Non-Coding CK19 RNA in Peripheral Blood and Tissue of Breast Cancer Patients

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Abstract- Breast carcinoma is the major cause of cancer-related death in women. The incidence of this carcinoma is rising and there are many attempts to decrease this problem. The aim of this study was detection of full-length cytokeratin 19 (CK19) mRNA, in peripheral blood and tissue of breast cancer patients in early stage of cancer. In this study, RT-PCR (reverse transcriptase-polymerase chain reaction) technique was used for detection of CK19 mRNA in peripheral blood and tissue of breast cancer patients. Primers were established to amplify the CK19 as a tumor marker. Moreover, CYFRA 21-1 subunit of CK19 protein was measured in the serum of patients. CK19 mRNA was detected and sequenced. It is shown that the most released CK19 mRNAs in blood and tissue of cancer patients are non-coding RNA. The mutated forms of mRNA are the incomplete transcripts of protein-coding gene as a long non-coding RNA (lncRNA) that could regulate gene expression. Moreover, small non-coding RNA (ncRNA) as fragments of CK19 is mostly observed in this experiment. They may play a role in tumorogenesis and their biologic exact function in breast cancer should be further elucidated.

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Keywords: Breast cancer; CK19; Non-Coding RNA; Tumor marker

Introduction

Breast cancer is known as a well characterized, rising and life threatening disease in the world (1-3). In the early stages of breast cancer, tumor cells are secreted into the blood (4). These circulating tumor cells (CTCs) are clinically important and they are hematogenus rout for metastasis (5). In this regard, finding an appropriate tumor marker is very crucial (6). Tumor marker is defined as a molecule that changes in non-cancerous (benign) or cancerous conditions qualitatively or quantitatively and this changes could be detected. Tumor markers usually are nucleic acids (DNA or RNA) or protein molecules. They can be applied in cancer diagnosis, risk estimation of recurrence or death, prediction and monitoring response to anticancer drugs (7). In an ideal condition, a diagnostic tumor marker must be highly sensitive and specific for the kind of tumor (8).

One of the most interesting tumor markers in breast cancer is CK19 (cytokeratin19) (9). CK19 is belonging to cytokeratin family (CKs) that owned string biopolymers in the cytoskeleton of eukaryotic cells (10). CK19 proteins is expressed on simple and stratified epithelium and separated from it in different cancer disease (11,12). It is reported that CK19 is a sensitive and specific marker for breast cancer (9). It can also be detected in both RNA and protein forms. RT-PCR (reverse transcriptase-polymerase chain reaction) is reported as a useful method for detection of CK19 RNA even in the early stage of breast cancer (13).

On the other hand, At the protein level, a piece of CK19 protein in the sera, called CYFRA 21-1, can be measured by a sandwich ELISA assay (CYFRA21-1 ELISA) using specific monoclonal antibodies for CK19 protein (11). Both fragment and full-length CK19 proteins have been detected in breast cancer cell lines and bone marrow of breast cancer patients with immunoassay tests (12).

In this study, CK19 molecular marker in blood and tissue of breast cancer patients was detected. Detection of CK19 marker, as a protein fragment, and also as small and long full-length RNA fragment was assessed.

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Materials and Methods

Blood and tissue samples

From all patients and healthy donors, written consent letter was obtained for participation in the experiment. The local ethical committee of Pasteur Institute of Iran approved the protocol. Breast cancer patients were referred from Tehran, Milad hospital. Peripheral blood and tissue samples were obtained from 32 patients with primary breast cancer (stage I) before chemotherapy. For each patient 5 ml of the peripheral blood and tissue samples was collected. Normal blood samples from 32 healthy individuals were also obtained. The blood was aliquot into two glass tubes. In one tube, 250 µl sodium citrate (0.032 g/ml) was added; this tube was used for RNA extraction. The second tube, which had no sodium citrate, was used for serum isolation.

CYFRA 21-1 ELISA

The blood samples were allowed to be clotted. They were centrifuged at 4000 rpm for 15 minutes for serum isolation. The serum was isolated and stored at -80°C. CYFRA 21-1 was measured by ELISA (CanAg, FUJIREBIO, Diagnostic Inc.) according to the manufacturer's instructions.

Total RNA extraction from blood and tissue

Total RNA was isolated by AccuZol reagent (Bioneer Inc, Seoul) according to the manufacturer's instructions. The isolated RNA was dissolved in distilled water and stored at -80°C until use. The concentration of RNA was measured at 260 nm with Picodrop (Ltd Cambridge, UK). RNA integrity was controlled by RT-PCR using primers for Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) amplification.

One Step RT-PCR assay

Reverse transcription of RNA and PCR amplification was carried out using one-step AccuPower™ RT-PCR PreMix kit (Bioneer, Seoul). The RT-PCR reaction was carried out in an Eppendorf DNA thermal cycler. The sequences for each set of primers (GAPDH, CK19 full-length and fragment) are given in table 1. The RT-PCR reaction was done according to the manufacture instructions. In summary, 1 µg of total RNA template and 10 pmol of reverse primer were mixed and incubated for 5 min at 70 °C. The mixture was placed on ice. The incubated mixture and the forward primer were transferred to AccuPower™ RT-PCR PreMix tube and fill up with diethylpyrocarbonate (DEPC), Distilled water (DW) up to total volume of 20 µl. After vortex mixing, cDNA synthesis was performed at 42 °C for 60 min. Reverse transcriptase (RTase) inactivation was done at 94 °C for 5 min. The cycling protocol of PCR reaction consisted of a 94 °C for 1 min (denaturation), 56°C for 30 sec (annealing) and 72°C for 1 min (extension), repeated for 30 cycles. The final extension was carried out at 72 °C for 10 minutes.

