Guanine Nucleotide Binding Protein β 1: A Novel Transduction Protein with a Possible Role in Human Breast Cancer

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Abstract. *To our knowledge, this is the first study to examine the relationship between guanine nucleotide binding protein β-1 (GNB1) mRNA expression and clinicopathological parameters. Furthermore, the correlations between GNB1, Rictor and the mammalian target of rapamycin (mTOR) were also investigated. Materials and Methods: Breast cancer tissues (n=136) and normal tissues (n=31) underwent reverse transcription and quantitative polymerase chain reaction. Transcript levels were correlated with clinicopathological data. Results: Higher mRNA transcript levels of GNB1 were found in the breast cancer specimens in paired samples (p=0.0029). The mRNA expression of GNB1 increased with TNM stage (TNM1 vs. TNM2/3/4, p=0.036), tumour grade (grade 2 vs. 3, p=0.006), in ductal tumours (p=0.0081), and was associated with adverse patient outcomes (mortality vs. disease-free survival: 4.9 vs. 0.01, p=0.027). GNB1 was positively-correlated with mTOR (r=0.525, p<0.000001) and Rictor (r=0.388, p=0.0000606). Conclusion: These observations may suggest that GNB1 plays an important role in the mTOR-related anti-apoptosis pathway and can potentially be targeted in the treatment of human breast cancer.*

The role of autocrine, paracrine and endocrine factors in cancer risk and oncogenesis has been the focus of much study. The well-attested role of obesity as a risk factor for certain types of cancers is believed to be at least partly mediated by various adipocytokines (1, 2). Similarly, the association of inflammation with carcinogenesis is believed to be mediated by products of the COX2 pathway (3). These and numerous other examples can be cited as the *raison d'être* for the research of transduction systems in relation to oncogenesis.

Guanine nucleotide binding protein beta polypeptide-1 (GNB1, or Gβ1) integrates signals between receptors and effector proteins and regulates certain signal transduction receptors and effectors (4).

We hypothesised that GNB1 is involved in the anti-apoptosis pathway mediated by the mammalian target of rapamycin (mTOR), and thus may play a role in human carcinogenesis. To our knowledge, this is the first study to examine the relationship between the mRNA expression of *GNB1* and clinicopathological parameters. Furthermore, the correlation between *GNB1*, mTOR, and other components of the mTOR pathway were also investigated.

Materials and Methods

Samples. Institutional guidelines including ethical approval and informed consent were adhered to. Immediately after surgical excision, a tumour sample was obtained from the tumour area while another was taken from the associated non-cancerous tissue (ANCT) within 2 cm from the tumour area, without affecting the assessment of tumour margins. Breast cancer tissues (n=136) and normal background tissues (n=30) were collected and stored at –140˚C in liquid nitrogen until the commencement of this study. This cohort has been the subject of a number of completed and on-going studies (5, 6).

All the patients were treated according to local guidelines, following discussions in multidisciplinary meetings. Patients undergoing breast-conserving surgery also underwent radiotherapy. Hormone-sensitive patients were given tamoxifen. Hormone-insensitive cases, high-grade cancer, and node-positive cases were treated with adjuvant therapy. Clinicopathological data (Table I) were collected from the patient charts, and were stored in an encrypted database. Median follow-up was 120 months (June 2004).

RNA extraction kits and reverse transcription kits were obtained from AbGene Ltd. (Epsom, Surrey, UK). PCR primers were designed using Beacon Designer (Premier Biosoft International Ltd., Pal Alto, CA, USA) and synthesised in-house. Custom-made hot-start Master Mix for quantitative PCR was from AbGene Ltd (7).

