

# Upregulation of MICA on high-grade invasive operable breast carcinoma

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The MHC class I chain-related gene A (MICA) is frequently expressed on the surface of intestinal epithelium and by many epithelial tumours. MICA is a stress-induced antigen which was identified as an activator of natural killer cells via interaction with the NKG2D receptor. We have raised a rabbit polyclonal antibody against a synthetic peptide that recognises denatured MICA on both Western blots and in formalin-fixed paraffin-embedded sections. In the present study this antibody was used to undertake a definitive study of 530 breast cancer cases with mean follow up of 7 years to determine the prognostic significance of MICA expression. To detect any association between MICA expression and NK infiltration, whole sections of 50 tumours were also analysed for CD56 staining. Univariate analysis showed significant relationships between MICA expression and histological grade ( $P = 0.006$ ), lymph node stage ( $P = 0.013$ ), Nottingham Prognostic Index (NPI,  $P = 0.002$ ), the presence of vascular invasion ( $P = 0.045$ ) and tumour type ( $P = 0.023$ ). Upregulation of MICA was more often found in histological grade 3, poor prognosis (NPI >5.4) tumours. Association of high MICA expression with NK cell infiltration was not demonstrated, as very few NK cells were present in whole breast sections. Our results suggest that induced expression of MICA may be an indicator of poor prognosis in breast carcinoma and is indicative of a tumour environment that has undergone stresses such as apoptosis, necrosis, or hypoxia.

**Keywords:** human, breast carcinoma, MICA, immunohistochemistry, prognosis

## Introduction

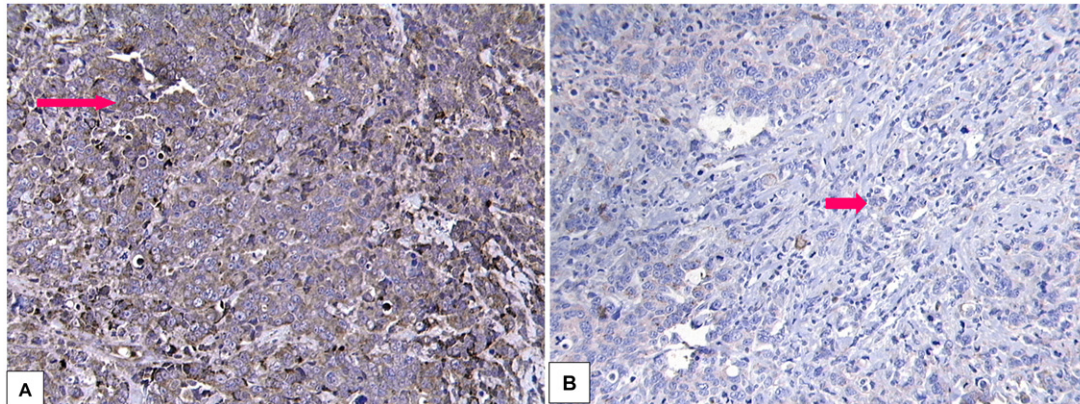
Breast cancer is the most frequent cancer in women (23% of all cancers) and represents the second leading cause of cancer overall when both sexes are considered together (1). Breast cancer is well recognized to be both biologically and clinically heterogeneous, therefore, understanding the biology of breast cancer and assessment of certain prognostic factors are important in predicting disease outcome and treatment strategies.

We have a longstanding interest in the tumour interface and whether the immune response *in situ* plays any role in tumour progression. An overwhelming amount of data from animal models and from human cancer patients indicates that a

functional cancer immunosurveillance process exists that acts as an extrinsic tumour suppressor. However, it has also become clear that the immune system can facilitate tumour progression by altering the immunogenic phenotype of tumours as they develop. This process has been termed "cancer immunoediting". Initiation of the anti-tumour immune response occurs when the immune system becomes alerted to the presence of the growing tumour. An effective immune response may result in tumour elimination. Alternatively, due to the inherent genetic instability of tumours, it may lead to the selection of tumour variants that are resistant to immune attack (2). Thus the loss or over-expression of molecules that confer resistance to immune attack may be independent markers of tumour progression (3, 4). The immune response may select for resistant tumour variants that over-express complement inhibitory molecules which inactivate complement and downregulate "inflammation", for loss of HLA, the receptor for T cell recognition (5), or tumours may shed major histocompatibility complex class I chain-related (MIC) proteins, which have been shown to downregulate NK and T cell immunity (6, 7).

The major histocompatibility complex class I chain-related (MIC) proteins represent a novel family of highly glycosylated, membrane-anchored MHC class I-like molecules. Although they share similar structure to classical class I heavy chains, MICA and MICB do not associate with  $\beta 2$ -microglobulin or transporter associated with antigen processing (TAP) expression (8). In normal tissue, MICA has been shown to be mostly restricted to the gastrointestinal tract but was shown to be stress inducible in a range of cell lines. However expression of MICA is upregulated by a range of primary tumours including lung, kidney, prostate, breast and colon (9, 10).