To ensure that genomic DNA is not in RNA samples, total RNA extraction samples have passed a PCR reaction instead of RT-PCR reaction. They didn't show any CK19 band.

Two step RT-PCR by Pfu polymerization

Step 1- cDNA synthesis: Extracted total RNA (1 µg) was used in Accupower® Cyclescript RT PreMix kit (Bioneer, Inc. Seoul). The reverse transcription reaction was composed of 3 steps: Step1 was performed at 25 °C, 30 sec, for primer annealing. Step 2 was performed at 45 °C, 4 min for cDNA synthesis. The step 3 was done at 55 °C, 30 sec for melting secondary structure of RNA template and cDNA synthesis. These steps repeated 12 times and then, the heat inactivation step performed at 95 °C, for 5 min.

Step2- PCR amplification: PCR amplification was performed by Accupower® pfu PCR PreMix kit (Bioneer, Inc. Seoul) to secure high-fidelity PCR products. P5 and P6 primers were used and the total volume of the reaction mixture was 50 µl. In summary, 100 ng of cDNA template and 10 pmol of P5 and P6 primers were added to Accupower® Pfu PCR PreMix tube.

Table 1. Sequences of primers used for amplification of full-length and fragment of CK19.

Primer	Nucleotide Sequence	Product
P1: GAPDH-F	5'-GGTCGGAGTCAACGGATTTG-3'	318bp
P2: GAPDH-R	5'-ATGAGCCCCAGCCTTCTCCAT-3'	
P3:Fragment F	5'-ATGAAAGCTGCCTTGGAAGA-3'	136bp
P4:Fragment R	5'-TGATTCTGCCGCTCACTATCAG-3'	
P5: Full-length F	5'-CGGGATCCCCATGACTTCCTAAGCTATCGC-3'	1201bp
P6: Full-length R	5'-GGAATTCTCAGAGGACCTTGGAGGCAG-3'	

F: forward primer R: reverse primer

Amplification was carried out with a Mastercycler personal eppndorf®AG, Hamburg, Germany. PCR reaction was initiated with a 5 min denaturation at 94 °C and terminated with a 15 min extension at 72 °C. The cycling protocol included of denaturation at 94 °C for 30 sec, annealing at 56 °C for 40 sec and extension at 72 °C for 1 min, repeated for 30 cycles.

Tumor marker detection was evaluated by both one and two step RT-PCR amplification to confirm the real polymerization of mRNA in blood and tissue of subjects.

Samples were also subjected to amplification for full-length CK19 marker, with the same primers. This PCR was done to avoid positive RT-PCR due to pseudogenes amplification.

Sequencing

The RT-PCR products from two-step assay were sequenced by Bioneer Inc. The nucleotide sequence of RT-PCR products was confirmed by sequencing and PCR amplification using Pfu polymerase (Fermentas) in both rounds was done to secure high-fidelity PCR products.

Statistical analysis

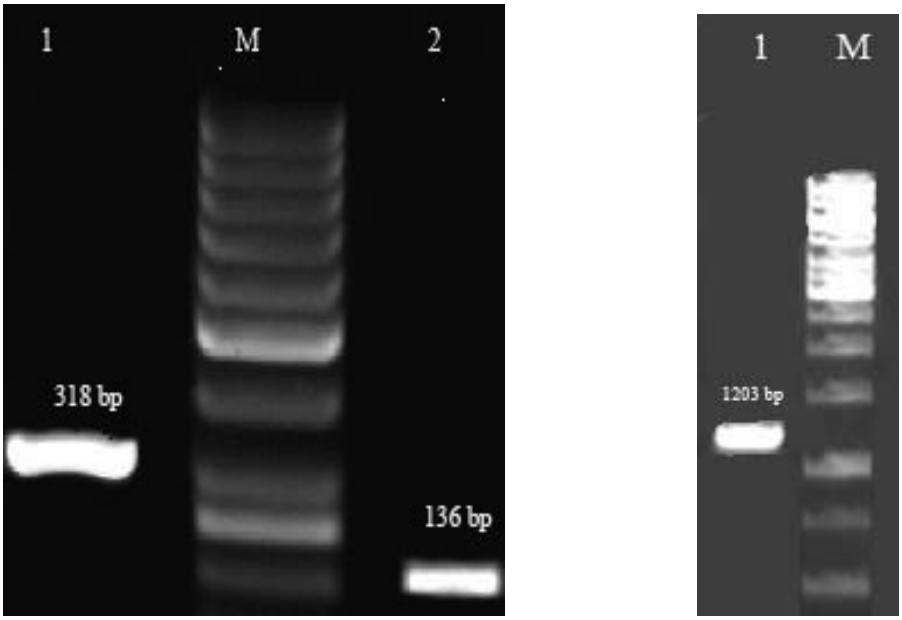
The relationship between positive results of CK19 in patients and normal subjects were subjected to ANOVA and student's t-test for statistical analysis and a *P*-value less than 0.05 was considered significant.