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Table I. *Clinical data describing the patient cohort.*

Parameter	Category	Number
Node status	Node positive	45
	Node negative	65
Tumour grade	1	16
	2	34
	3	49
Tumour type	Ductal	80
	Lobular	11
	Medullary	3
	Tubular	1
	Mucinous	1
	Other	5
TNM staging	1	55
	2	32
	3	5
	4	3
Clinical outcome	Disease-free	71
	With local recurrence	6
	Alive with metastasis	5
	Died of breast cancer	14
Follow-up	Median (in months)	120
	Range (in months)	100 to 150

Tissue processing, RNA extraction and cDNA synthesis. Approximately 10 mg of cancerous tissue were homogenised. A larger amount of ANCT (20-50 mg) was used as its high fat content made it difficult to obtain a sufficient RNA concentration for analysis. The concentration of RNA was determined using a UV spectrophotometer (Wolf Laboratories, York, UK) to ensure adequate amounts of RNA for analysis. Reverse transcription was carried out using a reverse transcription kit (AbGene) with an anchored oligo (dT) primer using 1 mg of total RNA in a 96-well plate to produce cDNA. The quality of cDNA was verified using ß-actin primers (primers 5'-ATGATATCGCCGCGCTCGTC-3' and 5'-CGCTCGGTGAGGATCTTCA-3').

Quantitative analysis. Transcripts of cDNA library were determined using real-time quantitative PCR based on the Amplifluor technology. The PCR primers were designed using Beacon Designer software, but an additional sequence, known as the Z-sequence (5'-ACTGAACCTGACCGTACA-3'), which is complementary to the universal Z-probe (Intergen Inc., Oxford, UK) was added to the primer. The primers used are detailed in Table II.

The reaction was carried out under the following conditions: 94˚C for 12 min and 50 cycles of 94˚C for 15 s, 55˚C for 40 s, and 72˚C for 20 s. The levels of each transcript were generated from a standard that was simultaneously amplified within the samples. Normalisation was carried out against cytokeratin 19 (CK19). With every run of the PCR, a negative and positive control were employed, using a known cDNA sequence (podoplanin) (7).

Statistical analysis. Analysis of the data was performed using the Minitab 14.1 statistical software package (Minitab Ltd. Coventry, UK) using a custom-written macro (Stat2005.mtw). Independent variables were compared using the Mann-Whitney *U*-test while paired variables were compared using the two-sample *t*-test. The transcript levels within the breast cancer specimens were compared to those of the ANCT and correlated with clinicopathological data collected accrued during follow-up. Median duration of follow-up was over 10 years.

p-Values less than 0.05 were considered significant whereas *p*-values between 0.05 and 0.10 were considered marginally significant.

Correlations between the expressions of the molecules were studied using Pearson product moment correlation test.

For purposes of the Kaplan-Meier survival analysis, the samples were divided arbitrarily into high and low transcription groups, with the value of the moderate prognostic group as defined by NPI serving as the dividing line. Survival analyses were performed using the SPSS version 12.0.1 (SPSS Inc., Chicago, IL, USA).

Results

Significantly higher mRNA transcript levels of *GNB1* were found in the breast cancer specimens compared to normal glandular tissue in paired samples (p=0.0029). The expression of *GNB1* mRNA was demonstrated to increase with increasing TNM stage (from 0.01 to 15.9) and this reached statistical significance when comparing TNM1 *vs.* TNM2/3/4 (p=0.036). Furthermore, the expression levels of *GNB1* increased with increasing tumour grade and this reached statistical significance when comparing grade 2 *vs.* grade 3 (p=0.006). GNB1 expression was found to be higher in ductal tumours compared with non-ductal tumours (p=0.0081). Patients who developed recurrent disease or died from breast cancer had higher expression levels of *GNB1* than those who were disease-free after a median follow-up period of 10 years (p=0.066; in ductal cell tumours: p=0.017). Those who died from breast cancer had significantly higher *GNB1* levels than those who remained disease-free (mortality *vs.* disease free survival: 4.9 *vs.* 0.01, p=0.027), which was even more significant within patients diagnosed with ductal cell tumours (33.9 *vs.* 0.01, p=0.0009) (Table III).

The Kaplan-Meier survival plot analysis suggests that lower levels of *GNB1* mRNA expression are associated with better overall (p=0.071) and disease-free survival (p=0.012) (Figures 1 and 2).

GNB1 was positively correlated with mTOR (r=0.525, p<0.000001) and Rictor (r=0.388, p=0.0000606) (Table IV).