MIC proteins function as ligands for the stimulatory C-type lectin-like NKG2D receptor, first identified on NK cells and subsequently shown to be expressed on  $\gamma \delta$  T cells and CD8+  $\alpha \beta$  T cells (11). This NKG2D signalling has been shown to have an important role in NK and T cell-mediated innate responses to tumours. *In vitro* studies have shown that the interaction of NKG2D with MICA and MICB molecules on epithelial tumour cells triggers the cytolytic responses of NK and  $\gamma \delta$  T cells independent of antigen presentation (4). This NKG2D signalling has been shown to be strong enough to overcome inhibitory signalling by MHC-specific receptors in some cases where the target cells express normal levels of MHC class I (4, 5).

**Figure 1**

**Peptide blocking of MICA.** Immunohistochemical staining of serial breast sections stained with rabbit anti-MICA polyclonal antibody without (A) and with (B) preincubation with the Z167-BSA MICA peptide (20x magnification).

There are only a few studies characterising MICA expression and histopathological characteristics. These have been based on small sample sizes and studies on cell lines (10). Vetter *et al.* reported the expression of MIC in 31 of 40 primary cutaneous melanomas and in 13 of 20 metastatic lesions (12).

Our previous study on 449 colorectal carcinomas showed a significant correlation between higher levels of MICA expression and improved disease-specific survival, with independent prognostic significance in multivariate analysis (13). In order to evaluate the prognostic value of MICA expression in patients with breast carcinoma, we have undertaken an analysis of 530 paraffin-embedded invasive breast cancers using polyclonal antisera to MICA. This may provide insight into the importance of MICA in breast carcinoma and may demonstrate a role for MICA in immune editing (2).

## Results

### MICA antiserum

#### Western blot

Western blotting with commercially available anti-MICA antibody gave a single band of approximately 65 kDa in MICA transfected CHO and HEK293 cells, but no band in untransfected cells. An identical profile was obtained using our rabbit antiserum (13).

#### Peptide blocking of antiserum on tissue sections

A similar analysis was carried out in a series of breast tumour sections. These were prepared and incubated with rabbit anti-MICA polyclonal antibody with or without Z167-BSA conjugate. Strong staining of breast tumours was observed with anti-MICA polyclonal antibody that was completely inhibited when the antibody was preincubated in the presence of peptide conjugate (Figure 1).

#### MICA expression in breast carcinomas

Previous studies have indicated that, contrary to expectations, tissue heterogeneity does not negatively influence the predictive power of the TMA results (14). To assess reproducibility of the scoring technique, a pilot study of 20 cases was performed on full-size tissue sections. A comparison of tissue arrays and

original full-size sections showed concordance in intensity and the area of positivity of MICA staining.

The present study included 530 primary operable invasive breast carcinomas from patients aged from 27 to 70. Patient characteristics are given in Table 1. Analysis of MICA expression in the arrayed 530 breast tumours revealed that 97% of the breast cancers showed positive staining for MICA, with 38% of samples demonstrating strong intensity of MICA expression and 37% moderate intensity. The immunohistochemical pattern of MICA expression in the invasive breast carcinoma cells was somewhat heterogeneous; it combined both granular cytoplasmic staining and cell membrane staining.

No staining of stroma (stromal extracellular matrix) or nuclei was observed, whereas adjacent normal epithelial cells, endothelial cells and some tumour infiltrating leukocytes showed positivity for MICA (Figure 2A). Ninety seven percent of the breast cancers showed positive staining for MICA. Thirty-eight percent ( $n=200$ ) of samples demonstrated strong intensity of MICA expression whereas moderate and weak intensity was observed in 37% ( $n=196$ ) and 22% ( $n=119$ ) of tumour samples respectively (Table 2; Figure 2, panels B-D). A variable proportion of MICA-positive cells was also observed; 53% ( $n=283$ ) of the tumours showed extensive expression of MICA (>75% positive cells), whereas 11% ( $n=56$ ) of cases demonstrated MICA immunoreactivity in less than 25% of tumour cells (Table 3).

#### Natural killer cell (CD56+ cell) infiltration in breast carcinoma

As MICA is recognised by NK cells expressing NKG2D, co-localisation of NK cells within a random series of tumours expressing variable levels of MICA was assessed. The whole sections were used to assess NK infiltration (CD56+ cells), as the tissue arrays were selected from areas of tumours rather than infiltration areas. A relative paucity of natural killer cells (CD56+ cells) was found inside the cancer component of the whole breast tumour samples (Figure 3A). Staining with monoclonal antibody to CD56 showed an absence of infiltrate in 20% of cases ( $n=10$ ) and a minimal infiltrate in half of the tumours ( $n=27$ ), whereas 16% and 10% of tumours had a CD56+ score of 2 ( $n=8$ ) and 3 ( $n=5$ ) respectively (Figure 3B). Consequently no significant association was found between NK infiltration and MICA expression ( $P=0.553$ ), or with prognostic indicators and patient outcome in these 50 breast tumours (data not shown).

