Analysis of potential markers for detection of submicroscopic lymph node metastases in breast cancer

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Summary We have developed sensitive assays for cytokeratin (K) 8, 16, 19, stromelysin 3 (ST3), MUC1 and maspin mRNAs using reverse transcription polymerase chain reaction (RT-PCR) and used these to assess lymph node status in patients undergoing surgery for breast cancer. In addition the RT-PCR assays were tested against lymph nodes from non-cancer patients to determine their specificity. Despite high sensitivity RT-PCR assays for K8, K16, K19, ST3 and maspin were not found to be useful as markers of submicroscopic disease as transcripts of these genes were detected in the great majority of control lymph nodes tested. Expression of MUC1 was also not found to be useful as it was both insensitive and non-specific. The importance of assessing potential markers against an adequately sized control population is demonstrated, as failure to do so can lead to erroneous conclusions.

Keywords: breast cancer; lymph node metastases; RT-PCR; molecular markers

Following potentially curative surgery for breast cancer the presence of histologically evident tumour cells in the axillary lymph nodes is used to select high-risk patients for adjuvant therapy; however, 30% of histologically node-negative patients also develop metastatic disease. More accurate staging, in particular detection of occult metastatic disease, may enable effective treatment strategies to be extended to more high-risk patients.

Polymerase chain reaction (PCR) is up to 100 times more sensitive than conventional techniques in detecting circulating tumour cells and submicroscopic metastases (Ghossein and Rosai, 1996). However, the extreme sensitivity of PCR also confers an inherent disadvantage to produce false positive results. Furthermore, the central question of whether PCR-detected metastases reliably predicts relapse remains unanswered for many tumour types. Whilst lacking specific markers expressed by breast cancer cells a number of research groups have used cytokeratins 18 and 19, epithelial mucin (MUC1), carcinoembryonic antigen (CEA), CD 44 and maspin as transcript markers for the detection of submicroscopic metastases in lymph nodes, bone marrow or peripheral whole blood by reverse transcription PCR (RT-PCR) (Matsumura and Tarin, 1992; Datta et al, 1994; Gerhard et al, 1994; Noguchi et al, 1994, 1996a, 1996b; Schoenfeld et al, 1994, 1996, 1997; Brown et al, 1995; Mori et al, 1995; Gunn et al, 1996; Luppi et al, 1996; Yun et al, 1997; Eltahir et al, 1998; Lockett et al, 1998). However, there appears to be conflicting data regarding the specificity of some of these cell type specific markers in particular MUC-1 (Noguchi et al, 1994; Hoon et al, 1995), CD44 (Matsumura and Tarin, 1992; Eltahir et al, 1998) and K19

(Traweek et al, 1993; Schoenfeld et al, 1994, 1996; Burchill et al, 1995; Krismann et al, 1995; Gunn et al, 1996; Dingemans et al, 1997; Yun et al, 1997; Eltahir et al, 1998). Maspin expression has been previously reported as being a specific marker for breast cancer (Luppi et al, 1996); however, there have been no larger confirmatory studies to assess the accuracy and reproducibility of these findings. Although discrepancies in specificity may be attributed to RT-PCR methods employed including primer design, single-step PCR, two-stage PCR or signal detection by Southern blotting, clearly there is need to establish which markers may have potential in the diagnosis of minimal residual disease in breast cancer.

In an attempt to define a suitable cell type-specific marker for RT-PCR detection of submicroscopic lymph node metastases in breast cancer, we have examined a panel of candidate genes, with particular emphasis on sensitivity and specificity of gene expression in tissue from patients with and without breast cancer.

MATERIALS AND METHODS

Patients and tissue collection

Ethics committee approval to undertake this study was obtained. All patients undergoing either mastectomy or wide excision and axillary dissection were eligible for inclusion into the study. Fully informed written consent for collection of tissues was obtained. Mastectomy or wide excision specimens with axillary nodes were collected fresh from the operating theatre and lymph nodes were dissected prior to examination of the tumour on a clean UV irradiated chopping board with a sterile surgical blade to prevent epithelial cell contamination. Lymph nodes were bisected, with half submitted for routine histology and half taken for examination by RT-PCR. After lymph node dissection specimens of tumour and normal breast were taken from each specimen. Tumour grade

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(Bloom and Richardson, 1957), size and the presence of ductal carcinoma in situ (DCIS) were recorded. Oestrogen and progesterone receptor status was assessed by immunohistochemistry using the 1D5 monoclonal and polyclonal antibody respectively (Dako). As a control group, lymph nodes were collected from patients undergoing surgery for histologically confirmed benign colorectal disease. Routine steps were taken in control specimens to avoid any luminal contamination prior to lymph node dissection.