Discussion

GNBs (also termed as G-proteins) have been also studied extensively with regards to their role in the intracellular signaling pathways of many significant receptors, which are termed G-protein coupled receptors (GPCRs). There are three known types of G-proteins, termed guanine nucleotide binding polypeptides alpha, beta and gamma, (Gα, Gβ, and Gγ) which exist as heterotrimers in association with GPCRs (4).

In their inactive state, they are bound to moieties of guanine diphosphate (GDP), localized in the cell membrane in association with the intracellular aspect of the GPCR.

Table II. *Primers used in real-time polymerase chain reaction (RT-PCR) analysis.*

GNB1 F1	ACGAGTCTGACATCAATGC
GNB1 ZR1	ACTGAACCTGACCGTACAAGCACGAA GGTCAAACAG
mTOR F1	CTGCAGAAGAAGGTCACT
mTOR ZR1	ACTGAACCTGACCGTACAAAGGAGAT GGAACGGAAG
Raptor F1	TGAACACCGGACCATGAC
Raptor ZR1	ACTGAACCTGACCGTACACAATGAGG TTTCCCTGAAG
Rictor F1	AACTTGCAAAACAGTGTGAA
Rictor ZR1	ACTGAACCTGACCGTACAATATCACAG CCTTGTTTGGT
Beta-actin Forward	ATGATATCGCCGCGCTCGTC
Beta-actin Reverse	CGCTCGGTGAGGATCTTCA

Table III. *Median GNB1 mRNA expression levels (median actual copy number normalized against CK19) in a cohort of 136 patients with breast cancer; Comparison between subgroups with different tumour grade, Nottingham prognostic index (NPI), and TNM stage.*

Patient and tumour characteristics	Median(s)	95% CI	*p*-Value
Tumour grade			
1 *vs.* 2	0.04 *vs.* 0.00	–0.00 to 0.16	0.3387
1 *vs.* 3	0.04 *vs.* 0.18	–0.57 to 0.03	0.2174
2 *vs.* 3	0.00 *vs.* 0.18	–0.30 to –0.01	0.0064
NPI			
1 *vs.* 2	0.035 *vs.* 0.074	–0.135 to 0.025	0.8061
1 *vs.* 3	0.035 *vs.* 0.2	–1.1 to 0.0	0.4167
2 *vs.* 3	0.074 *vs.* 0.2	–3.6 to 0.0	0.4269
TNM			
1 *vs.* 2	0.01 *vs.* 0.22	–0.28 to 0.00	0.1683
1 *vs.* 3	0.01 *vs.* 15.9	–33.7 to 0.1	0.0593
1 *vs.* 4	0.01 *vs.* 1.23	–36.31 to 0.50	0.0822
1 *vs.* 2/3/4	0.01 *vs.* 0.28	–0.34 to –0.00	0.0361
2 *vs.* 3	0.22 *vs.* 15.9	–33.9 to 0.3	0.1900
2 *vs.* 4	0.22 *vs.* 1.23	–36.24 to 4.72	0.2271
3 *vs.* 4	15.9 *vs.* 1.23	–36.4 to 616.9	1.0000
Survival			
DF *vs.* LR	0.028 *vs.* 0.151	–2.800 to 0.199	0.4999
DF *vs.* DR	0.028 *vs.* 0.048	–1.070 to 0.331	0.9000
DF *vs.* D	0.028 *vs.* 4.9	–31.8 to 0.0	0.0271
DF *vs.* LR/DR/D	0.028 *vs.* 0.50	–2.99 to –0.01	0.0656

SD: Standard deviation, DF: disease-free survival, LR: local disease recurrence, DR: distant disease recurrence, D: death from breast cancer, CI: confidence interval.

When the GPCR is activated, usually due to association of an agonist molecule with the receptor site, it fosters dissociation of the GDP molecule from the G-protein complex. Gα binds with a guanine triphosphate (GTP) , and triggers a change in the conformation of the molecule. The GTP-bound Gα also dissociates from Gβ, and Gγ (Gβγ). In this state, Gα and Gβγ activate downstream intracellular pathways, and remain active until the GTP is hydrolyzed by the intrinsic GTPase activity of Gα (4, 8).