Results

CYFRA 21-1 fragments of CK19 protein was measured in patient and normal sera. The results for CYFRA 21-1 fragment indicated that there was no significant difference between CYFRA 21-1 fragments of CK19 in sera of normal and patients (*P*=0.738) (Data not shown).

At first, total RNA extraction of subjects was used for GAPDH RT-PCR by P1-P2 primers (Figure 1a). Thereafter, the positive subjects are used for specific RT-PCR by specific primers for CK19 gene. CK19 fragment was detected in blood of patients and normal subjects by using P3 and P4 primers (Figure 1a). Expression of CK19 fragment in patients was statistically significant (*P*=0.019). The results are shown in table 2.

On the other hand, different RNA expression of CK19 in blood and tissue of patients were observed. Expression of CK19 from blood and tissue was compared. The results are summarized in table 3.



a) **b)**

Figure 1. RT-PCR products

a) Lane 1= GAPDH band, M= DNA molecular size marker (50bp marker), Lane 2= CK19 fragment

b) Lane 1= CK19 full-length, M= DNA molecular size marker (1kb marker)

Table 2. Detection of CK19 fragment in blood of patients and normal subjects.

	Blood samples	CK19 Fragment
	Positive	Negative
Normal	27	5
Patient	32	0

Chi-square=5.424, *P*-value = 0.0199 (significant)

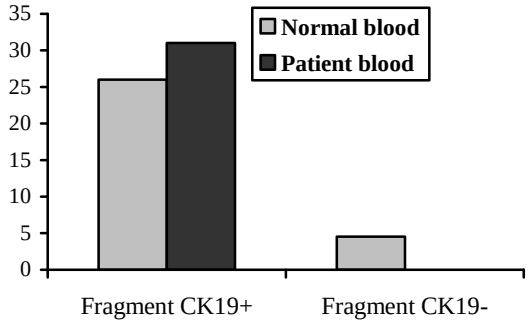


Figure 2. Detection of CK19 fragment is shown in patients and normal blood.

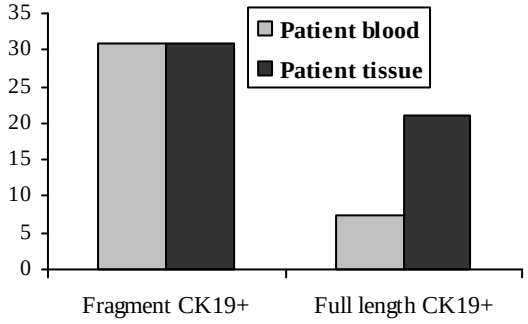


Figure 3. Full length and fragment of CK19 detection in blood and tissue of patients.

Table 3. The comparison of CK19 marker in blood and tissue of patients.

Patient samples	Fragment CK19 positive	Full-Length CK19 positive
Blood	32	9
Tissue	32	22

Chi-square=3.743, *P*- value=0.053 (non-significant)

There was no statistically significant difference between CK19 expression in blood and tissue of breast cancer patients (*P*=0.053).

In figure 3, CK19 small and long full-length fragment expression in blood and tissue subjects is demonstrated.

The same expression of CK19 fragment was shown in blood and tissue of patients but CK19 full-length expression in tissue was higher than blood. This difference was not statistically significant; therefore the expression of full-length CK19 in tissue is the same as blood. There was no expression for full-length CK19 in normal patients.

The sequences of expressed CK19 gene in blood and tissue samples were also compared. Alignment was done by multiple alignments using vector NTi-11.5 Advance® software.

The CK19 gene was sequenced from blood of five patients; one tissue was also sequenced. The schematic of the sequenced mutated genes, aligned with CK19 gene is represented in figure 4.