Markers

In common with other research groups we have developed sensitive assays for cytokeratins (K) 8 and 19 and MUC1 mRNAs using RT-PCR. In addition we have also investigated expression of stromelysin 3 (ST3), cytokeratin 16 (K16) and maspin. ST3 is a matrix metalloproteinase implicated in mammary carcinoma progression. In node-positive patients with infiltrating ductal carcinoma, multivariate analysis has revealed that ST3 level is a strong, independent prognostic parameter for disease-free survival (Ahmad et al, 1998). K16 has been reported as having expression limited to skin and breast tissue (Adams et al, 1995), making it a good potential marker of submicroscopic spread of breast cancer.

To control for the presence of epithelial cell contamination in the control population we used an assay for cytokeratin 20 (K20) which is a sensitive and specific gastrointestinal epithelial cell-specific marker (Gunn et al, 1996; Yun et al, 1997).

Cell lines

Both MCF-7 and T47-D breast cancer cell lines were used to develop the assays. These cell lines are known to express MUC1 (Abe and Kufe, 1993) and K19 (Moll et al, 1982). Additionally MCF-7 has been reported to express maspin (Luppi et al, 1996). Both cell lines were grown and maintained in RPMI-1640 supplemented with 10% fetal calf serum at 37°C in 5% carbon dioxide.

RNA extraction

Bisected lymph nodes and tissue samples from the resected specimen were collected into 1.5-ml microcentrifuge tubes containing 500 µl of 4 M guanidinium solution and manually homogenized using sterile DNA-free techniques. Total RNA from tissue samples and cell lines was extracted using a modification of the acid-guanidine isothiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). RNA samples were measured by spectrophotometry at 260 nm and stored at -80°C until required. Rigorous steps were taken to avoid epithelial cell contamination, by physical separation of the component stages of specimen dissection, RNA extraction, cDNA synthesis, PCR and PCR product electrophoresis. All specimens were accompanied by a reagent only negative control.

Reverse transcription

RNA samples were treated with RNase-free DNase 1 (Gibco BRL) prior to cDNA synthesis. cDNA was synthesized from a maximum of 2.5 µg of DNase-treated total RNA, with 200 units of M-MLuV Reverse Transcriptase (GibcoBRL) primed with random hexamers (Boehringer Mannheim), using the manufacturer's method in a total volume of 10 µl. Reverse transcriptase

minus controls were prepared for each DNase-treated RNA sample. One-twentieth of synthesized cDNA was used for PCR.

Oligonucleotide primers

Custom PCR primers (GibcoBRL) were designed as follows:

K8 F	GCG GCA GCT GCG TGA GTA
K8 R	GCT GAG GCC GGG GCT TGT GAG
K16 outer F	TCA ATG ACC GCC TGG CCT CTT A
K16 outer R	CAG GGC CAG TTC GTG CTC ATA
K16 inner F	CAA CGC CGA CCT GGA AGT G
K16 inner R	CAA TGG TGG CCG CAA TGA T
K19 F	CCA AGA TCC TGA GTG ACA TGC GAA G
K19 R	TGC AGC TCA ATC TCA AGA CCC TGA A
Maspin F	CAA GTG GGT GCT AAA GGT GAC
Maspin R	CAA AGT GGC CAT CTG TGA G
MUC1 F	CGT CGT GGA CAT TGA TCC TAC C
MUC1 R	GGT ACC TCC TCT CAC CTC CTC CAA
ST3 F	GGC GTG CCC GAC CCA TCT
ST3 R	CGG CCC TCG TGC ACC TCA GTA A
K20 F	AGA CCA AGG CCC GTT ACA G
K20 R	ACG ACC TTG CCA TCC ACT ACT TC

K19 primers were designed to span exons 4 and 5, hence spanning the shortest intron of the gene sequence, as well as incorporating mismatches between the pseudogene and cDNA sequences in the 3' pentamers (Bader et al, 1988; Gunn et al, 1996). K16 primers were designed to span exons 1 and 3 at the 5' end thus avoiding the known sequence of K16 pseudogene, with nested primers designed to span exons 1 and 2. β -actin PCR was performed on each specimen as an endogenous external control for RNA extraction and cDNA synthesis.

