

In situ hybridisation and S₁ mapping show that the presence of infiltrating plasma cells is associated with poor prognosis in breast cancer

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Summary In order to identify potential markers of prognosis in breast cancer, representative cDNA libraries were constructed using RNA isolated from primary breast tumour tissue associated with good and poor prognosis. Cross-screening of these libraries repeatedly identified cloned mRNA species associated with the immune system, in particular B-cells, in libraries derived from tumours of poor prognosis. We have used one of these, a κ_{IV} light chain cDNA probe, in two complementary studies to investigate the relationship between immunoglobulin gene expression and prognosis. The results obtained using a combination of S₁ mapping, RNA blotting and *in situ* hybridisation demonstrate that the presence of plasma cells, as defined by infiltrating cells which express high levels of immunoglobulin κ -chain mRNA, is associated with a poor prognosis.

A large proportion of women with operable primary breast cancer have occult micrometastases at presentation. The most accurate indicator of the presence of metastases is the extent of axillary lymph node involvement assessed by a pathologist after complete axillary dissection. However the introduction of breast conservation has reduced axillary dissections and the prognostic information derived from the determination of nodal involvement. This has led to a search for useful prognostic markers of metastases present within the primary tumour tissue such as histological grade (Richardson & Bloom, 1957), steroid receptor proteins (Howell *et al.*, 1984), milk fat globule antigens (Wilkinson *et al.*, 1984), *neu* oncogene expression (Slamon *et al.*, 1987) and cell surface glycoproteins (Leatham & Brooks, 1987). However, none of these markers is sufficiently accurate for clinical use, and new, more precise markers of prognosis would be of great value in order to plan appropriate treatments, and for the psychological management of the patient and her family.

In a series of experiments designed to identify novel markers of poor prognostic significance we constructed large cDNA libraries in bacteriophage λ gt10 using poly(A)-containing RNA isolated from primary breast tumour tissue associated with good and poor prognosis. Cross-screening of these libraries repeatedly identified mRNA sequences associated with the immune system, in particular B-cells, in the cDNA library constructed from poor prognosis tumour tissue, suggesting a potential correlation between the presence of infiltrating cells of the immune system and poor prognosis. Here we describe S₁ mapping, RNA blotting and *in situ* hybridisation studies using a cloned human immunoglobulin light κ -chain cDNA hybridisation probe. These provide evidence that the presence of plasma cells, as defined by infiltrating cells producing high levels of immunoglobulin κ -chain mRNA, is associated with poor prognosis in primary mammary carcinoma.

Materials and methods

Reagents

[α -³²P]dCTP (800 Ci mmol⁻¹) and [α -³²P]CTP (800 Ci mmol⁻¹) were obtained from New England Nuclear (Boston, MA, USA). Restriction endonucleases and

modifying enzymes were purchased from Boehringer Corporation Ltd. (Lewes, UK) or Amersham International plc (Amersham, UK). The cloning vectors pUC13, M13 mp10 and M13 mp11 were purchased from Pharmacia (Uppsala, Sweden) whilst pGEM 1 and pGEM 2 were from P & S Biochemicals (Liverpool, UK). All other materials were from sources described elsewhere (Edbrooke *et al.*, 1985; Pemble *et al.*, 1986).

Patients and pathological material

Primary breast tumour tissue for RNA extraction was obtained from patients involved in clinical trials at The Christie Hospital and Holt Radium Institute, Manchester. For retrospective studies, formalin fixed, paraffin embedded breast carcinoma tissue was obtained from the Department of Pathology, Norfolk & Norwich General Hospital, Norwich. Tissue for RNA extraction was frozen in liquid nitrogen immediately after excision. For *in situ* hybridisation studies formalin fixed tissues were processed and embedded in paraffin wax in routine pathology laboratories, without special precautions to prevent RNA degradation, then stored at ambient temperature for up to 11 years.

RNA isolation

Total RNA was extracted from frozen tumour tissue essentially as described by Chirgwin *et al.* (1979). Briefly, up to 1 g of frozen tissue was ground to powder under liquid N₂ and then homogenized in 10 ml of guanidine isothiocyanate buffer (50 mM Tris/HCl pH 7.6, containing 10 mM EDTA, 2% (w/v) sodium lauryl sarcosine, 0.01% (v/v) β mercaptoethanol, and 4 M guanidine isothiocyanate). Debris was cleared from the homogenate by centrifugation at 10,000 g for 10 min at 10°C, and the supernatant overlaid onto a 3 ml CsCl cushion (5.7 M CsCl, 100 mM EDTA pH 8.0). This was centrifuged in a Beckman SW40 rotor at 30,000 rpm for 18 h at 20°C. Following centrifugation, the supernatant was discarded and the RNA pellet rinsed with 70% (v/v) ethanol and resuspended in 1 ml of guanidine HCl buffer (7 M guanidine HCl, 10 mM sodium iodoacetate, 20 mM sodium acetate, 1 mM dithiothreitol, 20 mM EDTA pH 7.0). RNA was precipitated directly from this buffer by addition of half a volume of absolute ethanol, at -20°C overnight. RNA was recovered by centrifugation, and reprecipitated twice before being resuspended in 10 mM Tris HCl pH 8.0, 1 mM EDTA and stored at -70°C.

