The ubiquitin-binding protein p62 is expressed in breast cancers showing features of aggressive disease

Phil Rolland, Zahra Madjd, Lindy Durrant, Ian O Ellis¹, Rob Layfield² and Ian Spendlove

Academic and Clinical Department of Oncology, Institute of Infections and Immunity, University of Nottingham, Nottingham City Hospital NHS Trust, Hucknall Road, Nottingham NG5 1PB, UK

¹Department of Histopathology, University Hospitals Nottingham, Nottingham NG5 1PB, UK

²School of Biomedical Sciences, University of Nottingham, Nottingham NG7 2UH, UK

(Requests for offprints should be addressed to I Spendlove; Email: ian.spendlove@nottingham.ac.uk)

Abstract

p62 is a multi-functional protein, which induces nuclear factor- κ B (NF κ B) activation through multiple upstream signalling pathways, including those triggered by the epidermal growth factor (EGF) family of receptors. We hypothesised that p62 overexpression increased EGF family receptor expression and worse outcome in breast cancer would be associated. We stained a tissue microarray representing 523 breast cancers using a commercial guinea pig anti-human p62 sera and standard immunohistochemical methods to address this. Out of $n=106$ tumours, 20.3% stained positively. p62 expression correlated with grade ($P=0.010$) and distant metastasis ($P=0.04$) and EGF receptor (EGFR) ($P=0.012$), HER2 ($P=0.016$), HER3 ($P=0.007$) and HER4 (0.002) expressions. Though expression correlated with reduced 5-year survival (58.5 vs 73.6%), there was no association with overall disease specific survival. p62 expression may represent a marker of activation of the NF κ B pathway.

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Introduction

p62 (also known as Sequestosome 1) has been characterised as an immediate early response gene, indicating a role in cellular proliferation and differentiation. It encodes a multi-domain protein which has a functional role in the signalling cascades from a range of cell-surface receptors acting as a scaffolding protein in their linkage to nuclear factor- κ B (NF κ B) activation; via I κ B ubiquitination (Lee *et al.* 1998, Karin 1999, Sanz *et al.* 1999, Geetha & Wooten 2002, Seibenhener *et al.* 2004, Wooten *et al.* 2005). It has an important function in promoting survival signals, including proliferation, differentiation and induction of anti-apoptotic genes (Ciani *et al.* 2003, Seibenhener *et al.* 2004). Alterations in the nature of p62 have been shown to occur in benign disease states, such as Paget's disease of bone and conditions characterised by intracellular inclusions including Parkinson's, Alzheimer's and Wilson's diseases (Kuusisto *et al.* 2002, 2003, Stumtner *et al.* 2002, Muller *et al.* 2004, Cavey *et al.* 2005). Abnormal

expression has been documented in various neoplasms including gastrointestinal, prostate and breast cancers (Stumtner *et al.* 1999, Thompson *et al.* 2003, Qian *et al.* 2005, Kitamura *et al.* 2006).

Breast cancer progression is strongly associated with the expression of the EGF family of receptors (EGFR, HER2, HER3 and HER4), which have been targeted therapeutically (Atalay *et al.* 2003). These receptors display a range of homo- and heterodimerisation in the presence of multiple ligands and one of the most potent oncogenical receptor is the heterodimerisation of HER2 and HER3 (Gullick 1996, Srinivasan *et al.* 2001). Signalling from multiple members of the EGFR family has been shown to result in the activation of NF κ B and this has been implicated in the constitutive activation of NF κ B seen in a range of cancers including breast (Bhat-Nakshatri *et al.* 2002, Le Page *et al.* 2005, Zhang *et al.* 2006). p62 expression may be implicated in the regulation of this pathway.

We hypothesised that p62 overexpression would be associated with breast tumours exhibiting the clinico-pathological features of aggressive disease, overexpression of the EGF receptor family members and poorer prognoses. We stained a tissue microarray (TMA) representing 523 breast cancer cases (all having pre-existing EGF receptor family expressions data available from previously published work; [Abd El-Rehim *et al.* 2004b](#)) immunohistochemically in order to investigate this.

Materials and methods

This research was approved by the Nottingham Local Research Ethics Committee and its design and reporting seeks to adhere to the REMARK recommendations ([McShane *et al.* 2005](#)).