Polymerase chain reaction

PCR was carried out in a Hybaid Touchdown PCR machine (Hybaid, Middlesex, UK) with an initial denaturation at 94°C and final extension at 72°C common to all PCR reactions. Individual assays were as follows:

K8:	35 cycles of 94°C 30 s, 61°C 30 s, 72°C 30 s
K16 outer:	40 cycles of 94°C 30 s, 59°C 30 s, 72°C 30 s
K16 nested:	35 cycles of 94°C 30 s, 59°C 30 s, 72°C 30 s
K19:	35 cycles of 94°C 30 s, 62°C 30 s, 72°C 30 s
Maspin:	35 cycles of 94°C 30 s, 50°C 30 s, 72°C 30 s
MUC1:	40 cycles of 94°C 30 s, 59°C 30 s, 72°C 30 s
ST 3:	35 cycles of 94°C 30 s, 56°C 30 s, 72°C 30 s
K20:	35 cycles of 94°C 30 s, 62.5°C 30 s, 72°C 30 s

The PCR mix consisted of 150 µM of each dNTP, 1 µM of each primer, 1 × PCR buffer (Qiagen) and 0.5 units of *Taq* DNA polymerase (Qiagen) in a total volume of 10 µl. All PCR assays included a DNA-positive control and a no-template negative control. Five microlitre aliquots of the resulting PCR products were examined on 0.5 µg ml⁻¹ ethidium bromide stained, 2% agarose/TAE gels for presence or absence of PCR products.

Analysis

Sensitivity of the assays was calculated on the group of patients with histologically proven breast cancer by comparison of patients with histologically evident lymph node metastases and patients

Table 1 Patients with histologically confirmed breast cancer

Marker	Number of patients	Histologically positive (%)	Marker positive (%)	Sensitivity of marker % (95% CI)
K 8	36	13 (36%)	35 (97%)	100 (75.3–100)
K 16	53	22 (42%)	38 (72%)	100 (84.6–100)
K 19	36	13 (36%)	35 (97%)	100 (75.3–100)
Maspin	9	9 (100%)	9 (100%)	100 (66.4–100)
MUC 1	18	10 (56%)	11 (61%)	60 (26.2–87.8)
ST 3	39	15 (38%)	37 (95%)	100 (78.2–100)

Table 2 Lymph nodes from patients with breast cancer

Marker	Number of lymph nodes	Histologically positive nodes (%)	Marker positive nodes (%)
K 8	313	56 (18%)	255 (81%)
K 16	476	90 (19%)	108 (23%)
K 19	313	56 (18%)	221 (71%)
Maspin	90	66 (73%)	50 (55%)
MUC 1	159	41 (26%)	28 (18%)
ST 3	342	69 (20%)	239 (70%)

Table 3 Non-cancer control patients

Marker	Number of patients	Marker positive	Specificity of marker % (95% CI)
K 8	8	8	0 (0–36.9)
K 16	22	10	54.5 (32.2–75.6)
K 19	11	9	18.2 (2.3–51.8)
K 20	35	0	100 (90.0–100)
Maspin	13	11	15.4 (1.9–45.5)
MUC 1	10	4	60 (26.2–87.8)
ST 3	9	9	0 (0–33.6)

Table 4 Lymph nodes from non-cancer patients

Marker	Number of lymph nodes	Marker positive nodes (%)
K 8	54	52 (96%)
K 16	146	26 (18%)
K 19	61	41 (67%)
K 20	249	0 (0%)
Maspin	113	54 (48%)
MUC 1	90	44 (49%)
ST 3	64	41 (64%)

Figure 1 Ethidium bromide stained agarose gels of RT-PCR products of potential markers for detection of submicroscopic spread of breast cancer. Lanes 1–10 represent ten lymph nodes from a patient without cancer (A) K8, (B) K16, (C) K19, (D) Maspin, (E) ST3. (F) K20, demonstrating lack of epithelial cell contamination in the control lymph nodes with PCR product present only in the normal colonic epithelia (Lane N)

with marker-positive disease. Specificity was determined against patients with benign colorectal conditions confirmed on histology. As all assays were expected to be more sensitive than histology, specificity could only truly be determined by comparison against a population with no histological evidence of cancer. For both sensitivity and specificity 95% confidence intervals (95% CI) were used to control for sample size (Merrie et al, 1998) as calculated using CIA software (Gardner and Altman, 1989).