The poly(A)-containing RNA was isolated from total

cellular RNA by affinity chromatography on oligo-dT cellulose, essentially as described by Craig *et al.* (1976), except that column equilibration and binding was performed in 10 mM HEPES pH 7.5 containing 500 mM lithium chloride, and 0.2% (w/v) SDS, while elution was with 10 mM HEPES pH 7.5 containing 0.2% (w/v) SDS at 60°C.

Construction and screening of mammary carcinoma cDNA libraries in λ gt10

Representative cDNA libraries were prepared from total poly(A)-containing RNA isolated from breast carcinoma tissue. Double stranded cDNA was synthesised from 2 μ g poly(A)-containing RNA, according to conditions described by Riley *et al.* (1986). Subsequent modification of the cDNA, ligation to EcoRI restricted λ gt10 DNA and packaging into λ gt10 phage particles was as detailed by Huynh *et al.* (1985).

Cross-screening of the cDNA libraries using 32 P-labelled cDNA was carried out at low density ($1-2 \times 10^3$ pfu/9 cm plate) using the hybridisation conditions described by Benton & Davis (1977). Individual differentially expressed recombinant phage plaques were picked, amplified in liquid culture, and cDNA inserts excised and subcloned directly into the EcoRI site of pUC 13 using procedures described by Maniatis *et al.* (1982). EcoRI excised inserts from this subclone were then ligated into M13 vectors (Mp10, Mp11) for DNA sequence analysis (Sanger *et al.*, 1977) and S_1 mapping experiments. Inserts were also recloned into Gemini vectors (pGEM 1, pGEM 2) for preparation of RNA transcripts for *in situ* hybridisation.

RNA blotting and S_1 mapping

Analysis of poly(A)-containing RNA by RNA blotting was carried out as described by Taylor *et al.* (1984). Probes were labelled with [α - 32 P]dCTP by nick translation (Rigby *et al.*, 1977).

S_1 mapping experiments were performed employing conditions modified from Berk & Sharp (1977), using a single stranded 32 P-labelled M13 DNA probe, prepared essentially as described by Myers *et al.* (1985). Hybridisation reactions were incubated at 52°C for 18 h, and S_1 nuclease digestion was at 37°C for 1 h. Protected fragments were analysed by electrophoresis on 6% (w/v) polyacrylamide urea gels.

In situ hybridisation

Tumour blocks were sectioned (5 μ) on a microtome and sections layered onto clean, sterile, poly-L-lysine coated glass slides (Huang *et al.*, 1983). These were baked at 37°C overnight prior to storage at room temperature in a dry, dust-free box. Sections stored in this manner have been successfully used over a period of 6 months. Prior to use sections were dewaxed by sequential immersion of slides in xylene, followed by stepwise rehydration through alcohol.

Further pre-treatment of sections prior to *in situ* hybridisation, was performed as detailed by Hoefler *et al.* (1986).

Radiolabelled sense and anti-sense RNA probes were synthesised from HindIII linearised pGEM 1 (cRNA) and pGEM 2 (mRNA) constructs, by incorporation of [α - 32 P]CTP into transcripts from the T7 promoter, as modified by Promega (technical bulletin) from Melton *et al.* (1984). RNA probe (4 ng), corresponding to 2×10^6 cpm, was applied to each section in 20 μ l hybridisation buffer. Hybridisation was performed as described by Hoefler *et al.* (1986). After washing and dehydration, the slides were coated in Ilford liquid nuclear track emulsion (K5), dried and exposed at 4°C in light-tight boxes for 2–10 days. Slides were developed at 20°C in Kodak D19 developer, and fixed in 5% (w/v) sodium thiosulphate. Sections were counterstained with haematoxylin and eosin prior to microscopic examination.

Results

Identification of κ -mRNA in RNA preparations from primary breast carcinoma

We have constructed a number of representative λ gt10 cDNA libraries ($2-3 \times 10^6$ recombinants) from poly(A)-containing RNA isolated from primary breast carcinomas containing oestrogen and progesterone receptors or no receptors, and from primary breast carcinomas which on the criteria of size, grade, lymph node involvement, extracellular HMFG staining, and receptor status, are indicative of good or bad prognosis (see Table I). Direct cross-screening of these libraries with 32 P-labelled cDNA probes using strategies to identify cloned mRNA species prevalent for example in the tumour of potentially bad as opposed to good prognosis, repeatedly resulted in the identification of immunoglobulin κ -light and γ -heavy chain mRNA sequences, data consistent with the view that there was significant infiltration by B-cells of the 'poor' prognosis tumour relative to that of 'good' prognosis (Collis, 1988).