**Table 1**  
Clinicopathology of patients.

Patient and Tumour Characteristics	Percentage (Number)
Age (mean: 54 years)	
<40	7% (38)
41-50	29% (152)
51-60	36% (193)
>61	28% (147)
Menopausal status	
Pre-menopausal	37% (187/505)
Post-menopausal	63% (318/505)
Histological Grade	
Grade 1 (well differentiated)	21% (113)
Grade 2 (moderate)	35% (184)
Grade 3 (poor differentiated)	44% (233)
LN status	
Negative	64% (339)
Positive	36% (191)
NPI	
Good	36% (186/517)
Moderate	52% (270/517)
Poor	12% (61/517)
Tumour type	
Group 1	6% (31)
Group 2	21% (115)
Group 3	12% (63)
Group 4	61% (321)
Vascular invasion	
None or probable	69% (360/519)
Definite	31% (159/519)
Distant metastases	
Absent	88% (465)
Present	12% (65)
Any recurrence	
Absent	76% (403)
Present	24% (127)
Regional recurrence (axillary LN)	
Absent	91% (481)
Present	9% (49)
Local recurrence (breast)	
Absent	90% (477)
Present	10% (53)

**Comparison of MICA expression with patient and tumour characteristics**

The level of expression of MICA was assessed by three scoring methods, namely the intensity of the staining, the percentage of positive cells and the H-score. For the H-score determination, cut-off values were calculated to define groups showing strong (H-score >200), moderate (H-score = 100-200) and low (H-score <100) expression of MICA (Figure 4).

Analysis utilising the H-score method revealed statistically significant relationships between MICA expression and histological grade, lymph node stage, vascular invasion and Nottingham Prognostic Index (NPI) (Table 4). Grade 3 carcinomas in general expressed higher levels of MICA than lower grade lesions ( $P = 0.006$ ). Moreover, higher levels of MICA expression were more often seen in patients with axillary lymph node positive disease ( $P = 0.011$ ).

Similarly a significant relationship was found between NPI groups and MICA expression as assessed by the H-score method ( $P = 0.002$ ); good prognostic group tumours (NPI <3.4) were more likely to have lower levels of MICA. However, there was no significant relationship between MICA expression and tumour size, menopausal status or patient age at the time of diagnosis (Table 4).

**Table 2**  
Intensity of MICA expression.

Intensity of Expression	% of Tumours (Number)
None	3% (15)
Weak (+)	22% (119)
Moderate (++)	37% (196)
Strong (+++)	38% (200)
<b>Total</b>	<b>530</b>

**Table 3**  
Proportion of MICA-positive breast carcinoma cells.

Proportion of MICA-positive Cells	Percentage of Cases (Number)
<25%	11% (56)
25-50%	13% (71)
51-75%	23% (120)
>75%	53% (283)
<b>Total</b>	<b>530</b>

In addition, a similar significant association was found between the *intensity* of MICA expression and histological grade ( $P = 0.02$ ), as well as with lymph node stage ( $P = 0.02$ ), whereas no correlations were found between the *percentage* of MICA positive cells and pathological prognostic factors or patient characteristics as shown in Table 4.

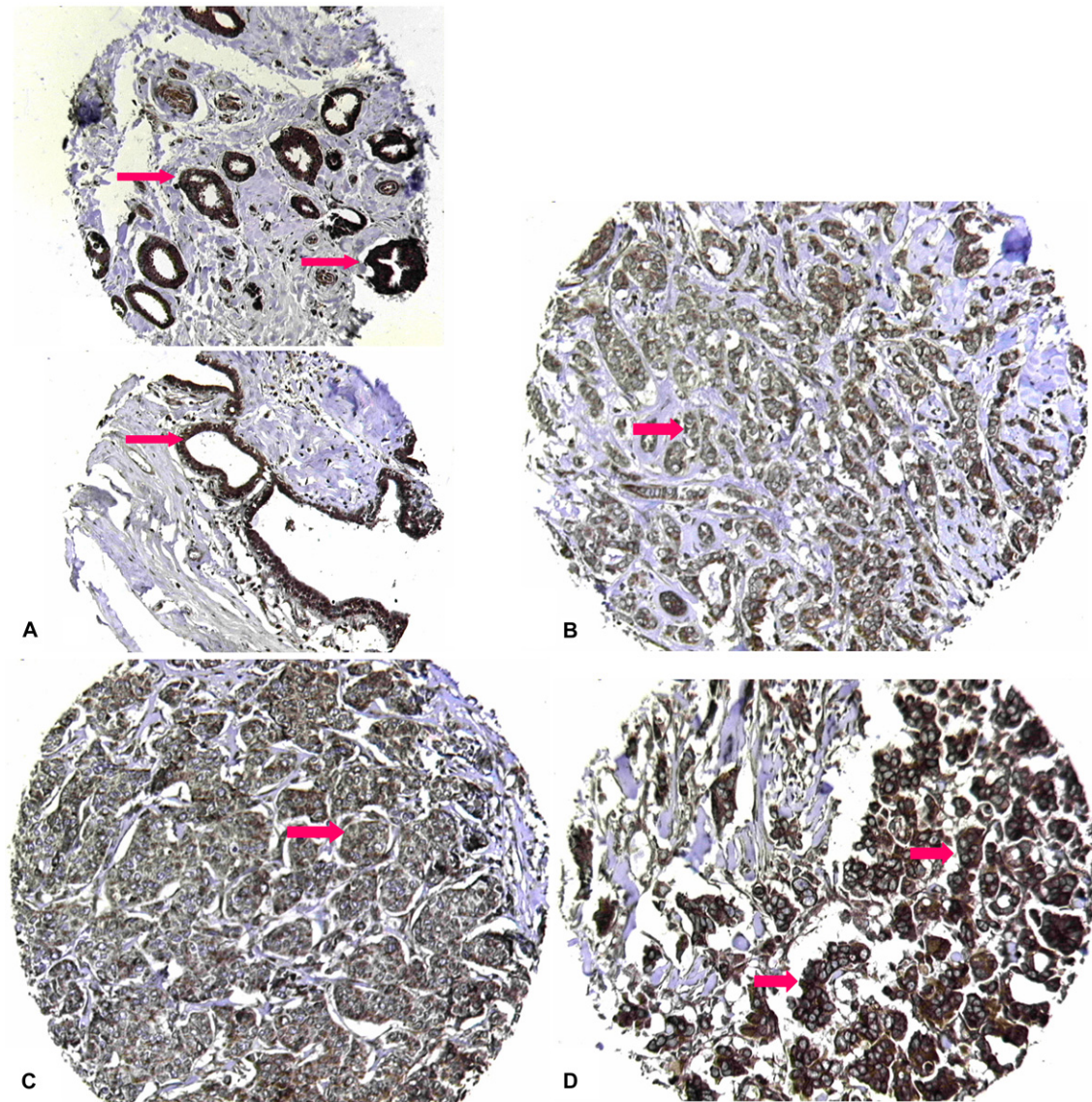
**Comparison of MICA expression with patient outcome**