RESULTS

RT-PCR assays for K8, K16, K19, maspin and ST 3 were found to be 100% sensitive compared to histology (Table 1), but with considerable variation in the proportion of positive lymph nodes from patients with breast cancer (Table 2). However, these markers were not specific (Table 3), with transcripts of these genes detected in many of the control lymph node samples tested

Table 5 Control patients and results from reported breast markers

Reference	Marker	Tissue	Number of patients	Marker positive	Specificity of marker % (95% CI)
Matsumura and Tarin, 1992	CD44	PBL	4	0	100 (39.8–100)
Eltahir et al, 1998	CD44	PBL	10	4	60 (26.2–87.8)
Gerhard et al, 1994	CEA	BM/PBL	56	0	100 (93.6–100)
Mori et al, 1995	CEA	LN	5	0	100 (47.8–100)
Brown et al, 1995	DF 3	PBL	4	0	100 (39.8–100)
Brown et al, 1995	K 18	PBL	4	4	0 (0–60.2)
Schoenfeld et al, 1994	K 19	LN	11	0	100 (71.5–100)
Datta et al, 1994	K 19	PBL	10	0	94.9 (82.7–99.4)
		BM	29	2	
Schoenfeld, 1996	K 19	LN	20	0	100 (83.2–100)
Luppi et al, 1996	K 19	PBL	17	5	70.6 (44–89.7)
Noguchi et al, 1996a	K 19	LN	(10)*	(0)*	–
Schoenfeld et al, 1997	K 19	PBL/BM	25	0	100 (86.3–100)
Eltahir et al, 1998	K 19	PBL	5	0	100 (47.8–100)
Lockett et al, 1998	K 19	LN	9	0	100 (66.4–100)
	c-myc				
	PIP				
Luppi et al, 1996	Maspin	PBL	17	0	100 (83.9–100)
		BM	4		
Noguchi et al, 1994	MUC 1	LN	(10)*	(0)*	–
Hoon et al, 1995	MUC 1	PBL	8	7	12.5 (3.1–52.7)
		LN	(8)*	(4)*	
Eltahir et al, 1998	MUC 1	PBL	23	21	8.7 (1.1–28.0)

Specificity of previously reported markers for the detection of submicroscopic spread of breast cancer. BM, bone marrow; LN, lymph nodes; PBL, peripheral blood leucocytes. *number of lymph nodes only, patient numbers not reported.

(Table 4, Figure 1). Expression of MUC1 was found to be both poorly sensitive and non-specific. K20 expression was not evident in any of the control lymph nodes assessed (Tables 3 and 4).

Initial assessment of K16 using a limited number of controls showed only two out of 52 lymph nodes from eight patients to be K16 RT-PCR-positive, however, extension of the control assessment to 146 nodes from 22 patients showed that 26 lymph nodes in ten patients without epithelial malignancy were K16 RT-PCR-positive (Table 3).

DISCUSSION

There have been several studies attempting to define markers for the detection of submicroscopic disease in breast cancer, with apparently convincing results for CEA, K19 and maspin (Table 5). However, data from studies assessing the detection of disease in colon and lung cancer have raised questions with regard to the tissue specificity of these markers (Adams et al, 1995; Burchill et al, 1995; Hoon et al, 1995; Krismann et al, 1995; Denis et al, 1997; Dingemans et al, 1997; Eltahir et al, 1998).