In order to investigate the possibility that levels of immunoglobulin mRNA expression might prove a useful prognostic factor, we have used one of these cloned sequences (phZH2) as a hybridisation probe to quantitate and localise the site of immunoglobulin mRNA expression in breast cancer. The cloned cDNA inserted in phZH2 (Figure 1) comprises a 256 bp fragment of κ light chain mRNA, containing an open reading frame, encoding part of the variable region (V_k), J-region (J_k) and part of the constant region (C_k). Comparison of the deduced amino acid sequence with other available K-chain amino acid sequences in the V_k regions demonstrated amino acid sequence homology with κ subgroup IV (see Klobeck *et al.*, 1985; Marsh *et al.*, 1985). We have used this κ_{IV} cDNA sequence, recloned into

Table I Clinical parameters used to define tumours of potentially good and bad prognosis

	Poor ^a					Good ^a				
	13	19	44	76	78	60	92	127	130	132
Patient no.	13	19	44	76	78	60	92	127	130	132
Age	54	53	67	54	73	64	49	58	37	76
Menopausal status	Pre	Pre	Post	Pre	Post	Post	Pre	Post	Pre	Post
Tumour size (cm)	10	6	6	8	2	6	3	3	2	1.5
Node involvement	+	—	+	+	—	—	—	—	—	—
Pathology ^b	IDC	IDC	IDC	IDC	IDC	IDC	IDC	IDC/ILC	IDC	IDC/ILC
Grade ^c	III	III	III	III	III	I	I	ND	I	ND
Receptors ER/PR	-/-	+/-	+/+	-/-	-/-	+/+	+/+	+/-	-/+	+/+
Extracellular milk fat globule antigen (ECS) ^d	—	—	—	—	—	+	+	+	+	—
Rime to relapse (m)	12	10	7	1	7	—	—	3	—	—
Time to death (m)	21	32	—	14	14	—	—	—	—	—

^aSelected on basis of tumour size, grade, nodal involvement, ECS with HMFG and disease free interval; ^bInfiltrating duct carcinoma (IDC). Mixed infiltrating duct/infiltrating lobular carcinoma IDC/ILC; ^cRichardson & Bloom (1957); ^dWilkinson *et al.* (1984). We took ECS to be a good prognostic feature.

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5'  THR LEU THR ILE CER SER LEU GLN ALA GLU ASP ALA ALA VAL TYR TYR CYS GLN GLN TYR
    ACT CTC ACC ATC AGC AGC CTG CAG GCT GAA GAT GCG GCA GTT TAT TAC TGT CAG CAA TAT 60
                                Vk
    VAL SER THR PRO ARG ALA PHE GLY PRO GLY THR LYS VAL GLU ILE LYS ARG THR VAL ALA
    GTG AGT ACT CCT CGT GCA TTC GGC CCA GGG ACC AAG GTG GAA ATC AAA CGA ACT GTG GCT 120
                                Jk
    ALA PRO SER VAL PHE ILE PHE PRO PRO SER ASP GLU GLN LEU LYS SER GLY THR ALA SER
    GCA CCA TCT GTC TTC ATC TTC CCG CCA TCT GAT GAG CAG TTG AAA TCT GGA ACT GCC TCT 180
                                Ck
    VAL VAL CYS LEU LEU ASN ASN PHE TYR PRO ARG GLU ALA LYS VAL GLN TRP LYS VAL ASP
    GTT GTG TGC CTG CAG AAT AAC TTC TAT CCC AGA GAG GCC AAA GTA CAG TGG AAG GTG GAT 240
    ASN ALA LEU GLN SER
    AAC GCC CTC CAA TCG 255
    3'
    
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Figure 1 Nucleotide sequence of immunoglobulin κ_{IV} light chain mRNA cloned in *phZH2*. Nucleotide sequence analysis was performed on both stands and across all restriction sites used in the sequencing strategy as described in **Materials and methods**. Deduced amino acid sequence encoding variable region (V_k), J-region (J_k), and constant region (C_k) is indicated.

appropriate vectors, to investigate by RNA blotting, S_1 mapping and *in situ* hybridisation the relative levels, and site of synthesis of κ -chain mRNA in RNA isolated from primary breast carcinomas, which, on a number of criteria (see Table I) were predicted to be of 'good' or 'bad' prognosis. This selection was found later to be reasonably accurate since on follow up all the tumours with features of a poor prognosis have resulted in relapse in all patients and death in 4 out of 5 within 32 months, whereas those with features of a good prognosis are alive 30–40 months after mastectomy, of which only one has relapsed (see Table I).

Initially the levels of κ -chain mRNA expressed in tumour RNA were assessed by RNA (Northern) blotting relative to actin mRNA in a small group of tumours. Total poly(A)-containing RNA was separated on the basis of size by gel electrophoresis, blotted onto Biodyne membranes and co-probed with ^{32}P -labelled *phZH2* and pAM-91 (actin) cDNA probes. A typical result (Figure 2), demonstrates the presence of an intense band of the expected size of κ -chain mRNA (1100ntds) in three RNA preparations all from tumours of bad prognosis, the presence of the same band in the control tonsil RNA preparation, and the presence of low levels only of κ -chain mRNA in the two good prognosis tumours examined in this instance. The actin mRNA (1600ntds) was present at approximately equivalent levels in all RNA preparations.