When MICA expression was compared with recurrence of carcinoma (either local, regional or distant), no significant relationships were noted ( $P$ -values for H-scores shown in Table 4). No correlation was seen between the expression of MICA as assessed by H-score and overall survival of the 530 invasive breast cancer patients, with mean follow-up of 7 years, by Kaplan-Meier analysis (log rank = 0.181, Figure 5). We did not observe correlations between any patient, tumour characteristics and tumours showing MICA expression only in their cell membrane (30/530).

**Discussion**

Of the 530 samples examined 97% were shown to express MICA. Only 15 tumours (about 3%) did not show any staining with MICA antibody which may reflect poor fixation of these tissues. About forty percent of these showed a high level of expression whereas only about 20% were weakly positive. Similarly 53% of samples showed a high percentage of cells staining for MICA. This trend towards high intensity and percentage staining may represent the consequences of stress in the tumour environment. In the present study normal breast epithelium near the tumour margins was seen to stain positively for MICA, although Groh *et al.* 1999 (10) found no MICA

Figure 2



**MICA staining in normal breast epithelium and breast tumours.** Expression of MICA in normal breast epithelium (A). Weak (B), moderate (C) and strong (D) expression of MICA in invasive breast carcinomas (40x magnification).

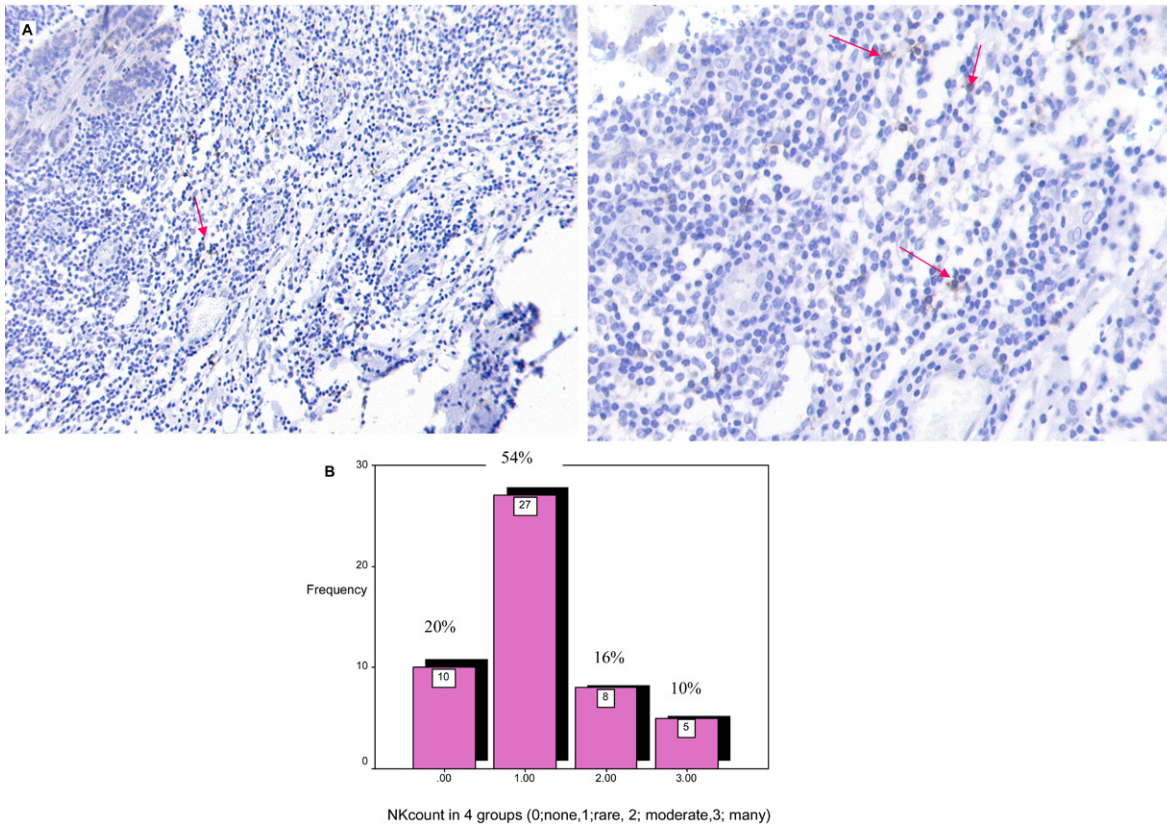
staining in normal breast tissue with the monoclonal antibody 6D4. This finding may be explained by the fact that our anti-MICA polyclonal antibody recognises denatured proteins and could therefore detect intracellular antigens. Indeed, the observed staining was mostly cytoplasmic. Alternatively, stresses which are locally encountered in the tumour microenvironment such as heat shock and transformation may induce MICA expression on the cell surface of adjacent normal epithelial cells.