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          1                                                  50
CK19      (1) ATGACTTCCTACAGCTATCGCCAGTCGTCGGCCACGTCGTCCTTCGGAGG
Blood1    (1) ATGACTTCCTACAGCTATCGCCAGTCGTAGGCCAAGTTGTCCTTCTGGGG
Blood2    (1) ATGACTTCCTACAGCTATCGCCAGTCGTAGGCCAAGTAGTCCTTCTGGGG
Blood3    (1) ATGACTTCCGACAGCTATCGCCAGTCGTAGGCCAAGTAGTCCTTCTGGGG
Blood4    (1) ATGACTTCCGACAGCTATCGCCAGTCGTAGGCCAAGTAGTCCTTCTGGGG
blood5    (1) ATGACTTCCTACAGCTATCGCCAGTCGTAGGCCACCTCGTCCTTCGGAGG
Tissue    (1) ATGACTTCCTACAGCTATCGCCAGTCGTAGGCCAAGTAGTCCTTCTGGGG
Consensus (1) ATGACTTCCTACAGCTATCGCCAGTCGTAGGCCAAGTAGTCCTTCTGGGG
             51                                                 100
CK19      (51) CCTGGGCGGCGGCTCCGTGCGTTTTGGGCCGGGGGTCGCCTTTCGCGCGC
Blood1    (51) CCTGGGTGGTGGCTCCGTGAGTTTTGTGGCAGAGGTTGCCTTTCGCGCGC
Blood2    (51) CCTGGGTGGTGGCTCCATGAGTTTTGTGGCAGAGGTTGCCTTTCGCGCGC
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  Blood3   (51) CCTGGGTGGTGGCTCCGTGAGTTTTGTGGCAGAGGTTGCCTTTCGCGCGC
  Blood4   (51) CCTGGGTGGTGGCTCCGTGAGTTTTGTGGCAGAGGTTGCCTTTCGCGCGC
  blood5   (51) CCTGGGCGGCGGCTCCATGCGTTTTGGGGCAGGGGTCGCCTTTCGCGCGC
  Tissue   (51) CCTGGGTGGTGGCTCCGTGAGTTTTGTGGCAGAGGTTGCCTTTCGCGCGC
Consensus  (51) CCTGGGTGGTGGCTCCGTGAGTTTTGTGGCAGAGGTTGCCTTTCGCGCGC
           101                                 150
   CK19  (101) CCAGCATTCACGGGGGCTCCGGCGGCCGCGGCGTATCCGTGTCCTCCGCC
  Blood1 (101) TCAGCATGCACTGGGCCTCTGGAGGCTGCGGCGTGTCCGTGTCCTCCGCC
  Blood2 (101) TCAGCATANNCTGGGCCTCTGGAGGCTGCGGCGTGTCCGTGTCCTCCGCC
  Blood3 (101) TCAGCATGCACTGGGCCTCTGGAGGCTGCGGCGTGTCCGTGTCCTCCGCC
  Blood4 (101) TCAGCATGCACTGGGCCTCTGGAGGCTGCGGCGTGTCCGTGTCCTCCGCC
  blood5 (101) CCAGCATTCACCGGGACTCCGGCGGCCGCGGCGTGTCCGTGTCCTCCGCC
  Tissue (101) TCAGCATGCACTGGGCCTCTGGAGGCTGCGGCGTGTCCGTGTCCTCCGCC
Consensus (101) TCAGCATGCACTGGGCCTCTGGAGGCTGCGGCGTGTCCGTGTCCTCCGCC
           151                                 200
   CK19  (151) CGCTTTGTGTCCTCGTCCTCCTCGGGGGCCTACGGCGGCGGCTACGGCGG
  Blood1 (151) CGCTTCGTGTCT--GTCCTCGTC------CTCCTTGGGGGGCTACGGCGG
  Blood2 (151) CGCTTCGTGTCT--GTCCTCGTC------CTCCTTGGGGGGCTACGGCGG
  Blood3 (151) CGCTTCGTGTCT--GTCCTCGTC------CTCCTTGGGGGGCTACGGCGG
  Blood4 (151) CGCTTCGTGTCT--GTCCTCGTC------CTCCTTGGGGGGCTACGGCGG
  blood5 (151) CGTTTCGTGTCCTCGTCCTCCTCGGTGGCCTACGGCGGGGGCTACGGCGG
  Tissue (151) CGCTTCGTGTCT--GTCCTCGTC------CTCCTTGGGGGGCTACGGCGG
Consensus (151) CGCTTCGTGTCT GTCCTCGTC     CTCCTTGGGGGGCTACGGCGG
           201                                 250
   CK19  (201) CGTCCTGACCGCGTCCGACGGGCTGCTGGCGGGCAACGAGAAGCTAACCA
  Blood1 (193) CGTCTTGGCCGTGTCCTACGGGCTGCTGGCGGGCAACGAGAAGCTCAATA
  Blood2 (193) CGTCTTGGCCGTGTCCTACGGGCTGCTGGCGGGCAACGAGAAGCTCAATA
  Blood3 (193) CGTCTTGGCCGTGTCCTACGGGCTGCTGGCGGGCAACGAGAAGCTCAATA
  Blood4 (193) CGTCTTGGCCGTGTCCTACGGGCTGCTGGCGGGCAACGAGAAGCTCAATA
  blood5 (201) CGTCCTGACCGCGTCCGACGGGCTGCTGGCGGGCAACGAGAAGCTAACCA
  Tissue (193) CGTCTTGGCCGTGTCCTACGGGCTGCTGGCGGGCAACGAGAAGCTCAATA
Consensus (201) CGTCTTGGCCGTGTCCTACGGGCTGCTGGCGGGCAACGAGAAGCTCAATA