In order to increase the sensitivity of our analyses, conserve RNA, and to determine the relative level of expression of κ_{IV} mRNA to total κ -mRNA, we have performed S_1 mapping using a 327 ntd uniformly ^{32}P -radiolabelled M13 derived cDNA probe containing M13

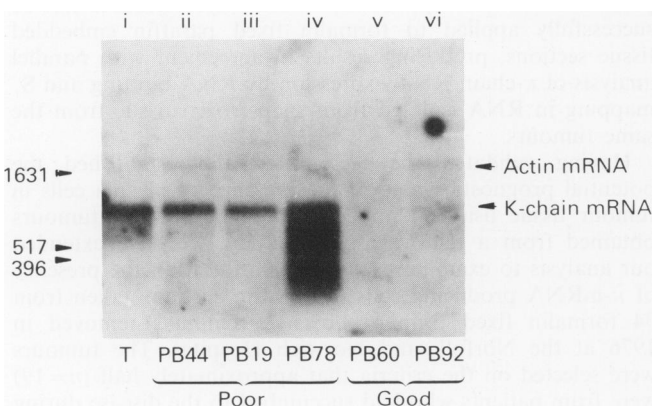


Figure 2 Identification of immunoglobulin κ -chain mRNA in RNA isolated from primary human breast carcinoma by RNA blotting. Total poly (A) containing RNA (1 μ g per track) isolated from (i) human tonsil (T), and (ii–vi) five human primary breast carcinoma of good (PB60, PB92) and bad (PB44, PB19, PB78 – see Table I) prognosis, was analysed by RNA blotting for the presence of immunoglobulin κ -light chain mRNA and actin mRNA (see text and **Materials and methods**). The position of DNA size markers of 1631, 517, 396bp, and κ -light chain and actin mRNA is indicated.

polylinker sequence, and sequence encoding $V_{\kappa_{IV}}$, J_k and C_k (see Figure 3AI). The results (Figure 3BI) compare κ -mRNA levels in poly(A)-containing RNA isolated from five bad prognosis, and five good prognosis tumours (see Table I) with RNA from a tonsil (positive control), benign breast and normal breast. In the tonsil control track, a number of diffuse protected bands are apparent, the two most prominent being of 183 and 175 ntds, representing complete protection of the C_k and J_k region of the probe, and limited S_1 nuclease digestion of part of the 5' end of the J_k region respectively. This was expected taking into account J region duplication (Hieter *et al.*, 1982) and junctional variation during V–J recombination (Weigert *et al.*, 1980). Some larger but minor species were also present. These represent κ_{IV} mRNA (256 ntds) and protection of limited sequence in the V_k region in addition to J_k and C_k sequence (194 ntds). Comparative analysis of all breast RNA preparations, demonstrated that relative κ -mRNA levels in the five 'bad' prognosis tumours were 5–30-fold higher than κ -mRNA levels in the normal breast RNA preparations. In a comparison of the 'good' prognosis tumours, three showed barely detectable levels of κ -mRNA, and two had κ -mRNA levels 2–4-fold higher than normal breast RNA. κ_{IV} mRNA was identified at low levels, in tonsil and two 'bad' prognosis tumours (PB13, PB44). The absence of detectable κ_{IV} mRNA in the remaining RNA preparations probably reflects the low levels of total κ -mRNA of which κ_{IV} mRNA is a minor component.

In addition to our analysis of the relative levels of κ -mRNA, a potential marker for plasma cells in the tumour tissues, we have also examined by S_1 mapping in the same RNA preparations (see Figure 3AII), the relative levels of T-cell receptor β -chain mRNA (Collins *et al.*, 1985), an equivalent marker for T cells. The results (Figure 3BII), using the same RNA samples used for κ -mRNA analysis, show the expected protected T cell receptor mRNA band of 479 ntds in the tonsil RNA preparation, identifying the presence of T cell receptor mRNA. The protected band was barely discernible in the benign and normal breast RNA preparations, and of the remainder, only in one 'good' prognosis RNA preparation were T cell receptor mRNA levels significantly increased (2–3-fold) compared with normal breast. Analysis of T cell receptor mRNA levels in RNA preparations from bad prognosis tumours, showed a small 2–3-fold increase, and in one instance a 10–15-fold increase in T cell receptor mRNA levels relative to normal breast. Overall T cell receptor mRNA levels were elevated in tumour tissue, but there was no particular bias between good and bad prognosis tumour RNA preparations.

Retrospective analysis of κ -mRNA expression by in situ hybridisation

The results described above on a relatively small number of clinically well defined breast tumour preparations, provide evidence for increased infiltration of primary breast tumours, where the prognosis is poor, by cells of the immune system, in particular B cells. The approach whilst revealing, is time consuming, too complex for the routine pathology laboratory, and inappropriate for retrospective studies where available material is limited to formalin fixed paraffin embedded blocks. We have therefore employed *in situ* hybridisation using ^{32}P -labelled κ -chain cRNA hybridisation probes to identify κ -mRNA producing cells in paraffin embedded formalin fixed tissue sections. Initially the validity of the technique was established using tonsil, rapidly processed post-operatively. As can be seen the morphology of the section is retained in spite of prolonged hybridisations (Figure 4a), and the κ -chain cRNA probe hybridises strongly to discrete regions of the tonsil tissue (as judged by autoradiography) consistent with the presence of a cuff of mature plasma cells around reactive follicles. In contrast the ^{32}P -labelled κ -chain mRNA probe showed no hybridisation to