It was hypothesized that NK infiltration of the tumours would be focal and thus TMA analysis was considered unsuitable for this assessment. Rather, a selection of whole sections from breast tumours expressing variable levels of MICA were identified and used to assess NK infiltration. Analysis of these 50 whole tumour sections for NK cells did not reveal a significant increase in infiltration of NK cells. Using tissue microarrays constructed

specifically from inflammatory areas of tumours with a variety of MICA expression could provide further support of this finding. It should also be noted that NKT cells express CD56, whilst in this study we did not explicitly discriminate between NK and NKT cells.

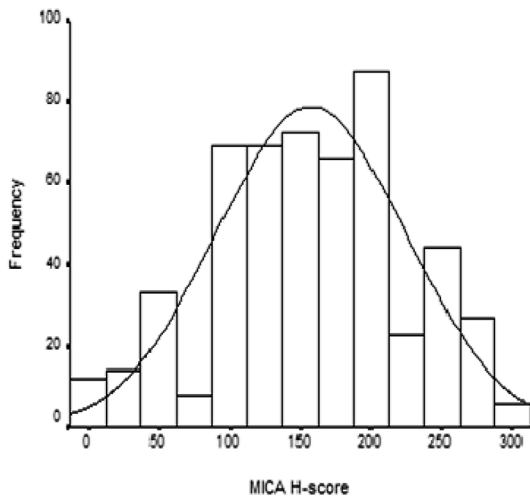
Data about MICA in tumours and normal tissue is only emerging and in the literature there appears to be no systematic analysis about the expression of MICA on a protein level, especially in normal tissues. However, it seems that very few tissues constitutively express MICA on the cell surface; rather it is stored in cytoplasmic granules where it can be rapidly exported to the cell surface under conditions of cell stress (15, 16). The majority of breast tumours in this study showed strong cytoplasmic expression of MICA, and cell surface expression could not be distinguished. MICA can be expressed in the cytoplasm and expressed on the cell surface due to malignant

**Figure 3**



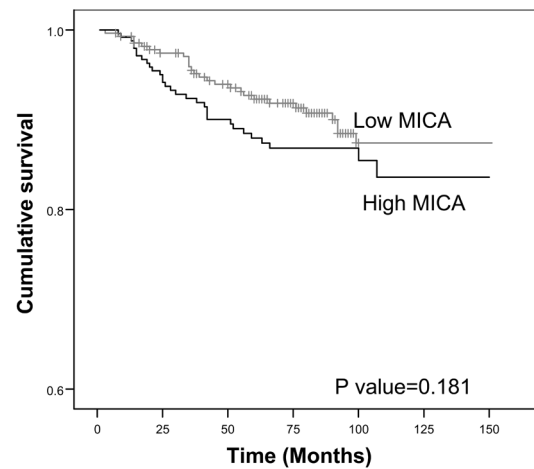
**CD56 staining representative of NK infiltration in the tumour area.** (A) Representative whole section showing a paucity of NK cells in the tumour. (B) Number of tumours in each category of NK (CD56+) staining. The CD56 stained sections were categorised based on the number of positive cells in a high power field as follows: grade 0 (none), grade 1 (rare, <5); grade 2 (moderate, 5-10) and grade 3 (many, >10).

**Figure 4**



Histogram showing distribution of MICA H-score values.

**Figure 5**



Kaplan-Meier plot comparing MICA expression with patient outcome.

**Table 4**  
Association of MICA expression in invasive breast carcinoma with clinicopathological parameters (Pearson chi-square).

