Technical Report

Improved method for the detection of cytokeratin 19-positive cells in the peripheral blood of breast cancer patients

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To determine the correlation of the levels of circulating tumor cells (CTC) in breast cancer patients with clinical parameters like disease-free survival and response to therapy, we employed a previously reported quantitative real-time reverse-transcription polymerase chain reaction (QRT-PCR) assay for cytokeratin-19 (Ck19; Ann Oncol 2001; 12: 39–46). Our preliminary results based on this assay (Protocol A) prompted us to optimize the experimental conditions. Here we report an improved assay (Protocol B), which more sensitively and specifically detects CTC in breast cancer patients.

Laboratory Investigation (2004) 84, 658–661, advance online publication, 5 April 2004; doi:10.1038/labinvest.3700086

Keywords: breast cancer; micrometastasis; circulating tumor cells; real-time RT-PCR; cytokeratin 19; peripheral blood

To determine the correlation of the levels of circulating tumor cells (CTC) in breast cancer patients with clinical parameters like disease-free survival and response to therapy, we employed a previously reported quantitative real-time reverse-transcription polymerase chain reaction (QRT-PCR) assay for cytokeratin-19 (Ck19).¹ Our preliminary results based on this assay (Protocol A) prompted us to optimize the experimental conditions. Here we report an improved assay (Protocol B), which more sensitively and specifically detects CTC in breast cancer patients.

For quantification of Ck19-positive (Ck19+) cells by QRT-PCR, we first had to establish a standard curve using serial dilutions of the Ck19+ human epithelial breast cancer cell line SK-BR-3 (American Type Culture Collection # HTB-30, Manassas, VA, USA)² spiked into the Ck19-negative (Ck19-) human leukemia cell line HL-60 (American Type Culture Collection # CCL-240, Manassas, VA, USA).³ Concentrations in the samples were 0–10⁵ SK-BR-3 diluted in enough HL-60 for a total of 10⁶ cells per sample. Total RNA was extracted using the

RNeasy Mini kit (Qiagen, Valencia, CA, USA) and reverse transcription of 1 µg RNA was performed using the Omniscript Reverse-Transcriptase kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions for both procedures. In Protocol A, we used the sequence and concentration of the Ck19 primers (primers A and B, Figure 1) and Ck19 TaqMan probe as reported by Aerts *et al* (Figure 1).¹ The Ck19 TaqMan probe is modified at the 5'-end with FAM dye and at the 3'-end with TAMRA dye. In all, 2½ µl of the cDNA solution was used in a 25 µl PCR reaction containing the following: 1 × PCR buffer (Qiagen, Valencia, CA, USA), 200 µM of each dNTP (Roche, Indianapolis, IN, USA), 900 nM each of primers A and B, 200 nM Ck19 TaqMan probe, and 2.5 U HotStarTaq DNA polymerase (Qiagen, Valencia, CA, USA). We also adapted the previously reported annealing temperature¹ and, using the iCycler (Bio-rad, Hercules, CA, USA), QRT-PCR was performed with the following thermal parameters: 15 min at 95°C and 50 cycles of 30-s denaturation at 94°C and 1 min annealing at 60°C. To account for the difference in each reverse transcription reaction, parallel QRT-PCR reactions were performed for the housekeeping gene glyceraldehyde phosphate dehydrogenase (GAPDH). For each GAPDH QRT-PCR reaction, 2.5 µl cDNA solution was used in a 25 µl reaction containing the following: 1 × PCR buffer (Qiagen, Valencia, CA, USA), 200 µM of each dNTP (Roche, Indianapolis,

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Received 19 September 2003; revised and accepted 04 February 2004; published online 5 April 2004

1 ACTACAGGAC CATCCAGGAC CTGCGGGACA AG/ATTCTTGG TGCCACCATC GAGAACTCCA
Ck19 primer D Ck19 primer A
61 GGATTGTCTT GCAGATGAC AAAGCCCGTC TGGCTGCAGA TGACTTCCGA ACCAA/GTTTG
121 AGACGGAACA GGCTCTGGC ATGAGCGTGG AGGCGACAT CAAAGGCTG CGCAGGTGC
Ck19 primer B
181 TGGATGAGCT GACCTGGC AGGACCGAOC TGGAGATGCA GATCGAAGGC CTGAAGGAAG
Ck19 primer C

Figure 1 Specific location of the primers and TaqMan probe on the Ck19 mRNA. Primers are underlined and the probe is represented in bold letters. The junction between exon 1 and exon 2 is represented by '/' and the junction between exon 2 and exon 3 is represented by '/' as described.¹

IN, USA), 900 nM sense GAPDH primer (CCCACTCTCCACCTTTGAC), 900 nM antisense GAPDH primer (CATACCAGGAAATGAGCTTGA-CAA), 200 nM GAPDH TaqMan probe (FAM-CTGGCATTGCCCTCAACGACCA-TAMRA), and 2.5 U HotStarTaq DNA polymerase (Qiagen, Valencia, CA, USA). The thermal parameters for GAPDH QRT-PCR were the same as the parameters used for Ck19.