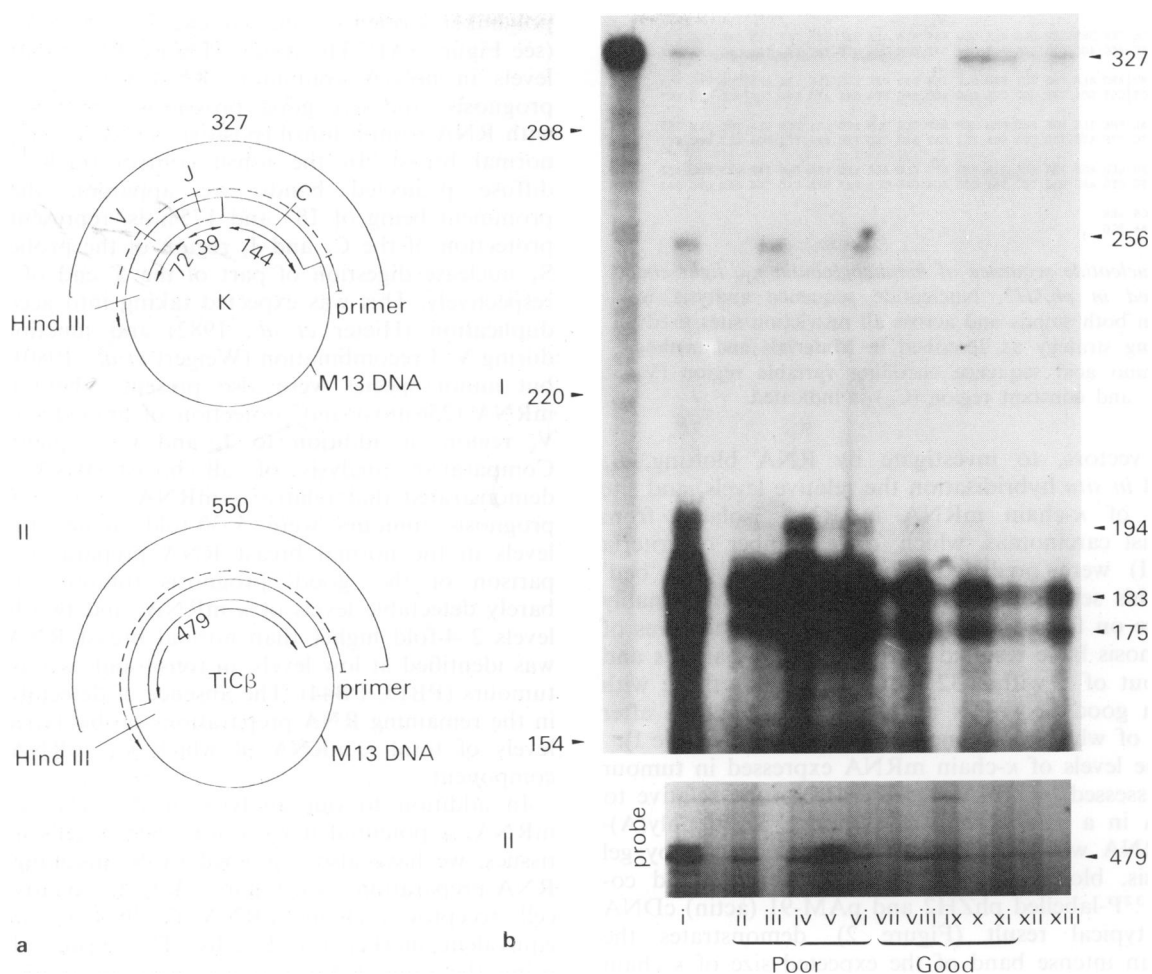


Figure 3 *S*₁ analysis of the relative amounts of immunoglobulin κ -light chain mRNA and T cell receptor mRNA in RNA isolated from human primary breast carcinoma. (A) Single stranded ³²P-labelled cDNA probes specific for immunoglobulin κ -chain mRNA species (I) and TiC β chain mRNA of the T cell receptor (II) were generated by recloning phZH₂ cDNA (256 bp) and 479 bp EcoRI fragment of PB400 into M13 (see Collins *et al.*, 1985, and **Materials and methods**), resulting in cDNA hybridisation probes of 327 and 550 ntds respectively. (B) *S*₁ nuclease analysis of poly (A) containing RNA (500 ng per track) using (I) a κ _{IV} cDNA probe, or (II) a T cell receptor β -chain cDNA probe was carried out as described in **Materials and methods**. Samples were analysed in the following order; track (i) tonsil RNA, tracks (ii-vi) poor prognosis tumour RNA (PB76, 13, 78, 19, and 44 respectively), tracks (vii-xi) good prognosis tumour RNA (PB 132, 130, 127, 92 and 60 respectively) track (xii) benign breast carcinoma RNA, and track (xiii) normal breast RNA. The relative mobility of Hinf I restricted pAT153 size markers of 298, 220 and 154 pb, and the size of bands resistant to *S*₁ nuclear digestion are shown.