Tumour and Patients Characteristics	Percentage of Positive Cells ( <i>P</i> -value)	Intensity ( <i>P</i> -value)	H-score ( <i>P</i> -value)
Age (mean: 54 years) <40 41-50 51-60 >61	0.49	0.23	0.17
Menopausal status* Pre-menopausal Post-menopausal	0.60	0.11	0.33
Histological Grade Grade 1 (well differentiated) Grade 2 (moderate) Grade 3 (poor differentiated)	0.10	<b>0.02</b>	0.006
LN status Negative Positive	0.40	<b>0.02</b>	0.011
Tumour size (mm) <10, 11-20, 21-30, 31-40, 41-50.	0.97	0.50	0.41
NPI Good Moderate Poor	0.13	0.23	0.002
Vascular invasion None or probable Definite	0.60	0.76	0.04
Distant metastases Absent Present	0.28	0.67	0.43
Any recurrence Absent Present	0.88	0.56	0.44
Regional recurrence (axillary LN) Absent Present	0.79	0.74	0.17
Local recurrence (breast) Absent Present	0.80	0.21	0.24

\*Percentage of total number of recorded cases.

transformation. However, if MICA is only expressed within the cytoplasm, and not at the cell surface, it cannot interact with NKG2D on NK cells.

There have been a number of small studies examining the expression of MICA on tumours. The most extensive of these has been carried out in skin cancer where 70% of primary tumours and 60% of metastatic tumours were found to be positive for MICA (12). Other studies on cell lines derived from different histotypes have not shown correlation between MICA expression and invasiveness or metastatic potential (17). However, our study of breast tumours has demonstrated significant correlations between MICA expression and tumour grade, type, lymph node staging and vascular invasion. Similarly, patients with poor prognosis tumours were more likely to have high levels of MICA on their tumours than those of good prognosis.

Our group has previously shown that MICA expression is an independent marker of good prognosis in colorectal cancers (13). This may be because the colon is a naturally more inflammatory environment; indeed MICA expression is mostly restricted to the gastrointestinal tract where it is constitutively expressed. Similarly, we have observed different results

regarding expression of HLA in breast and colorectal carcinomas. It has been shown that loss of MHC class I is an independent indicator of good prognosis in breast cancers (18), whereas colon tumours with low level expression of MHC class I were found to confer a significantly poorer prognosis (19).

The MICA gene is highly polymorphic, with over 50 alleles described (20). The  $\alpha 2$  domain has the greatest number of polymorphic positions, including positions 142, 151 and 156 which are located in the peptide sequence used to generate our antiserum (21). However, of the 15 MICA allelic variants commonly identified in Caucosoid populations (22, 23) only the alleles MICA\*011 and MICA\*012, each constituting <3% of the total allelic diversity, are predicted to differ in sequence in the region of our peptide. The amino acids at positions 140-160 of the remaining 13 alleles (MICA\*001, 002, 004, 006, 007, 008, 009, 010, 016, 017, 018, 019 and 047) all show complete sequence homology with our peptide, and should therefore all be recognized by our antiserum. The use of this antiserum was validated by Western blotting of MICA transfected cell lines, and specificity of the antiserum demonstrated by peptide blocking studies. Furthermore, we observed some

immunohistochemical staining in all tumours examined, indicating the presence of MICA in almost all cases.

MICA is a stress-induced molecule that has been associated with immune surveillance, providing a marker of "altered self" to the immune system. One of the primary mediators of MICA recognition are NK cells, which recognise MICA expression via the NKG2D receptor. This recognition has been shown to reduce the threshold required to trigger the NK cell, and results in a more sensitive and efficient immune response (24). The observed correlation between increased MICA expression and a poor prognostic outcome raises a number of issues. If immune surveillance is occurring in these breast tumours, one might expect that tumours expressing high levels of MICA would be a target for NK and T cell lysis and thus eradicated. However, the opposite was observed, with the most aggressive tumours expressing higher levels of MICA. The simple explanation may be that dedifferentiation results in expression of MICA, but that in the absence of other proinflammatory signals there is no infiltration of immune effector cells and therefore MICA expression has no influence on tumour survival. Alternatively, if MICA is only expressed within the cytoplasm and not at the cell surface it cannot interact with NKG2D on NK cells.

It may be the case that aggressive breast tumours comprise variants which are resistant to the immune attack by other motifs, independently of MICA expression, and that the higher expression of MICA observed in these cases just reflects the local environmental stresses around these tumours, which might be expected to be greater in poor prognosis tumours. However, in support of the immunosurveillance hypothesis which proposes an interplay between the immune system and tumour cells resulting in either elimination of the tumour or tumour escape (2), recent reports have identified a mechanism by which tumours appear to be able to subvert this surveillance mechanism. Soluble forms of MICA were identified from tumours and shown to cause downregulation of NKG2D by engaging with the receptor on tumour infiltrating lymphocytes (TILs), impairing T cell function (6, 7). Indeed, our analysis of CD56+ cell infiltration in 50 whole sections of invasive breast tumour showed minimal or no infiltrate in nearly 75% of the cases evaluated, with just 10% of the cases demonstrating frequent CD56+ cells present. Tumours over-expressing MICA may therefore fall into two groups: (i) Tumours with high cell surface expression of MICA, which are targets for NK / T cell attack and have a good prognosis, and (ii) tumours that have both high levels of cell surface expression but also release soluble MICA, downregulating the immune response, resulting in a poor prognosis. The combined effects of these 2 groups would fail to show a significant influence on overall survival. The development of ELISA assays to measure sMICA may help to differentiate these two hypothetical phenotypes (25).