Quantification for both Ck19 and GAPDH is based on the TaqMan principle.⁴ The cycle number where the FAM signal crosses the threshold is designated as the Ct value. For each PCR run, the baseline and threshold values were calculated using the default settings of the iCycler software (version 3.0a). To construct the standard curve, the assay was performed three times on three different days. In one assay, the samples were measured in triplicate. The intra-assay coefficient of variation (CV) was 8% and the interassay CV was 14%. The average of all Ct values obtained was used to normalize the results using the following equation: $\Delta Ct = (\text{GAPDH Ct of sample} / \text{average of all GAPDH Cts}) \times \text{Ck19 Ct of sample}$. The standard curve was calculated using linear regression analysis (Microsoft Excel). In our hands, Protocol A resulted in an unexpectedly low sensitivity. It was only able to reliably detect 1000 SK-BR-3 cells in a pool of 10^6 cells (Figure 2).

To determine the specificity of Protocol A in the clinical setting, we tested samples from 20 healthy volunteers. In total, 10 ml of peripheral blood from

Figure 2 Comparison of the sensitivity of Protocol A and Protocol B. Standard curves represent the correlation between ΔCt Ck19 values and the starting amount of SK-BR-3 cells diluted in HL-60. ΔCt Ck19 = (GAPDH Ct of sample/average of all GAPDH Cts) $\times$ Ck19 Ct of sample. Error bars represent the s.d. of all replicates.

healthy volunteer (HIC # 10614 to BAB), obtained in heparinized tubes (Becton Dickinson, Franklin Lakes, NJ, USA), were subjected to RBC lysis using ice-cold RBC lysis buffer (0.16 M NH_4Cl ; 0.01 M KHCO_3 ; 10 mM Na_2EDTA (all from JT Baker, Phillipsburg, NJ, USA). Nucleated cells were then collected and washed twice with phosphate-buffer saline prior to RNA extraction. cDNA synthesis and QRT-PCR were performed as described for the cell lines. The assay was performed twice in two different days. The interassay CV was 9%. The amount of Ck19+ cells in each sample was calculated by interpolation from the standard curve after normalization. Our results show that a significant proportion (75%; 15 out of 20) of the samples from healthy volunteers had detectable levels of Ck19+ cells, and in most of them the amount detected was significant (range 0–93507; Table 1).

To improve the sensitivity and specificity of the assay, we optimized the reaction by varying its

Table 1 Number of circulating Ck19+ cells in healthy volunteers ($N=20$) and breast cancer patients ($N=16$) using Protocol A and Protocol B

Sample number	Number of Ck19+ cells	
	Protocol A	Protocol B
N01	289 $\pm$ 26	0
N02	670 $\pm$ 60	4
N03	1278 $\pm$ 115	0
N04	60 $\pm$ 5	0
N05	0	0
N06	0	0
N07	4	0
N08	480 $\pm$ 43	0
N09	0	0
N10	566 $\pm$ 51	0
N11	0	0
N12	2	0
N13	93 507 $\pm$ 8416	0
N14	33 816 $\pm$ 3043	6
N15	3400 $\pm$ 3306	0
N16	4806 $\pm$ 433	0
N17	2	0
N18	0	0
N19	2	0
N20	856 $\pm$ 77	0
Px1	3	0
Px2	3	210 $\pm$ 2
Px3	0	5
Px4	4	0
Px5	238 $\pm$ 21	0
Px6	2748 $\pm$ 247	0
Px7	10 555 $\pm$ 950	16
Px8	45 709 $\pm$ 4114	1
Px9	743 $\pm$ 67	79 $\pm$ 1
Px10	610 $\pm$ 55	0
Px11	62 637 $\pm$ 5637	95 $\pm$ 1
Px12	0	0
Px13	33 185 $\pm$ 2987	1
Px14	46 939 $\pm$ 4225	0
Px15	1790 $\pm$ 161	0
Px16	2699 $\pm$ 243	0

N = healthy volunteer; Px = patient.

components and by varying the annealing temperature (data not shown). Despite all these alterations however, we were still not able to make significant improvement on the sensitivity and specificity. In the process of optimizing the PCR conditions, we visualized the PCR products in 1.5% agarose stained with ethidium bromide. Figure 3a shows that using Protocol A to amplify Ck19 in the Ck19-negative-HL-60 cells resulted in the amplification of two PCR products, the expected 101-bp product and an additional 298-bp product. Since the signal from the TaqMan probe was also seen when we used HL-60 alone without spiking with SK-BR-3 (Figure 2), these products must therefore be the source of the high background signal observed in the results from Protocol A. These products were always seen despite our efforts for optimization.