tonsil tissue, thereby demonstrating the specificity of the hybridisation probe (Figure 4b). Application of the same approach to formalin fixed paraffin embedded sections representative of good (PB60) and bad (PB78) prognosis primary breast tissues, in this instance using sections cut from blocks prepared without special precautions in a routine hospital pathology environment, showed results consistent with data obtained by RNA blotting and *S*₁ mapping of RNA isolated from the same tissues (see Figures 2 and 3). Hybridisation of the ³²P-labelled κ -chain cRNA probe to the bad prognosis tissue was intense and localised to single cells scattered throughout the section, but focussed in the stroma immediately surrounding although generally not invading the tumour foci (Figure 4c). No hybridisation was observed to any cells on examination of sections of good prognosis tissue (PB60) which were processed in parallel to the bad prognosis (PB78) using the same cRNA probe preparations, hybridisation and autoradiographic solutions (Figure 4d). In all experiments the control ³²P-labelled κ -mRNA hybridisation probe showed no hybridisation to any section when analysed in parallel and developed after an identical period of exposure. In some instances regions of 'yellowing' were seen over single cells. Examination under dark field microscopy showed no evidence for silver grains in these regions, which we presume therefore may reflect an artefact of the methodology. On the basis of the data

presented it would appear that *in situ* hybridisation can be successfully applied to formalin fixed paraffin embedded tissue sections, producing results in agreement with parallel analysis of κ -chain RNA expression by RNA blotting and *S*₁ mapping in RNA isolated from snap frozen tissue from the same tumours.

Having validated the technology and established the potential prognostic value of the presence of plasma cells in tumour tissue using a limited number of primary tumours obtained from a relatively recent study, we have extended our analysis to examine by *in situ* hybridisation the presence of κ -mRNA producing cells in paraffin sections taken from 34 formalin fixed primary breast carcinomas removed in 1976 at the Norfolk and Norwich Hospital. The tumours were selected on the criteria that approximately half ($n=19$) were from patients who had succumbed to the disease during a 10 year post-operative period, and the remainder were from patients who were alive, and in some instances had no recurrent disease, during the same period ($n=15$) – see Table IIA, B. Sections were cut from all the blocks, and the number of κ -mRNA producing cells in each grouping examined by *in situ* hybridisation. Sections were scanned under light microscopy, and the area of tissue within a 2.6 mm² field of view containing the greatest number of κ -mRNA producing cells scored blind. After a 48 h exposure period, out of the 15 blocks examined from the surviving

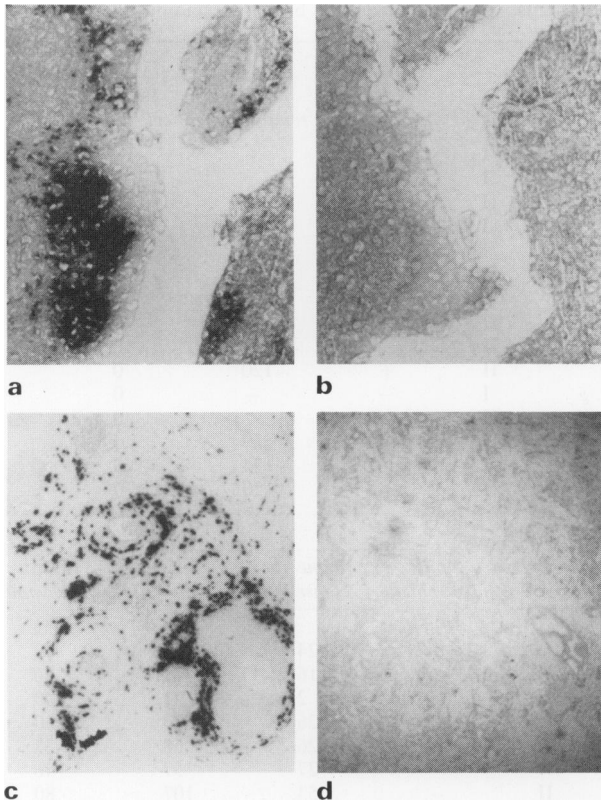


Figure 4 Identification of cells expressing immunoglobulin κ -chain mRNA by *in situ* hybridisation. Immunoglobulin κ -chain cDNA cloned in pHZ₂ (256bp) was subcloned into pGEM1 and pGEM2, and used to generate ³²P-labelled cRNA and mRNA hybridisation probes as described in **Materials and methods**. These were then used for *in situ* hybridisation to (a) human tonsil (cRNA), (b) human tonsil (mRNA), (c) poor prognosis primary breast carcinoma - PB78 (cRNA), and (d) PB60 - good prognosis primary breast carcinoma (cRNA). Sections were exposed for 48 h.

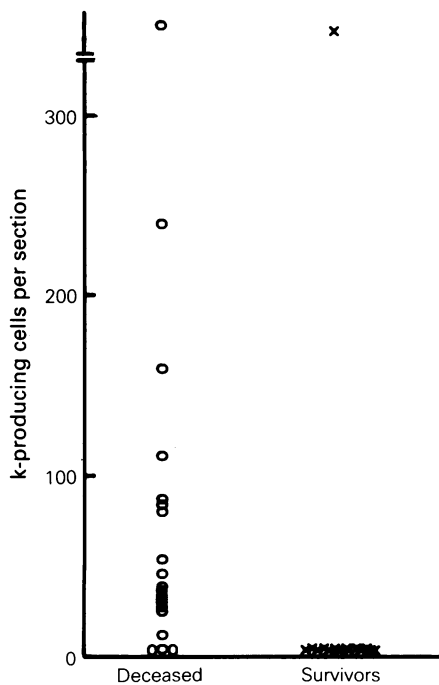


Figure 5 Comparison of the maximum number of immunoglobulin κ -chain producing cells present in a 2.6 mm² field of view as judged by *in situ* hybridisation (see **Figure 4**, and text) in sections from 11 year old formalin-fixed paraffin embedded primary breast carcinoma tissue from patients now deceased or still surviving (see Tables IIA, B). Exposure time was 48 h.

