status (0.411). Levels were higher in the HER2/*neu* overexpressing (p=0.010) and luminal B subtypes (p=0.007), relative to the luminal A subtypes. There was no significant difference in the expression levels of *RPL19* according to menopausal status (p=0.189), grade (p=0.955), nodal status (p=0.562), NPI (p=0.102) or stage of disease (p=0.265). No differences were noted in relation to either DFS or OS.

TUBG1. Gene expression levels did not differ significantly between HER2/*neu*-positive and negative patients (p=0.453), ER-positive and negative patients (p=0.496), or PR-positive and negative patients (p=0.818). Furthermore, no differences were noted across the intrinsic subtypes (p=0.083), in relation to clinicopathological variables or in relation to either DFS or OS.

Discussion

This work has confirmed the close relationship which exists between gene expresssion levels of HER2/*neu* and *GRB7*. Located at the minimal common region of amplification at the HER2/*neu* amplicon, *GRB*7 has been repeatedly demonstrated to co-amplify with HER2/*neu* (8,18,19). Our work has

demonstrated that *GRB7* is expressed at higher levels in patients with ER-negative breast cancer; this has particular relevance given reports by van Agthoven *et al* which have suggested that *GRB7* is associated with tamoxifen resistance and estrogen-independent proliferation of breast cancer cells *in vitro* (20); the same authors confirmed this association using RT-PCR on breast cancer tissue samples, and concluded that their data supported previously established associations for HER2/*neu* and *GRB7* with endocrine resistance in breast cancer, further adding to the rationale behind the examination of combinations of endocrine therapies with HER2/*neu*-targeting compounds. Furthermore, they postulated that their cell line data and other experimental evidence, suggests that *GRB7* may exert an active role in tamoxifen resistance of breast cancer independently of HER2/*neu* (21). GRB7 protein is known to bind strongly to HER2/*neu* via its SH2 domain, and it has been previously suggested that coamplification of the two genes is connected with extramucosal tumour invasion in oesophageal cancer (22). Intriguingly, recent work by Nencioni *et al* has shown that GRB7 is rapidly upregulated in response to HER2/*neu* inhibition with lapatinib, and may offer one explanation for acquired resistance to this latter agent, with accumulation of *GRB7* in response to HER2/*neu* signalling inhibition resulting in increased breast cancer cell aggressiveness and metastatic disease progression (23).

The relationship between *GRB7* and prognosis has been investigated extensively and, as noted earlier, the gene is located on the Oncotype Dx assay (24) used to predict breast cancer recurrence. In addition, Vinatazer *et al* (25) demonstrated a correlation between higher levels of GRB7 protein expression and lower disease-free and overall survival, a finding also reported by Cobleigh *et al* (26) and, more recently, by Nadler *et al* (27). These findings correlate well with the results of the present work which has demonstrated a significant relationship between higher levels of *GRB7* and poorer disease-free survival up to 5 years, and taken together suggest that *GRB7* holds greater clinical significance than acting simply as a surrogate marker for expression or amplification of HER2/*neu*.

Arriola *et al*, using RT-PCR on 27 breast cancer samples, found that *RARA* was one of 4 genes significantly more highly expressed in HER2/*neu*/*TOP2A* co-amplified versus HER2/*neu*-amplified breast cancers (the other three were *CASC3, CDC6* and *SMARCE1*) (28). To our knowledge, however, this is the first work to detail gene expression levels of *RARA* by RT-PCR across intrinsic subtypes, and between ER- and HER2/*neu*-positive and -negative breast cancers. In attempting to sub-classify patients beyond the traditional hormone receptor positive/negative and intrinsic subtype classification systems, it is interesting to note that levels of *RARA* were significantly positively correlated with those of HER2/*neu*, and were significantly greater in those patients classified as ER-positive or HER2/*neu*-positive, relative to their negative counterparts. There has been much success with targeting *RARA* in acute promyolocytic leukemia, with approximately 80% of patients achieving remission when treated with all-trans retinoic acid (ATRA), a synthetic derivative of vitamin A. Although Fitzgerald *et al*, demonstrated that the ER-negative, HER2/*neu*-positive cell-line SKBR3 was associated with high expression levels of RARA and response to retinoic acid (29), it appears that SKBR3 is an exception, with most of the work in this area concluding that the majority of the ATRA-resistant cell-lines are associated with moderate to high levels of the HER2/*neu* protein (30-32). In addition, functional work has shown that high expression levels of HER2/*neu* are associated with resistance to ATRA (30,33), but that a synthetic analogue of ATRA, 4-hydroxyphenyl-retinamide (4HPR), can repress HER2/*neu* expression, and thereby sensitise cells to ATRA.