Since Protocol A resulted in these two distinct products in our negative control cell line, we then proceeded to design a new primer pair using Primer3 software and re-optimized the PCR method. Since the new primer pair (primers C and D; Figure 1) flanks the regions immediately outside primers A and B, we were able to use the same Ck19 TaqMan probe. Figure 1 illustrates the location of the probe and all primers used on the Ck19 mRNA. For Protocol B, the modifications of the PCR described in Protocol A include the addition of 3 mM MgCl₂ and increasing the annealing temperature to 65°C. Visualization of the PCR products using primers C and D in Protocol B to amplify Ck19 in HL-60 did not result in any amplified product (Figure 3b). Using Protocol B on the same serial dilutions of SK-BR-3, we were able to improve the sensitivity to 10 SK-BR-3 in 10⁶ HL-60 (Figure 2). The assay was also performed three times on three different days with three replicates each day. The intra-assay CV was 0.4% and the interassay CV was 2%. In retesting the samples from the same healthy volunteers, our results also show a significant improvement on the specificity (Table 1). Only 10% (two out of 20) of the healthy volunteers had detectable Ck19+ cells and the values observed were considerably lower (range 0–6).

We also tested 16 patients diagnosed with American Joint Committee on Cancer Stage II (N = 7) or III

(N = 9) breast cancer. All patients had received chemotherapy but none of them had received treatment for at least 2 months when tested. For Protocol B, the samples from healthy volunteers and patients were assayed twice on two different days. The interassay CV was 1%.

Table 1 shows the comparison of the levels of CTC detected in these patients using either protocol. The two protocols yielded notably diverse results. As shown in Figure 4, a cutoff value, which differentiates between illegitimate expression in blood cells and gene expression in cancer cells, can easily be established using the results from Protocol B. By defining the maximum value (maximum value = 6, Table 1) measured in healthy volunteers as the cutoff,^{5–7} and defining this value as background signal, we consider a sample with Ck19+ cell count ≤ 6 to be negative for CTC. Similarly, a Ck19+ cell count > 6 was considered positive for CTC. Using Protocol B, we were able to identify four patients (patients # 2, 7, 9, and 11) who still have a significant amount of CTC despite their treatment.

Our *in vitro* spiking experiment showed that we were able to improve the sensitivity of the reported QRT-PCR assay for Ck19 using our dilution samples. In addition, *in vivo* studies using samples from healthy volunteers showed that we were able to improve the specificity. The two protocols also showed markedly different results in breast cancer patients (Table 1). The results from Protocol B, which permitted us to establish a cutoff value using the highest limit in the negative control group, allowed the differentiation between background signal and signal from CTC (Figure 4).

Figure 3 PCR amplification products after 30 cycles using (a) Ck19 primers A and B in Protocol A (101-bp) and (b) Ck19 primers C and D in Protocol B (231-bp) visualized using ethidium bromide staining on 1.5% agarose gel. Lane 1—marker; Lane 2—SK-BR-3; Lane 3—HL-60.

Figure 4 Number of circulating Ck19+ cells in healthy volunteers (N = 20) and breast cancer patients (N = 16) using (a) Protocol A and (b) Protocol B. Each healthy volunteer is represented by ▲ and each patient is represented by ■. The cutoff value shown as — — — represents the highest value in healthy volunteers. The amount of Ck19+ cells in each sample is calculated by interpolation from the standard curve after normalization.

We are currently using Protocol B to follow the effects of chemotherapy on the levels of CTC in Stage II and III breast cancer patients. Our current results show intra-patient consistency over time and preliminary evidence of response or nonresponse to treatment. The question of clinical correlation, however, can only be answered using a significant sample size and longer follow-up time. The changes in the protocol reported here can help gain more insight on the clinical relevance of the absolute quantity of CTC.

Acknowledgements

The authors thank the late Dr Barry Kacinski, Dr Maryann Flick, and Dr Harriet Kluger for a critical review of the manuscript; Ms Sofya Rodov for her technical assistance; Dr Jordan Pober for the use of the iCycler; and Dr Michael DiGiovanna for the patients' samples. We also thank all the patients and volunteers for their indispensable cooperation. This work was supported by grants from Aventis (BAB), NIH training grant to the Department of Therapeutic Radiology, Yale University (CA-09259-20) (ABA) and Summer Fellowship from University of New Haven (ES).

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