patients, hybridisation was undetectable in 14 sections and in only one instance was hybridisation apparent, in this instance to numerous single cells (Figure 5). In contrast, of the 19 blocks examined from patients now deceased, three showed no detectable κ -mRNA producing cells, whilst the remainder had positive cell counts ranging from 12 to 800 cells within the optimum field of view (Figure 5). Prolonged exposure (5-7 days) identified a second population of cells to which the probe hybridised weakly. Cell counts of this population showed no significant bias towards either the 'good' or 'bad' prognosis groupings.

The *in situ* experiments described above have also been performed using a λ light chain constant region cRNA probe. These results confirmed our observations using the κ -chain cRNA probe, but in addition, provided evidence that the ratio of plasma cells producing κ as opposed to λ light chain mRNA was unexpectedly high (4.5:1), when compared with the expected ratio (2:1) which we have found in normal tissue by *in situ* hybridisation.

Discussion

We have demonstrated using complementary but independent approaches that the presence of elevated levels of immunoglobulin κ -chain mRNA in breast tumour tissue is associated with poor prognosis. In particular, that cells enriched in κ -chain mRNA infiltrate tumours of 'poor' as opposed to 'good' prognosis.

Lymphoplasmacytic infiltration within tumours has been studied extensively and, according to the early literature, was generally thought to be a favourable prognostic sign. A review of earlier work (Underwood, 1974) reported that in 8 studies, five showed a correlation between cell infiltration and 'good' prognosis, whereas three were negative. Since that time there have been numerous additional conflicting reports of associations between infiltration and for example favourable prognosis (Black *et al.*, 1975; Dawson *et al.*, 1982; Stenkvist *et al.*, 1982), and poor prognosis (Roses *et al.*, 1982; Fisher *et al.*, 1983). However, a consistent theme in more recent studies has been the association of lymphoplasmacytic infiltration and histological features of poor prognosis such as poor grade, nuclear pleomorphism, tumour necrosis and lymph node invasion by tumour (Black *et al.*, 1975; Lauder *et al.*, 1977; Fisher *et al.*, 1983; An *et al.*, 1987; von Kleist *et al.*, 1987; Zuk & Walker, 1987).

Studies on lymphocyte subpopulations infiltrating breast carcinoma and benign lesion by immunocytochemistry using panels of monoclonal antibodies specific for various T cell subtypes and B cells, uniformly agree that T cells predominate, and that T cells were more abundant in malignant as opposed to benign tissue (see Schoorl *et al.*, 1976; Hsu *et al.*, 1981; Hurlimann & Soraga, 1985; Lwin *et al.*, 1985; An *et al.*, 1987; von Kleist *et al.*, 1987; Zuk & Walker, 1987). B cells have variably been reported to be absent or few in number in carcinoma, though Hurlimann and Saraga (1985) report that B cells can represent up to 48% of the total number of T cells, whilst Zuk and Walker (1987) provide evidence that the proportion of B cells increase relative to T cells in carcinoma as opposed to benign breast, with an overall B:T cell ratio as high as 1:2. In all studies the prognostic significance of T cell subpopulation and B cell distribution was unclear.

Our study on ten primary breast tumours representative of 'good' and 'bad' prognosis (see Table I), in agreement with previous studies, identified the presence of increased T cell infiltration in the tumour population relative to normal breast, as judged by the measurement of T cell receptor mRNA. Furthermore, no significant trend of increased T cell receptor mRNA in RNA from the 'bad' as opposed to 'good' prognosis tumour population was shown, and our data provided no comparative data on B or T cell numbers. However, levels of immunoglobulin κ -chain mRNA (a

Table II Clinical parameters of surviving and deceased 'Norwich' patients, who presented with breast carcinoma in 1976