Patients

A TMA representing 523 cases of primary operable invasive breast carcinoma, from patients aged 70 years or less, diagnosed between 1987 and 1998 from the Nottingham Tenovus Primary Breast Carcinoma Series was used. These well-characterised breast cancer cases have been treated by a conventional algorithm and this series has been successfully used to study the proteomics of breast cancer, being powerful enough to detect small variations in clinical outcome ([Madjd *et al.* 2003, 2004](#), [Abd El-Rehim *et al.* 2004a, 2005](#), [Putti *et al.* 2005](#)).

Patient characteristics including age and menopausal status along with information concerning recurrence and survival were maintained on a prospective database. Patients were followed up at 3-month intervals initially, then 6-months and annually for a median period of 76 months.

Tumours

The characteristics of these cancers including histological grade (grade 1=low; grade 2+3=high), lymph node stage, tumour size and Nottingham prognostic index (NPI) were prospectively recorded in the database. The tumours were classified into the following four prognostic types for analysis.

1. Excellent prognosis type (>80%, 10-year survival), including tubulo-lobular, tubular mucinous and invasive cribriform carcinoma.
2. Good types (60–80%, 10-year survival), including tubular mixed, mixed ductal with special type and alveolar lobular carcinoma.

3. Moderate prognosis type (50–60%, 10-year survival), including classical lobular, medullary, atypical medullary and lobular mixed carcinoma.
4. Poor prognosis types ($\leq 50\%$ 10-year survival), including ductal NST, solid lobular, mixed ductal and lobular carcinoma.

Treatment

Patient management was guided by NPI and oestrogen receptor (ER) status. The assays to determine the ER status of each tumour section were conducted by a dextran-coated charcoal technique at the Tenovus Institute, Cardiff. A cut-off of 10 fmol/mg protein indicated positivity. Those women with an NPI score ≤ 3.4 received no adjuvant therapy and those with an NPI score > 3.4 received tamoxifen if ER-positive ($\pm$ Zoledex if pre-menopausal) or CMF (cyclophosphamide, methotrexate, fluorouracil) if ER-negative and fit enough to receive it.

TMA preparation

The excised tumours were sliced, fixed immediately, placed in neutral buffered formalin and processed through to embedding in paraffin wax. Tumour samples were then arrayed as described previously ([Kononen *et al.* 1998](#)). Tumour biopsies with a diameter of 0.6 mm were taken from representative regions of each donor block using manual tissue arrayer (Beecher Instruments, WI, USA) and then precisely arrayed into a new recipient paraffin block. Contrary to expectation, tissue heterogeneity does not significantly influence the predictive power of the TMA results. Single cores were analysed in this study ([Bubendorf *et al.* 2001](#), [Torhorst *et al.* 2001](#)).

Immunohistochemistry

• Primary antibody

p62: A commercial polyclonal guinea pig anti-human p62 (C-terminal specific) antibody was used as the primary antibody (Research Diagnostics, INC New Jersey, USA). Its optimal dilution following titration on whole breast sections was found to be 1/1000.

• Peptide-blocking assay

To demonstrate the specificity of the antisera on immunohistochemical staining, BSA-conjugated peptide NYDIGAALDTIQYSKHPPPL (M1044-A8 Alta Bioscience) – the peptide to which it had been

raised – was pre-incubated with the anti-p62 antisera for 1 h at 4 °C prior to its addition to the sections. Titrations of peptide from 0.25 to 20 µg/ml were carried out at a constant, optimal concentration of antisera (1:1000 dilution).

- Staining protocol

Deparaffinised 4 µm thick sections of TMA and similar thickness whole sections of breast tumours (× 10) were immersed in 0.3% hydrogen peroxide for 15 min to block endogenous peroxidase activity. Microwave pretreatment in EDTA buffer (pH 8.5) was performed at 10 min high power and 10 min low power to retrieve antigenicity. The primary antibody was incubated on the slides for 60 min at a dilution of 1:1000. Primary antibody was omitted from the negative control, which was left incubating in normal swine serum (NSS). Sections of breast tumour found to express p62 acted as positive and negative controls. The sections were then incubated with a HRP-conjugated rabbit anti-guinea pig secondary antibody (Dakocytomation, Cambridge-shire, UK) for 45 min. 3,3'-Diaminobenzidine (Dako) was applied for visualisation. The sections were lightly counterstained with haematoxylin (Dako), dehydrated in alcohol, cleared with xylene (Genta Medica, York, UK) and mounted with distyrene, plasticiser and xylene (DPX; BDH, Poole, England).