CEA gene expression must be regarded with caution as a tumour specific marker, as it has been detected by nested RT-PCR in normal tissues. Jonas et al (1996) found 23% of controls without cancer had evidence of CEA expression in peripheral blood, which the authors propose may be due to venepuncture-induced skin contamination. More recently, Liefers et al (1998) used the nested CEA RT-PCR assay developed by Gerhard et al (1994) for the detection of disease in lymph nodes. However, only seven lymph nodes from two patients were used as controls and CEA expression was detected in these at high cycle numbers. These results raise doubt with regards to the 100% tissue specificity reported by Gerhard et al (1994) (Table 5).

The results from this study confirm a lack of tissue specificity for both MUC-1 and K19 gene expression. Two previous reports have suggested MUC-1 to be tissue specific (Noguchi et al, 1994, 1996a); however, there is no reported data on the number of control patients, and a total of only ten lymph nodes assessed. Our results concur with that of other groups in showing that MUC-1 is expressed in cells of lymphohaemopoietic origin (Hoon et al, 1995; Eltahir et al, 1998). Several studies in breast cancer have reported K19 to be a tissue-specific marker (Datta et al, 1994; Schoenfeld et al, 1994, 1996, 1997; Noguchi et al, 1996a; Eltahir et al, 1998; Lockett et al, 1998). However, there is now a substantial body of evidence to show that K19 can also be detected in peripheral blood and lymphatic tissue rendering it unsuitable as a specific marker of submicroscopic disease (Adams et al, 1995; Burchill et al, 1995; Krismann et al, 1995; Denis et al, 1997; Dingemans et al, 1997).

In contrast to Luppi et al (1996) we did not find that maspin proved to be a specific marker of occult tumour spread in breast cancer. Using the primers and conditions reported by Luppi et al, the maspin gene product could not be amplified in the T47-D and MCF-7 cell lines or in genomic DNA. Analysis of these primers revealed a 5 bp (base pair) self-dimer at the 3' end of the forward primer, a double 3 bp self-dimer in the reverse primer and a 4 bp pair dimer between the two primers, resulting in marked primer dimer formation and poorly specific priming of the maspin gene. Custom PCR primers (Gibco BRL) were subsequently redesigned using PrimerSelect (DNASTAR) with considerably less primer dimer formation and straightforward amplification of the maspin gene product. Using the redesigned primers maspin gene expression was found to vary markedly between tumours, and was evident in many control nodes. In addition to this the MCF-7 cell line did not display evidence of maspin expression (Figure 2).

Figure 2 Tissue specificity of maspin RT-PCR product, demonstrating a lack of tissue specificity and absence of expression in the MCF-7 cell line. SM, skeletal muscle; H, heart; KI, kidney; AD, adrenal; LI, normal liver; SP, spleen; CC, colon adenocarcinoma; CO, normal colon; SB, small bowel; HM, hepatic metastasis; T47-D and MCF-7, breast cancer cell lines; B, PCR control

Although K16 expression appeared less ubiquitous compared to other genetic markers, it also lacked specificity, as did ST3 and K8. With regards to K16, assessment of only ten lymph nodes as in previous studies (Noguchi et al, 1994, 1996a), would have resulted in a false assertion of 100% specificity. However, when the number of control nodes was increased to 154 from 23 patients, a more accurate determination of specificity was possible.

Although many previous studies have reported 100% specificity, analysis of 95% confidence intervals (Table 5) shows that the majority cannot make this claim with any degree of certainty. Use of a large control group increases the reliability of the determination of specificity, and examination of too few patients can result in false estimates.

We have adopted rigorous protocols to avoid and monitor for epithelial cell contamination. This is achieved by strategically separating component parts of the RT-PCR assay, the use of sterile DNA-free techniques, disposable consumables, reagent only negative controls, reverse transcriptase minus controls and PCR reagent only controls. The adherence to such methodology is essential for success of any RT-PCR assay. The absence of K20 in the control lymph nodes rules out the possibility of epithelial cell contamination from the gastrointestinal tract, confirming the validity of the positive results of the potential markers.

To date there have been no markers of submicroscopic spread of breast cancer identified that are both sensitive and specific. Many markers such as K19 and maspin have been initially reported as sensitive and specific, but little emphasis has been placed on determining assay specificity to a reliable level.

Markers of submicroscopic spread in breast cancer could have potential therapeutic impact, especially when combined with sentinel node assessment of axillary disease. As yet no such marker exists and future assays must be assessed by the use of control populations of sufficient size to reliably determine specificity as well as sensitivity.

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