G-proteins are a ubiquitous signaling mechanism found in many receptor-dependent pathways. A number of isoforms have been identified for each G-protein. At least five isoforms have been identified for Gβ, and 12 in the case of Gγ (4). There is evidence of specificity of certain Gβ-Gγ dimer combinations to certain GPCRs (9). This is believed to enable cells to express multiple instances of the G-protein complex in association with distinct GPCRs whilst maintaining good receptor-effector coupling and efficient signal transduction (10, 11).

The immunosuppressive and anti-proliferative actions of rapamycin were well-known. The mammalian target of rapamycin (mTOR) was discovered in humans in the 1990s. Since then, it has been found to exist in one of two multi-protein complexes (12).

The first mTOR complex (mTORC1) was found to have wide-ranging effects on metabolism, immunity, autophagy and cell proliferation (12). Furthermore, it is known to have a role in carcinogenesis (13). Antagonists mTORC1 have been studied as potential therapeutic agents, which have been clinically-proven in the case of renal cell carcinoma (14). However, such success as a therapeutic agent has not been replicated for other types of cancers. Certain studies have endeavored to demonstrate markers of rapamycin sensitivity in cancers other than renal cell carcinoma (15, 16). Other components of mTORC1 are mammalian lethal with sec-13 protein-8 (mLST8, also known as GβL), regulatory-associated protein of mammalian target of rapamycin (Raptor), DEP domain containing mTOR-interacting protein (DEPTOR), the Tel Two Interacting protein (TTI1)/Telomerase maintaining-2 (TEL2) complex, and proline-rich AKT substrate 40 kDa (PRAS40).

The second complex (mTORC2) is less well-documented, and is also referred to as the rapamycin-insensitive mTOR complex. Like mTORC1, it is composed of mTOR, mLST8, DEPTOR, and TTI1/TEL2 complex. In addition, it also contains rapamycin-insensitive companion of mTOR (Rictor) (17), mammalian stress-activated MAP kinase-interacting protein-1 (mSIN1), protein observed with Rictor-1 and -2 (Protor1/2), and proline rich protein-8 (PRR8) (13). mTORC2 has been found to have a role in regulation of cell survival and the cytoskeleton through the stimulation of various kinases, including AKT/PKB (protein kinase B). Through AKT/PKB, it is also thought to inhibit mTORC1 (18).

It has been suggested that selective targeting of components and functions of the mTOR pathway rather than employing dual inhibitors would potentially be a better therapeutic strategy in the treatment of neoplasia (19). Rapamycin analogues inhibit the activity of mTORC1, which include not

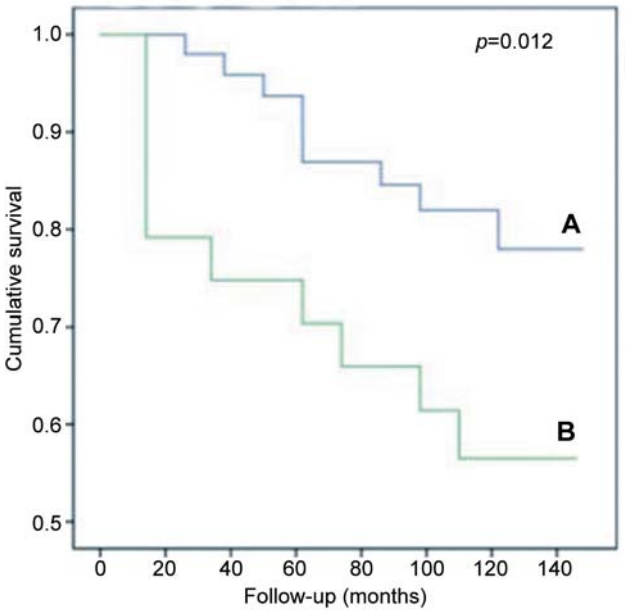

Figure 1. *Disease-free survival curve according to mRNA expression of GNB1. The population has divided into higher and lower transcription groups with the moderate prognosis group by the Nottingham prognosis index (NPI) serving as the dividing line. Curve A: Lower transcription group; Curve B: higher transcription group.*