- Evaluation of staining

On reviewing the distribution, variety and frequency of staining within cores we adopted a binary system of quantifying the expression of p62. Those cores with > 5% of cells staining with intensity ≥ 1 were deemed positive and others negative. The complete scoring was performed by one author (P R), a high level of concordance with a second author (Z M) having being achieved over an initial 100 cases.

- EGFR, HER2, HER3 and HER4

Immunohistochemical staining of these markers was performed as described previously using the standard streptavidin biotin complex (Abd El-Rehim *et al.* 2004b).

Statistical analysis

SPSS vs 12.0 was used for the statistical analyses (Chicago, IL, USA). Univariate associations of p62 staining with the clinicopathological parameters of breast cancer, the expression of the EGF family of receptors and 5-year survival were analysed by Pearson's χ^2 -test.

The effect of p62 expression upon overall disease-specific survival was analysed using Kaplan–Meier curves and the log rank test. To estimate the size and independence of effects, Cox's regression was employed; returning hazards ratios and 95% confidence intervals.

P values <0.05 indicated statistically significant differences.

Results

Clinicopathological characteristics

At the time of diagnosis, the patient age in this series of cases ranged from 27 to 70 years (mean = 53.5 years) and 61.2% (*n* = 301) were post-menopausal. Out of *n* = 168, 32.7% had tumours less than 1.5 cm in size, 63.8% (*n* = 329) were stage 1, 17.7% (*n* = 92) were low grade and 15.3% (*n* = 80) had distant metastases. Out of *n* = 312, 60.9% had a poor tumour type and most tumours, 54.1% (*n* = 272), were in the moderate NPI group ($> 3.4 \leq 5.4$). Out of *n* = 334, 67.1% were ER-positive. Recurrence of disease occurred in 24.7% (*n* = 129) of cases and the 5-year survival was 70.6% (*n* = 369) (Table 1).

Table 1 The major clinicopathological characteristics of the whole series and association of positive p62 expression with clinicopathological parameters in a univariate analysis using the Pearson's χ^2 -test

	No. of cases (%)		χ^2 (<i>P</i> =) (±)
	p62-positive (<i>n</i> = 106)	p62-negative (<i>n</i> = 417)	
Tumour size	<i>n</i> = 106	<i>n</i> = 408	
< 1.5 cm	31 (29.2)	137 (33.6)	
≥ 1.5 cm	75 (70.8)	271 (66.4)	0.718 (0.397)
Stage	<i>n</i> = 105	<i>n</i> = 411	
LN-negative	69 (65.7)	260 (63.3)	
LN-positive	36 (34.4)	151 (36.7)	0.218 (0.641)
Distant mets	<i>n</i> = 106	<i>n</i> = 417	
Absent	83 (78.3)	360 (86.3)	
Present	23 (21.7)	57 (13.7)	4.205 (0.040) (+)
Grade	<i>n</i> = 106	<i>n</i> = 413	
Low	10 (9.4)	82 (19.9)	
High	96 (90.6)	331 (80.1)	6.281 (0.012) (+)
Tumour type	<i>n</i> = 104	<i>n</i> = 408	
1	4 (3.8)	28 (6.9)	
2	15 (14.4)	90 (22.1)	
3	11 (10.6)	52 (12.7)	
4	74 (71.2)	238 (58.3)	6.115 (0.106)
Five year DSS	<i>n</i> = 106	<i>n</i> = 417	
Survived to	62 (58.5)	307 (73.6)	
5 years			
Died within	44 (41.5)	110 (26.4)	9.313 (0.002) (+)
5 years			

LN, lymph node status; Mets, metastases; DSS, disease-specific survival; NPI, Nottingham prognostic index; ±, positive/inverse associations.

p62 staining

Tissue staining with the p62 antisera decreased as peptide concentration increased, supporting the specificity of this antibody in staining for the protein it was raised against (Fig. 1b and c). The staining of the TMA cores reflected that observed in whole sections. The staining observed was granular, cytoplasmic and homogenous; nuclear staining was rarely observed and cell membrane staining did not occur (Fig. 2a). Out of $n=106$, 20.3% of cases stained positive for p62 and 79.7% ($n=417$) were p62-negative (Fig. 2b).