           251                                 300
   CK19  (251) TGCAGAACCTCAACGACCGCCTGGCCTCCTACCTGGACAAGGTGCGCGCC
  Blood1 (243) TGCAGAACCTCAGCGACCCTCTGGCCTCCTACCTGGACAAGGTGGGCGCC
  Blood2 (243) TGCAGAACCTCAGCGACCCTCTGGCCTCCTACCTGGACAAGGTGGGCGCC
  Blood3 (243) TGCAGAACCTCAGCGACCCTCTGGCCTCCTACCTGGACAAGGTGGGCGCC
  Blood4 (243) TGCAGAACCTCAGCGACCCTCTGGCCTCCTACCTGGACAAGGTGGGCGCC
  blood5 (251) TGCAGAACCTCAATAACCACCTGACGGCCTGGCTGGACAAGGTGCGCGCC
  Tissue (243) TGCAGAACCTCAGCGACCCTCTGGCCTCCTACCTGGACAAGGTGGGCGCC
Consensus (251) TGCAGAACCTCAGCGACCCTCTGGCCTCCTACCTGGACAAGGTGGGCGCC
           301                                 350
   CK19  (301) CTGGAGGCGGCCAACGGCGAGCTAGAGGTGAAGATCCGCGACTGGTACCA
  Blood1 (293) CTGGAGGCAGCCAACGGCAAACTGGAGGTGAAGATCCGCGACTGGTACCA
  Blood2 (293) CTGGAGGCAGCCAACGGCAAACTGGAGGTGAAGATCCGCGACTGGTACCA
  Blood3 (293) CTGGAGGCAGCCAACGGCAAACTGGAGGTGAAGATCCGCGACTGGTACCA
  Blood4 (293) CTGGAGGCAGCCAACGGCAAACTGGAGGTGAAGATCCGCGACTGGTACCA
  blood5 (301) CTGGAAGAGGTCACCTGGGCGCTGGGGGTGAAGGTCCATGGCTGGTACCA
  Tissue (293) CTGGAGGCAGCCAACGGCAAACTGGAGGTGAAGATCCGCGACTGGTACCA
Consensus (301) CTGGAGGCAGCCAACGGCAAACTGGAGGTGAAGATCCGCGACTGGTACCA
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            351                             400
   CK19   (351) GAAGCAGGGGCCTGGGCCCTCCCGCGACTACAGCCACTACTACACGACCA
  Blood1  (343) GAAGCAGGGGCCCGGGCCCTCCCGTGACTACAGCCACT-CTACAAGACTA
  Blood2  (343) GAAGCAGGGGCCCGGGCCCTCCCGTGACTACAGCCACT-CTACAAGACTA
  Blood3  (343) GAAGCAGGGGCCCGGGCCCTCCCGTGACTACAGCCACT-CTACAAGACTA
  Blood4  (343) GAAGCAGGGGCCCGGGCCCTCCCGTGACTACAGCCACT-CTACAAGACTA
  blood5  (351) GAAGCAGGGGCCTGGGTGATCCTGTGGCTACACCCACTACTTCAAGCCCA
  Tissue  (343) GAAGCAGGGGCCCGGGCCCTCCCGTGACTACAGCCACT-CTACAAGACTA
Consensus (351) GAAGCAGGGGCCCGGGCCCTCCCGTGACTACAGCCACT CTACAAGACTA
            401                             450
   CK19   (401) TCCAGGACCTGCGGGACAAGATTCTTGGTGCCACCATTGAGAACTCCAGG
  Blood1  (392) TCCAGGACCTGCGGTACAAGATTCTTGGTGCCACCATTGAGAACTCCAGG
  Blood2  (392) TCCAGGACCTGCGGTACAAGATTCTTGGTGCCACCATTGAGAACTCCAGG
  Blood3  (392) TCCAGGACCTGCGGTACAAGATTCTTGGTGCCACCATTGAGAACTCCAGG
  Blood4  (392) TCCAGGACCTGCGGTACAAGATTCTTGGTGCCACCATTGAGAACTCCAGG
  blood5  (401) TCAGGGACCTAGGCTGGGTGATGCCTGGAGCCACCATTGAGAACTCCAGG
  Tissue  (392) TCCAGGACCTGCGGTACAAGATTCTTGGTGCCACCATTGAGAACTCCAGG
Consensus (401) TCCAGGACCTGCGGTACAAGATTCTTGGTGCCACCATTGAGAACTCCAGG
            451                             500
   CK19   (451) ATTGTCCTGCAGATCGACAATGCCCGTCTGGCTGCAGATGACTTCCGAAC
  Blood1  (442) ATTGTCCTGGAGATCGACAACGCCCGTCTGGCTGCAGATGACTTCCGAAC
  Blood2  (442) ATTGTCCTGGAGATCGACAACGCCCGTCTGGCTGCAGATGACTTCCGAAC
  Blood3  (442) ATTGTCCTGGAGATCGACAACGCCCGTCTGGCTGCAGATGACCTCTGAAC
  Blood4  (442) ATTGTCCTGGAGATCGACAACGCCCGTCTGGCTGCAGATGACCTCTGAAC
  blood5  (451) ATTGTCCTGCAGATCGACAATGCCCGTCTGCCTGCAGATGACTTCCGAAC
  Tissue  (442) ATTGTCCTGGAGATCGACAACGCCCGTCTGGCTGCAGATGACTTCCGAAC
Consensus (451) ATTGTCCTGGAGATCGACAACGCCCGTCTGGCTGCAGATGACTTCCGAAC
            501                             550
   CK19   (501) CAAGTTTGAGACGGAACAGGCTCTGCGCATGAGCGTGGAGGCCGACATCA
  Blood1  (492) CAAGAGTGAGACGGAGCAGGCTCTGCGCATGAGCGCGGAGGCCGACATCA
  Blood2  (492) CAAGAGTGAGACGGAGCAGGCTCTGCGCATGAGCGCGGAGGCCGACATCA
  Blood3  (492) CAAGAGTGAGACGGAGCAGGCTCTGCGCATGAGCGCGGAGGCCGACATCA
  Blood4  (492) CAAGAGTGAGACGGAGCAGGCTCTGCGCATGAGCGCGGAGGCCGACATCA
  blood5  (501) CAAGATTGAGACGGAACAGGCTCTGCGCATGAGCGTGGAG-CCGACATCA
  Tissue  (492) CAAGAGTGAGACGGAGCAGGCTCTGCGCATGAGCGCGGAGGCCGACATCA
Consensus (501) CAAGAGTGAGACGGAGCAGGCTCTGCGCATGAGCGCGGAGGCCGACATCA