In conclusion, our results suggest that an induced expression of MICA may be an indicator of poor prognosis for breast carcinoma and is indicative of a stressed environment. However, a subset analysis of these tumours for the NK marker CD56 provided no evidence of increased infiltration of NK cells into these MICA positive tumours. This may represent the escape phase in immune editing of these tumours, resulting in a more aggressive phenotype with a poor prognostic outcome.

## Abbreviations

MICA, MHC class I chain-related gene A; NPI, Nottingham Prognostic Index

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## Materials and methods

### Generation of rabbit polyclonal anti-MICA antisera

A 21-mer peptide was generated based on the unique hydrophilic region of MICA amino acid positions 140-160 of the  $\alpha 2$  domain, as previously described (26). Briefly, the peptide, designated Z167 (MNVRNFLKEDAMKTKTHYHAM), was conjugated to KLH (keyhole limpet hemocyanin) at a 1:1 (w/w) ratio using 0.2% (4  $\mu$ l/ml) of glutaraldehyde for 1 h at room temperature with constant agitation. The conjugated peptide was then dialysed against PBS. Two New Zealand white rabbits were immunised subcutaneously with 100  $\mu$ g of conjugate three times at 14 day intervals and serum was collected and tested against Z167-BSA conjugate by ELISA.

### Western blot

Western blot was performed as described previously (13). CHO and HEK293 cells were transfected with full length MICA (allele \*002) cloned into pCR3.1 using Gene Juice (Merck Biosciences, UK) following the manufacturer's instructions. Cells were harvested and washed in PBS prior to lysis in standard Laemmli reducing sample buffer. Samples were separated by 10% SDS-PAGE and Western blotted onto PVDF membranes under standard conditions. The membranes were blocked in 5% Marvel™ in PBS containing 0.1% TWEEN 20 (PBST) for 1 h. The membranes were then washed and incubated with primary antibody for 1 h in PBST. The Western blot was developed with commercial monoclonal anti-MICA antibody at 1:300 dilution (R&D systems, Abingdon, UK), and rabbit polyclonal anti-MICA at 1:300 and 1:100 dilution. The membranes were washed and secondary antibody was added to the blot for 1 h prior to washing. Rabbit antisera was detected with a goat anti-rabbit HRP-conjugated secondary antibody.

Mouse monoclonal anti-MICA antibody was used as a positive control and detected with rabbit anti-mouse HRP-conjugated secondary antibody. Blots were developed with the ECL reagent (Amersham Pharmacia Biotech UK) and exposed to X-ray films. Blocking assays were carried out by incubating BSA-peptide conjugate with primary antibody for 1 h at 4°C prior to addition to the blotting membrane.

### Patients

The present study included 530 primary operable invasive breast carcinomas from patients aged from 27 to 70 (mean: 54 years) diagnosed between 1987 and 1992 and entered into the Nottingham/Tenovus Primary Breast Carcinoma Series. This is a large series of primary operable breast cancers treated in a uniform manner and has been used to study a wide range of potential prognostic factors and markers (27-29). These 530 tumours comprised those from a larger tissue array of 700 cases in which viable, non-necrotic tumour tissue was present and could be analysed after immunostaining. Patient characteristics, including age and menopausal status, were collected at diagnosis and information on local, regional and distant recurrence and survival was retrieved from a prospectively maintained database (Table 1). Patients were followed up at 3-month intervals for the first 18 months after treatment, every 6 months for the next 5 years, and then annually for a median period of 83 months (7 years). Data recorded for all patients in addition to those noted above included histopathological features such as invasive tumour size, histological grade (30), tumour type (31), lymph node stage, vascular invasion (32) and oestrogen receptor status (33). The Nottingham Prognostic Index (NPI) (34, 35) was also routinely assessed and recorded in the database. The NPI score was calculated for each patient based on the following equation:  $NPI = [0.2 \times \text{tumour size (cm)}] + \text{grade (1-3)} + \text{lymph node stage (1-3)}$ . This index predicts survival of patients with invasive breast cancer and is classified clinically in three groups: good (NPI less than or equal to 3.4), moderate (NPI greater than 3.4 and less than or equal to 5.4) or poor (NPI greater than 5.4) prognosis according to the score obtained (36-38).

Patient management was based on tumour characteristics by calculating the NPI. Patients with an NPI score less than or equal to 3.4 received no adjuvant therapy, those with a NPI score greater than 3.4 received tamoxifen if ER (estrogen receptor) positive ( $\pm$  Zoledex if pre-menopausal) or classical cyclophosphamide, methotrexate and 5-fluorouracil (CMF) if ER negative and fit enough to undergo chemotherapy treatment (39-41).