The evidence above suggests that HER2/*neu* amplification negatively impacts on that of *RARA*, and hence ER-negative, HER2/*neu*-positive patients tend not to respond to treatment with ATRA. In contrast, however, our results suggest a significant positive relationship between gene expression levels of HER2/*neu* and *RARA*, suggesting that a significant subgroup of HER2/*neu*-positive patients do in fact express *RARA*, and would thus be amenable to treatment with ATRA. Given that our results also suggest a positive relationship with ER-positivity, it seems reasonable to conclude that this group would largely consist of patients from the luminal B subtype, although it should be noted that levels of *RARA* were not significantly higher in this subgroup relative to levels for patients in either the luminal A or HER2/*neu* overexpressing subgroups.

RPL19 expression levels did not correlate with HER2/*neu* status, ER status, or with any of the other clinicopathological variables under study. With the exception of three other studies which have demonstrated coexpression with HER2/*neu* (8,34,35), no work has reported either positive or negative relationships between expression or amplification of *RPL19* and breast cancer, and it would thus appear that this marker is of questionable significance as a predictive or prognostic marker, as least in breast cancer; it has previously been associated with poorer prognosis in both prostate (36) and colorectal cancer (37).

Expression levels of *TUBG1* mRNA have been reported by Niu *et al*, using RT-PCR, to be increased from normal breast tissue, through to atypical ductal hyperplasia, ductal carcinoma *in situ* (DCIS) and invasive ductal carcinoma (IDC) of the breast, and furthermore these levels were demonstrated to correlate well with protein expression of TUBG1 as measured using IHC (38). Our work here has built on that of Niu *et al*, but has not demonstrated an association between expression levels of this gene, and those of HER2/*neu*, by RT-PCR. Furthermore, no association has been found with ER, PR- or HER2/*neu* status, and no differences were seen across subtypes or in relation to clinicopathological variables.

The association between *LASP1* and HER2/*neu* has been confirmed in a number of studies (12,35,39). The results reported above have reiterated this relationship using RT-PCR, whilst also demonstrating higher levels of this novel gene in the HER2/*neu* overexpessing subtype, and in those patients classified as HER2/*neu*-positive using IHC and FISH. This latter result does conflict with the only other analysis of LASP1 in relation to clinicopathological variables; Grunewald *et al* reported that protein expression of LASP1, as assessed using IHC, did not correlate with HER2/*neu* status (40). On the other hand, these authors stated that increased expression levels of LASP1 correlated with increased tumour size and rates of nodal positivity and, whilst neither of these findings were corroborated by our results, we did note a trend towards improved DFS in those patients with lower levels of *LASP1* gene expression.

This work has elucidated the relationship between HER2/*neu*, the intrinsic breast cancer subtypes, and a number of novel targets located at the HER2/*neu* amplicon on chromosome 17. In particular, we have demonstrated that gene expression levels of

RARA differ significantly across the subtypes, and our findings suggest that luminal B patients, in particular, might benefit from treatment with ATRA or a similar derivative. In addition, our findings have reiterated the relationships previously described between HER2/*neu* and *LASP1* and *GRB7*, and suggest that the latter may hold additional prognostic or predictive significance in addition to being a surrogate marker for HER2/*neu* expression.

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