            551                             600

   CK19   (551) ACGGCCTGCGCAGGGTGCTGGATGAGCTGACCCTGGCCAGGACCGACCTG
  Blood1  (542) ACGGCCTGCGCAGGGTGCTGGACGAGCTGACCCTGGCCATTACCGACCTG
  Blood2  (542) NCGGCCTGCGCAGGGTGCTGGACGAGCTGACCCTGGCCATTACCGACCTG
  Blood3  (542) ACGGCCTGCGCAGGGTGCTGGACGAGCTGACCCTGGCCATTACCGACCTG
  Blood4  (542) ACGGCCTGCGCAGGGTGCTGGACGAGCTGACCCTGGCCATTACCGACCTG
  blood5  (550) ACGGCCTGCG---GGTCCTGGCCAGGCTCAGCTCGCCCAGCACCCTGCAC
  Tissue  (542) ACGGCCTGCGCAGGGTGCTGGACGAGCTGACCCTGGCCATTACCGACCTG
Consensus (551) ACGGCCTGCGCAGGGTGCTGGACGAGCTGACCCTGGCCATTACCGACCTG
            601                             650
   CK19   (601) GAGATGCAGATCGAAGGCCTGAAGGAAGAGCTGGCCTACCTGAAGAAGAA
  Blood1  (592) GAGATGCAGATCTAAGGCCTGAAGGAAGAGCTGGCCTACCTGAAGAAGAA
  Blood2  (592) GAGATGCAGATCTAAGGCCTGAAGGAAGAGCTGGCCTACCTGAAGAAGAA
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  Blood3  (592) GAGATGCAGATCTAAGGCCTGAAGGAAGAGCTGGCCTACCTGAAGAAGAA
  Blood4  (592) GAGATGCAGATCTAAGGCCTGAAGGAAGAGCTGGCCTACCTGAAGAAGAA
  blood5  (597) AGAACGTTGATGTG-GATCTCCAGGCTCAGCGGGGCCAGCTCCTCAAACT
  Tissue  (592) GAGATGCAGATCTAAGGCCTGAAGGAAGAGCTGGCCTACCTGAAGAAGAA
Consensus (601) GAGATGCAGATCTAAGGCCTGAAGGAAGAGCTGGCCTACCTGAAGAAGAA
            651                             700
   CK19  (651) CCATGAGGAGGAAATCAGTACGCTGAGGGGCCAAGTGGGAGGCCAGGTCA
  Blood1  (642) CCATGAGAAGGAAATCAGTGGGCTGAGGGGCCAAGTGGGAGGCCAGGTCA
  Blood2  (642) CCATGAGAAGGAAATCAGTGGGCTGAGGGGCCAAGTGGGAGGCCAGGTCA
  Blood3  (642) CCATGAGAAGGAAATCAGTGGGCTGAGGGGCCAAGTGGGAGGCCAGGTCA
  Blood4  (642) CCATGAGAAGGAAATCAGTGGGCTGAGGGGCCAAGTGGGAGGCCAGGTCA
  blood5  (646) TGGTTCAGAAGTCATCTGCAGCCAGATGGGTGCTGTTGGTCATCAGGACA
  Tissue  (642) CCATGAGAAGGAAATCAGTGGGCTGAGGGGCCAAGTGGGAGGCCAGGTCA
Consensus (651) CCATGAGAAGGAAATCAGTGGGCTGAGGGGCCAAGTGGGAGGCCAGGTCA
            701                             750
   CK19  (701) GTGTGGAGGTGGATTCCGCTCCGGGCACCGATCTCGCCAAGATCCTGAGT
  Blood1  (692) GTGGGGAGGTGGATTCGGCTCAGGGCACCTATCTCGCCAAGATCCTGAGT
  Blood2  (692) GTGGGGAGGTGGATTCGGCTCAGGGCACCTATCTCGCCAAGATCCTGAGT
  Blood3  (692) GTGGGGAGGTGGATTCGGCTCAGGGCACCTATCTCGCCAAGATCCTGAGT
  Blood4  (692) GTGGGGAGGTGGATTCGGCTCAGGGCACCTATCTCGCCAAGATCCTGAGT
  blood5  (696) ATCCTGAAGTTCAATGGTGGCAGTGAAATCTTGTCCCATAGGTCCTGATG
  Tissue  (692) GTGGGGAGGTGGATTCGGCTCAGGGCACCTATCTCGCCAAGATCCTGAGT
Consensus (701) GTGGGGAGGTGGATTCGGCTCAGGGCACCTATCTCGCCAAGATCCTGAGT
            751                             800
   CK19  (751) GACATGCGAAGCCAATATGAGGTCATGGCCGAGCAGAACCGGAAGGATGC
  Blood1  (742) TACATGCGAAGCCAATACGAGGTCATGGCGGAGCAGAACTGGAAGGATGC
  Blood2  (742) TACATGCGAAGCCAATACGAGGTCATGGCGGAGCAGAACTGGAAGGATGC
  Blood3  (742) TACATGCGAAGCCAATACGAGGTCATGGCGGAGCAGAACTGGAAGGATGC
  Blood4  (742) TACATGCGAAGCCAATACGAGGTCATGGCGGAGCAGAACTGGAAGGATGC
  blood5  (746) GGCTTGAGTAGTGGGTGTAAGCCCATGGCCGAGCAGAACCGGAAGGATGG
  Tissue  (742) TACATGCGAAGCCAATACGAGGTCATGGCGGAGCAGAACTGGAAGGATGC