Univariate associations of p62 expression with clinicopathological factors, the EGF family of receptors and 5 year survival (χ^2)

When tumours staining positive for p62 were compared with known histological prognostic factors (stage, tumour size, grade, etc.), patient characteristics (age, menopausal status, etc.) and outcome (recurrence and 5-year survival); p62-positive staining was seen to be associated with higher grade (9.184; $P=0.010$) and the presence of distant metastases (4.205; $P=0.040$). p62-positive cases were less likely to survive to 5 years (58.5 vs 73.6%; 9.313; $P=0.002$; Table 1). p62-positive

tumours were more likely to express all of the EGF family of receptors than their negative counterparts (EGFR: $P=0.012$; HER2: $P=0.016$; HER3: $P=0.007$; HER4: $P=0.002$; Table 2).

Association of p62 expression with overall disease-specific survival

p62 expression did not predict the overall disease-specific survival (log rank 0.93; $P=0.34$) in univariate or multivariate analyses (Fig. 3; Table 3).

Discussion

In this breast cancer array of 523 tumours, a clear staining pattern of p62 expression was seen in 20% of cases. As there is a paucity of data concerning the immunohistochemical expression of p62 in normal and cancerous breast tissue, it is not possible to draw conclusions about whether this represents overexpression in some cancers or underexpression in others. Despite this, as earlier work by western blot on the 13 lysates out of 9 normal and 4 cancerous breast tissues concluded that the p62 protein was relatively overexpressed in tumours (Thompson *et al.* 2003), one might speculate that those tumours

Figure 1 (a) Whole section of breast cancer known to stain positive with 1/1000 dilution of p62 antisera co-incubated with no blocking peptide; positive control. (b) Whole section of breast cancer known to stain positive with 1/1000 dilution of p62 antisera co-incubated with blocking peptide at 1/1000 dilution showing reduced staining. (c) Whole section of breast cancer known to stain positive with 1/1000 dilution of p62 antisera co-incubated with blocking peptide at 1/50 dilution showing almost absent staining. (d) Whole section of breast cancer known to stain positive with 1/1000 dilution of p62 antisera with normal swine serum substituted for the p62 antisera; negative control.

Figure 2 (a) An example of a high-grade breast cancer core illustrating the cytoplasmic staining observed with p62 antisera at 1/1000 dilution at $\times 200$ and $\times 400$ magnification. Stroma, cell membrane and nucleus consistently exhibited no staining. (b) An example of a breast cancer core not reacting to the p62 antisera at 1/1000 and therefore classified as p62 negative at $\times 200$ magnification.

deviating most from normality would be the ones to express most p62 protein as evidenced by a positive reaction to the immunohistochemical staining. The univariate associations with advancing grade and the presence of metastases we observed in positively staining

Table 2 Association of positive p62 expression with the epidermal growth factor (EGF) family of receptors using the Pearson χ^2 test

Biomarker	p62-positive (n=106)	p62-negative (n=417)	χ^2 (P=)
EGFR- positive	n=99; 28 (28.3)	n=354; 60 (16.9)	6.35 (0.012)
HER2-positive	n=106; 50 (47.2)	n=401; 138 (34.4)	5.85 (0.016)
HER3-positive	n=93; 86 (92.5)	n=344; 278 (80.8)	7.152 (0.007)
HER4-positive	n=96; 84 (87.5)	n=344; 247 (71.8)	9.924 (0.002)

EGFR, epidermal growth factor receptor (clone 113, NCL-L-EGFR, Novocastra, Newcastle, UK); HER2, (Dako, Denmark); HER3, (Clone RTJ1, NCL-c-erbB-3, Novocastra, Newcastle, UK); HER4, (clone HFR1, RB-9045-R7, Neomarkers, CA, USA).