### Tissue preparation

After surgical excision, fresh tumours were incised and immediately placed in neutral buffered formalin and processed through to embedding in paraffin wax. Tissue microarray (TMA) technology was employed in this study to enable the consideration of a large number of tumours. This series of breast tissue microarrays were prepared from our series of primary operable breast carcinomas according to the method described by Kononen *et al.* (42). All cases were reviewed and the tumour area was marked on their H&E stained slides. Tissue arrays were then constructed by placing 0.6 mm diameter samples from 100 different tumour samples per single block in a 10 by 10 arrangement, with 1 mm spacing separating each specimen. The TMA blocks were constructed in three copies, each containing one sample from a different region of the tumour.

### Peptide blocking studies

A series of six whole sections of breast tumours from a single patient were incubated with purified polyclonal rabbit anti-MICA antiserum, which had been preincubated for 1 h at 4°C with increasing titrations of the immunizing Z167 peptide, so as to further demonstrate specificity of the antiserum for the peptide epitope.

### Immunohistochemistry

Immunohistochemical detection of MICA was performed by a routine streptavidin-biotin peroxidase technique employing the rabbit polyclonal anti-MICA antibody. After deparaffinisation in xylene and rehydration through graded alcohol, slides were immersed in methanol/hydrogen peroxide for 10 min to block endogenous peroxidase activity. Antigens were retrieved by incubating consecutive tissue sections in citrate buffer (pH 6.0) for 10 min at high power followed by 10 min at low power in an 800 W microwave oven. After washing with Tris-buffered saline (TBS, pH 7.4), tissue sections were incubated for 10 min with 100 µl of normal swine serum (NSS) to block non-specific antibody binding sites. The test sections were then treated with 100 µl of rabbit polyclonal anti-MICA at optimal dilution, which was found to be 1:60 (stock concentration 1 mg/ml), for 60 min at room temperature. After rinsing with TBS, sections were then incubated in biotinylated goat anti-mouse/rabbit IgG (Dako Ltd, Denmark) diluted 1:100 in NSS for 30 min, followed by preformed StreptABComplex (Dako Ltd, Denmark) for 60 min at room temperature. MICA localization was visualized using 3,3'-diaminobenzidine tetrahydrochloride (DAB, Dako Ltd, Denmark) followed by copper sulphate enhancement. The sections were lightly counterstained with haematoxylin (Dako Ltd, Denmark), dehydrated in alcohol, cleared in xylene (Genta Medica, York, UK) and mounted with DPX (Distyrene, Plasticiser and xylene; BDH, Poole, England).

The omission of primary antibody and its replacement with NSS was used as negative control (13, 18). Normal breast epithelium near the tumour margins, as well as tumour infiltrating leukocytes, were used as internal positive controls for MICA staining. A whole section of colorectal tissue which has been shown to be positive for MICA was also used as positive control.

To study NK infiltration, 50 full size tissue sections from the same series of tumours were randomly selected and stained with monoclonal antibody (mAb) to CD56 (Clone 1B6, Serotec). The optimal dilution used was 1/100, as suggested by the manufacturer and optimised on a control tissue (tonsil) before testing on tumour tissues. Staining of whole size tissue sections was similarly accompanied by a negative control with omission of the mAb. A positive control of lymphoid tissue (tonsil) was included to ensure efficacy of staining (not shown).

### Evaluation of immunostaining

The tissue sections were initially evaluated using a semi-quantitative system by one author (ZM) in a coded manner without knowledge of the clinical and pathological parameters. The results obtained were confirmed by 2 observers using a multi-headed microscope and a consensus agreement was achieved. Three scoring methods were used to assess MICA levels in tumour sections:

1. The intensity of the immunostaining was classified into four categories: 0 (no immunostaining present), 1 (weak immunostaining), 2 (moderate immunostaining) and 3 (strong immunostaining).

2. The percentage of positive cells was assessed semi-quantitatively and classified into four groups: 1 (<25% positive cells), 2 (25-50% positive cells), 3 (51-75% positive cells) and 4 (>75% positive cells).
3. The Histochemical score (H score) of immunoreactivity was obtained by multiplying the intensity and percentage scores (43, 44). Histochemical scores were sub-grouped into 3 groups of equal range for analysis and classified as: weak (<100), moderate (100-200) and strong (201-300).

CD56 stained whole sections were categorised according to the number of stained cells in a high power field as follows: grade 0 (none), grade 1 (rare, i.e. <5); grade 2 (moderate, 5-10) and grade 3 (many, >10). Initially the slides were scanned at 10x magnification to obtain a general impression of the overall distribution of the CD56 positive cells, in particular the maximal count. The CD56+ cells were then assessed semi-quantitatively at higher magnification and the final scores given.

### Statistical analysis

Statistical analysis of data was performed using the SPSS package (version 11 for Windows). Pearson chi-square and Pearson R tests were used for univariate analysis to determine the significance of associations between variables. For survival analysis, Kaplan-Meier curves were derived and the statistical significance of differences between the survival rates of groups with different MICA expression was assessed using the log-rank test. Survival was censored if the patient was still alive or died from other causes. *P* values <0.05 were identified as statistically significant.

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