Consensus (751) TACATGCGAAGCCAATACGAGGTCATGGCGGAGCAGAACTGGAAGGATGC
            801                             850
   CK19  (801) TGAAGCCTGGTTCACCAGCCGGACTGAAGAATTGAACCGGGAGGTCGCTG
  Blood1  (792) TGAAGCCTGGTTCACCAGCCGGACTGAAGAATTGAACCGGGAGGTCGCTG
  Blood2  (792) TGAAGCCTGGTTCACCAGCCGGACTGAAGAATTGAACCGGGAGGTCGCTG
  Blood3  (792) TGAAGCCTGGTTCACCAGCCGGACTGAAGAATTGAACCGGGAGGTCGCTG
  Blood4  (792) TGAAGCCTGGTTCACCAGCCGGACTGAAGAATTGAACCGGGAGGTCGCTG
  blood5  (796) TACAGCCTGGTTCCCCAGCTCTACAGGTGACCTCTTCCGGGCGTTCACTG
  Tissue  (792) TGAAGCCTGGTTCACCAGCCGGACTGAAGAATTGAACCGGGAGGTCGCTG
Consensus (801) TGAAGCCTGGTTCACCAGCCGGACTGAAGAATTGAACCGGGAGGTCGCTG
            851                             900
   CK19  (851) GCCACACGGAGCAGCTCCAGATGAGCAGGTCCGAGGTTACTGACCTGCGG
  Blood1  (842) GCCACACAGATCAGCTCCAGATGAGCCGGTCCAAGGTCGCTGACCTGCGG
  Blood2  (842) GCCACACAGATCAGCTCCAGATGAGCCGGTCCAAGGTCGCTGACCTGCGG
  Blood3  (842) GCCACACAGATCAGCTCCAGATGAGCCGGTCCAAGGTCGCTGACCTGCGG
  Blood4  (842) GCCACACAGATCAGCTCCAGATGAGCCGGTCCAAGGTCGCTGACCTGCGG
  blood5  (846) GCCAGGCGGAGCAGCTCCAGATGAGCAGGTCCGAGGTTACTGACCTGCGG
  Tissue  (842) GCCACACAGATCAGCTCCAGATGAGCCGGTCCAAGGTCGCTGACCTGCGG
Consensus (851) GCCACACAGATCAGCTCCAGATGAGCCGGTCCAAGGTCGCTGACCTGCGG
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              901                                950
    CK19   (901) CGCACCCTTCAGGGTCTTGAGATTGAGCTGCAGTCACAGCTGAGCATGAA
   Blood1  (892) CGCACCCTCCAGGGTCTTGAG------CTGCAGTCACGGCTGAGCATGAA
   Blood2  (892) CGCACCCTCCAGGGTCTTGAG------CTGCAGTCACGGCTGAGCATGAA
   Blood3  (892) CGCACCCTCCAGGGTCTTGAG------CTGCAGTCACGGCTGAGCATGAA
   Blood4  (892) CGCACCCTCCAGGGTCTTGAG------CTGCAGTCACGGCTGAGCATGAA
   blood5  (896) C-CTACCAGCAGCCCATTGGACCTGGCCAGCATTCACAGCTGCCACTGTA
   Tissue  (892) CGCACCCTCCAGGGTCTTGAG------CTGCAGTCACGGCTGAGCATGAA
Consensus  (901) CGCACCCTCCAGGGTCTTGAG      CTGCAGTCACGGCTGAGCATGAA
              951                               1000
    CK19   (951) AGCTGCCTTGGAAGACACACTGGCAGAAACGGAGGCGCGCTTTGGAGCCC
   Blood1  (936) AGCCGCCTTGGAAGCCACACTGGCAGAAACGGAGGCGCGCTTTGGAGTCC
   Blood2  (936) AGCCGCCTTGGAAGNNNCACTGGCAGAAACGGAGGCGCGCTTTGGAGTCC
   Blood3  (936) AGCCGCCTTGGAAGCCACACTGGCAGAAACGGAGGCGCGCTTTGGAGTCC
   Blood4  (936) AGCCGCCTTGGAAGCCACACTGGCAGAAACGGAGGCGCGCTTTGGAGTCC
   blood5  (945) AGCTGCCTTGGAAGACACACTGGCAGAAAAGGATGAGGACAATGGAAACC
   Tissue  (936) AGCCGCCTTGGAAGCCACACTGGCAGAAACGGAGGCGCGCTTTGGAGTCC
Consensus  (951) AGCCGCCTTGGAAGCCACACTGGCAGAAACGGAGGCGCGCTTTGGAGTCC
              1001                              1050
    CK19  (1001) AGCTGGCGCATATCCAGGCGCTGATCAGCGGTATTGAAGCCCAGCTGGGC
   Blood1  (986) AGCTGGCGCAGATCCAGCCGCTGATCAACTGTATTGAAGCCCAGCTGGGC
   Blood2  (986) AGCTGGCGCAGATCCAGCCGCTGATCAACTGTATTGAAGCCCAGCTGGGC
   Blood3  (986) AACTGGCGCAGATCCAGCCGCTGATCAACTGTATTGAAGCCCAGCTGGGC
   Blood4  (986) AACTGGCGCAGATCCAGCCGCTGATCAACTGTATTGAAGCCCAGCTGGGC