Figure 3 A Kaplan–Meier plot illustrating how disease-specific survival varies with p62 expression; the log rank test confirming no statistically significant differences.

tumours would support this thinking. Though we failed to find a concordant association with the overall survival, p62 expression did correlate with short-term (5-year) survival. This might be explained by its association with those tumours having distant metastases at presentation; a small but important group with a mean survival 45 when compared with 168 months for the series overall. p62 expression was shown not to be an independent prognostic factor and indeed its association with higher grade and indirectly stage (of which distant metastases is a subset) made it unlikely that it could be. Our observation that 20% of breast cancers positively express p62 was lower than the proportion seen in smaller series of prostate (91%) and digestive system cancers (100%), and possibly reflects site-specific variations in tumour biology, though methodological differences remain an alternative explanation. The distribution of staining observed and the associations we found with grade and

Table 3 Cox’s regressional multivariate analysis of p62 expression versus known independent prognostic factors in breast cancer

	Hazard ratio (95% confidence intervals)	Significance (P=)
Stage		
LN-negative		
LN-positive	2.35 (1.44–3.83)	0.01
Tumour size > 1.5 cm	4.93 (1.97–12.34)	0.01
Grade		
Low		
High	4.58 (1.11–18.97)	0.036
Positive p62 expression	1.30 (0.74–2.29)	0.366

LN, lymph node status.

metastatic disease were, however, the same for the three sites (Su *et al.* 2005, Kitamura *et al.* 2006).

A variety of signals from upstream receptors and pathways may be transduced to NF κ B activation by p62 connecting to atypical protein kinase C and activating inhibitor of NF κ B kinase B subunit (IKK β) by phosphorylation in its scaffolding role (Karin 1999, Sanz *et al.* 1999, Wooten *et al.* 2005). The freed NF κ B is subsequently translocated into the nucleus where it can promote cellular de-differentiation, proliferation, invasion, metastasis and reduced apoptosis (Barkett & Gilmore 1999, Chiarugi *et al.* 1999, Dong *et al.* 2002, Andela 2004). Recent studies demonstrating that the EGF family is able to activate the NF κ B-signalling pathway offer an explanation for the constitutive NF κ B-pathway activation seen in a range of tumours including breast cancer (Bhat-Nakshatri *et al.* 2002, Le Page *et al.* 2005, Zhang *et al.* 2006) and as there is a strong positive correlation between the expression of p62, HER3 and HER4 in this work, p62 expression may provide further mechanistic insight. Although HER3 is functionally unable to transduce a signal to the cytoplasmic side of the plasma membrane, as its kinase domain is inactive, it is one of the most potent oncogenic members of the EGF family. This is due to its ability to form heterodimers with HER2. This heterodimerisation process is common to all members of the EGF family, allowing a wide range of signalling possibilities and signal strengths depending on precise dimerisation and the ligands used (Wallasch *et al.* 1995, Gullick 1996, Roskoski 2004).

An associated function of p62 is its binding to poly-ubiquitinated proteins and its subsequent shuttling of them to the proteasome for degradation. This mechanism may be important in tumour biology and also in Paget's disease (Ciani *et al.* 2003, Goodsell 2003, Seibenhener *et al.* 2004). p62 is an important scaffold in the RANK-signalling pathway in osteoclasts and in Paget's disease mutations in the ubiquitin-associated domain that results in loss of function are believed to promote osteoclastogenesis via activation of NF κ B (Hocking *et al.* 2004, Cavey *et al.* 2005). Interestingly, the majority of primary bone cancers occurring in adulthood do so in patients with Paget's disease (Huvos 1986).

Autoantibodies to p62 have been observed more frequently in the sera of patients with gastrointestinal cancers that have metastasised (Su *et al.* 2005). This suggests that p62 may mutate into an autoantigen as cancer advances and preliminary work in prostate cancer supports this theory (Shi *et al.* 2005, Su *et al.* 2005). This is yet to be investigated in breast cancer but the close correlation of p62 with EGFR family, its

relationship with NF κ B and this potential to elicit an immune response make p62 an intriguing potential target for intervention.

In summary, we have demonstrated for the first time that p62 expression in breast cancer is associated with tumour progression, identifying a distinct set of tumours. The expression of p62 correlates with the expression of members of the EGF family, particularly HER3 and HER4 and may represent a marker of activation of the NF κ B pathway.

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