(A) Survivors									
Patient no.	Age at presentation	Menopausal status	Tumour size (cm)	Pathology	Grade	Node ^a involvement	Time to relapse (m)		In situ cell count
162902	37	Pre	1.0	IDC	I	-	-		0
391162	47	Peri	2.0	ILC	I	-	-		0
450368	31	Pre	4.0	IDC	II	+	11		0
484672	55	Peri	1-2 ^b	IDC	I	-	-		0
490319	78	Post	1.5	IDC	I	-	-		0
470718	47	Peri	3.0	Medullary CA.	II	-	-		500
253608	44	Pre	1-2 ^b	Medullary CA.	III	-	-		0
146001	66	Post	2-5 ^b	IDC	II	-	-		0
486849	43	Pre	1-2 ^b	IDC	II	-	-		0
230532	66	Post	1-2 ^b	ILC	II	+	120		0
093831	53	Peri	1.5	IDC	I	-	-		0
013029	55	Peri	1.5	ILC	II	+	-		0
489891	64	Post	2.0	ILC	II	+	9		0
013986	63	Post	2.0	IDC	I	-	134		0
405980	43	Peri	2-5 ^b	IDC	II	+	120		0
(B) Deceased									
Patient no.	Age at presentation	Menopausal status	Tumour size (cm)	Pathology	Grade	Node ^a involvement	Time to relapse (m)	Time to death (m)	In situ cell count
152202	62	Post	1.5	IDC	III	+		6	55
459639	64	Post	2.0	IDC	III	+	24	35	12
135646	56	Post	4.0	IDC	II	+	16	23	36
175169	66	Post	2.0	IDC	III	-	2	21	240
487326	55	Post	1.0	ILC	II	+	26	36	0
373311	58	Post	2.5	IDC	II	-	26	68	32
344370	60	Post	2.0	IDC	III	-	27	57	46
199775	66	Post	1.5	IDC	II	-	53	107	80
348408	51	Peri	2.0	IDC+ILC (mixed)	III IDC/ II ILC	+	40	110	24
340155	48	Peri	3.0	IDC	III	-	60	66	85
463179	65	Post	2.5	IDC	I	+	21	40	112
484818	67	Post	1-2 ^b	IDC+Paget's	II	+	27	38	0
427389	43	Pre	2-5 ^b	IDC	I	-	8	20	29
484848	60	Post	3.0	IDC	III	+		11	161
517453	58	Post	2.5 ^b	IDC	II	+	32	70	0
343639	61	Post	1-2 ^b	IDC	II	-		21	29
467777	52	Peri	1-2 ^b	IDC	II	+	41	46	85
057362	59	Post	2-5 ^b	IDC	II	+	19	31	800
048695	47	Pre	1.3	IDC	III	+	13	49	35

^aDetermined by pathological examination; ^bTumour size estimated or measured from histology section.

marker for 70% of resting B cells or plasma cells) were significantly elevated in RNA isolated from tumours of 'bad' as opposed to 'good' prognosis, relative to normal breast. *In situ* hybridisation using a ³²P-labelled κ -chain cRNA hybridisation probe supported data obtained on tumours PB78 (bad) and PB60 (good) obtained using S₁ mapping and RNA blotting. Moreover, *in situ* hybridisation demonstrated that the cells expressing κ -chain mRNA were not of tumour origin, were scattered throughout the stroma sometimes surrounding but not invading tumour foci, and were morphologically identical to cells of lymphoplasmacytic origin variously described in the literature. Since the κ -mRNA is very abundant in these cells, as determined by the relatively short exposure time, we would presume that we have identified plasma cells, as opposed to resting B cells. The latter would also express κ -chain mRNA, but at a very much reduced level (50-100-fold - see Kelly & Perry, 1986). In this respect the tonsil tissue controls are of importance. These not only define the specificity of the cRNA hybridisation probe, but also demonstrate that under the conditions of hybridisation used, plasma cells as opposed to resting B cells have been localised. The identification of an additional population of cells after prolonged exposure, may reflect the localisation of resting B cells in addition to the plasma cells.

Analysis of the distribution of plasma cells in archival, paraffin embedded formalin-fixed tissue, using blocks prepared without special precautions from tumours removed

from patients operated upon over ten years earlier, provided data which confirmed and extended the data generated via a detailed molecular analysis of κ -chain mRNA levels and localisation in the ten selected tumours of potentially 'good' and 'bad' prognosis. In this study, plasma cells were found in tumours from 84% of women who had relapsed and died, whereas only in one of the tumours (6%) from women who survived 10 years or more were plasma cells detected. It is of interest that the single tumour in the group of survivors in which plasma cell infiltration was considerable, was a medullary carcinoma, a tumour type associated with 'good' prognosis (Ridolfi *et al.*, 1977) with characteristic lymphoplasmacytic infiltration (Hsu *et al.*, 1981).

Clinically our studies would suggest that the presence of plasma cells in infiltrating duct carcinoma and mixed infiltrating duct and lobular carcinoma is associated with a poor prognosis. It would also appear, in agreement with previous studies (Ridolfi *et al.*, 1979; Hsu *et al.*, 1981) that plasma cells are present in medullary carcinoma of the breast, and that in these tumours the presence of plasma cells does not reflect a 'poor' prognosis.

Technically our studies emphasise the potential value of *in situ* hybridisation in routine pathology, and demonstrate that data may be generated after a period of 10 years from tissue fixed and embedded without special precautions to eliminate nuclease degradation of mRNA. The technology described in this paper relies on short-half life ³²P-labelled cRNA as the hybridisation probe. However, since the κ -chain mRNA is

highly abundant in plasma cells and therefore well within the detection limits of non-radiolabelled biotin-streptavidin and similar detection systems, the development of non-radiolabelled κ -chain cRNA hybridisation probes suitable for bulk synthesis and therefore routine use would not appear to be an insuperable objective. The use of defined hybridisation probes also should eliminate much conflicting evidence obtained from similar clinical studies obtained using antisera of differing specificity, and in addition, overcome problems associated with the use of monoclonal antibodies on formalin fixed paraffin sections.

A larger more detailed study to determine whether plasma cell infiltration is a true independent prognostic factor or

whether it is related to other histological features of prognosis in mammary tumours would now be of value. Such a study should include for comparison a parallel analysis of B and T cell markers using a panel of monoclonal antibodies.

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