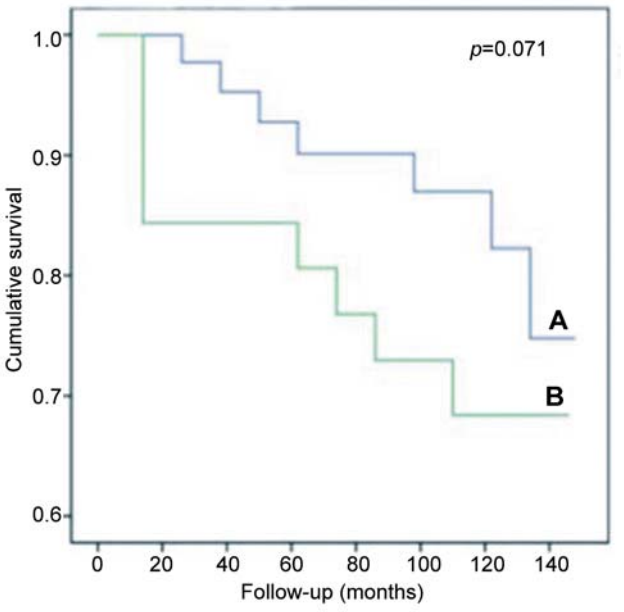

Figure 2. *Overall survival curve according to mRNA expression of GNB1. The population has divided into higher and lower transcription groups with the moderate prognosis group by the Nottingham prognosis index (NPI) serving as the dividing line. Curve A: Lower transcription group; Curve B: higher transcription group.*

Table IV. *Pearson product-moment correlations*

		Rictor	Raptor	GNB1
mTOR	Correlation coefficient	0.221	–0.0249	0.525
	p-Value	0.0189	0.812	0.0000000202
Rictor	Correlation coefficient		–0.0515	0.388
	p-Value		0.620	0.0000606
Raptor	Correlation coefficient			–0.0341
	p-Value			0.755

mTOR: Mammalian target of rapamycin, Rictor: rapamycin-insensitive companion of mammalian target of rapamycin, Raptor: regulatory-associated protein of mammalian target of rapamycin, GNB1: guanine nucleotide binding protein β-1.

only proliferative effects, but also its metabolic and immunity-related functions. Currently, mTOR kinase inhibitors are being studied as potentially less toxic alternatives, selectively targeting kinase effectors of both mTORC1 and mTORC2. Theoretically, a specific inhibitor of mTORC2 would have a more attractive toxicity profile, as it would affect only the forkhead box O (FOXO) signaling pathway out of the various AKT/PKB downstream effectors (20).

The correlation of GNB1 with mTOR and Rictor is suggestive of the presence of a GPCR complex affecting the mTOR pathway in the case of human breast cancer, potentially specific to the mTORC2 entity. This may suggest the possibility of selective targeting of the rapamycin-insensitive mTOR pathway in a manner distinct from the mTOR kinase inhibitors currently under trial. In view of the potential gains in improved toxicity profiles, this may merit further investigation.

Furthermore, the correlation of GNB1 with clinico-pathological parameters may recommend it as a prognostic indicator of outcome in human breast cancer, especially within the subset of patients diagnosed with ductal cell carcinoma.

Conclusion

To our knowledge, we are the first group to demonstrate an association between *GNB1* mRNA expression and the clinicopathological parameters of human breast cancer. Our study has shown a highly significant correlation between *GNB1*, *Rictor* and *mTOR* mRNA expressions using a robust real-time quantitative PCR methodology. Our findings in this cohort of breast cancer patients are lent greater strength by the long-term follow-up of at least ten years.

Whilst limitations related to sample size would have to be acknowledged regarding our study, it has yielded statistically significant results which could guide future avenues of research. Further research is required into the complementary roles of GNB1 and components of the mTOR pathway in the pathogenesis of breast cancer, including immunohistochemical studies confirming protein expression and distribution, and *in vitro* experiments further exploring the mechanisms involving these and other relevant molecules. Such research could guide more effective targeting of the mTOR pathway in human breast cancer, and may potentially identify further targets for therapeutic interventions.

Competing Interests

The Author(s) have no competing interests to declare.

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