   blood5  (995) AACAGAGGCATATACTGACACTGCTGTGGCCCATGGAAGTCCTGGTGGAT
   Tissue  (986) AGCTGGCGCAGATCCAGCCGCTGATCAACTGTATTGAAGCCCAGCTGGGC
Consensus  (1001) AGCTGGCGCAGATCCAGCCGCTGATCAACTGTATTGAAGCCCAGCTGGGC
              1051                              1100
    CK19  (1051) GATGTGCGAGCTGATAGTGAGCGGCAGAATCAGGAGTACCAGCGGCTCAT
   Blood1  (1036) GATGTGCGAGCTGATAGTGAGCGGCAGAATCAGGATTAACAGCAGTTCAT
   Blood2  (1036) GANGGGGAGGGGCATGGTTAGGGGCAGAATCAGGATAAACAGCAGTTGAT
   Blood3  (1036) GATGTGCGAGCTGATAGTGAGCGGCAGAATCAGGATTAACAGCAGTTCAT
   Blood4  (1036) GATGTGCGAGCTGATAGTGAGCGGCAGAATCAGGATTAACAGCAGTTCAT
   blood5  (1045) GCTGGGCATGCAGAAGGTGTCCCGCTGCCTCAAAATGCATGGAGGCTCAC
   Tissue  (1036) GATGTGCGAGCTGATAGTGAGCGGCAGAATCAGGATTAACAGCA-TTCAT
Consensus  (1051) GATGTGCGAGCTGATAGTGAGCGGCAGAATCAGGATTAACAGCAGTTCAT
              1101                              1150
    CK19  (1101) GGACATCAAGTCGCGGCTGGAGCAGGAGATTGCCACCTACCGCAGCCTGC
   Blood1  (1086) GGACATCAAGTCGCGGCTGGAGCAGGAGATCTCCACCTACCGCAGCCTGC
   Blood2  (1086) GGACATCAAGTGGCAGCTAGAGCAGAAGATCTCCANCGAGCGCAGCCTGC
   Blood3  (1086) GGACATCAAGTCGCGGCTGGAGCAGGAGATCTCCACCTACCGCAGCCTGC
   Blood4  (1086) GGACATCAAGTCGCGGCTGGAGCAGGAGATCTCCACCTACCGCAGCCTGC
  blood5  (1095) TGCCATCAAGTCGCGGCTGGAGCAGGCGGTCTGTGACTGGCGATAGCTGT
   Tissue  (1085) GGACATCAAGTCGCGGCTGGAGCAGGAGATCTCCACCTACCGCAGCCTGC
Consensus  (1101) GGACATCAAGTCGCGGCTGGAGCAGGAGATCTCCACCTACCGCAGCCTGC
              1151                              1200
    CK19  (1151) TCGAGGGACAGGAAGATCACTACAACAATTTGTCTGCCTCCAAGGTCCTC
   Blood1  (1136) TCGAGGGCCAGAAAGATCACTACAACAACCTGCCTGCCTCCAAGGTGCTC
   Blood2  (1136) AAGACGGCCAGAAAGATCACTACAACAACCTGTCCGCCTCCAAGGTCCTC
   Blood3  (1136) TCGAGGGCCAGAAAGATCACTACAACAACCTGTCTGCCTCCAAGGTCCTC
   Blood4  (1136) TCGAGGGCCAGAAAGATCACTACAACAACCTGTCTGCCTCCAAGGTCCTC
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blood5  (1145) AGGAAGTACATGGGGATCCCGAATCGGACCGGGCCGACTGCAGAGGCCTC
Tissue  (1135) TCGAGGGCCAGAAAGATCACTACAACAACCTGTCCGCCTCCAAGGTCCTC
Consensus  (1151) TCGAGGGCCAGAAAGATCACTACAACAACCTGTCTGCCTCCAAGGTCCTC

          1201
CK19  (1201) TGA
Blood1  (1186) TGA
Blood2  (1186) TGA
Blood3  (1186) TGA
Blood4  (1186) TGA
blood5  (1195) TGA
Tissue  (1185) TGA
Consensus  (1201) TGA
```

Figure 4. Schematic representation of newly reported non-coding *CK19* genes alignment (CK19-NM-002276.4). Residues in an alignment are colored according to vector-NTI-Advance-11 users Manual.

Blue on cyan: consensus residue derived from a block of similar residues at a given position

Yellow: consensus residue derived from a completely conserved residue at a given position

Furthermore, existence of two stop codons very nearly after the first codon (ATG) leading to no cytokeratin expression (Figure 5). Two nucleotide substitution, C A was observed. The existence of stop codons in the first part of CK19 RNA results non-coding CK19 RNA.

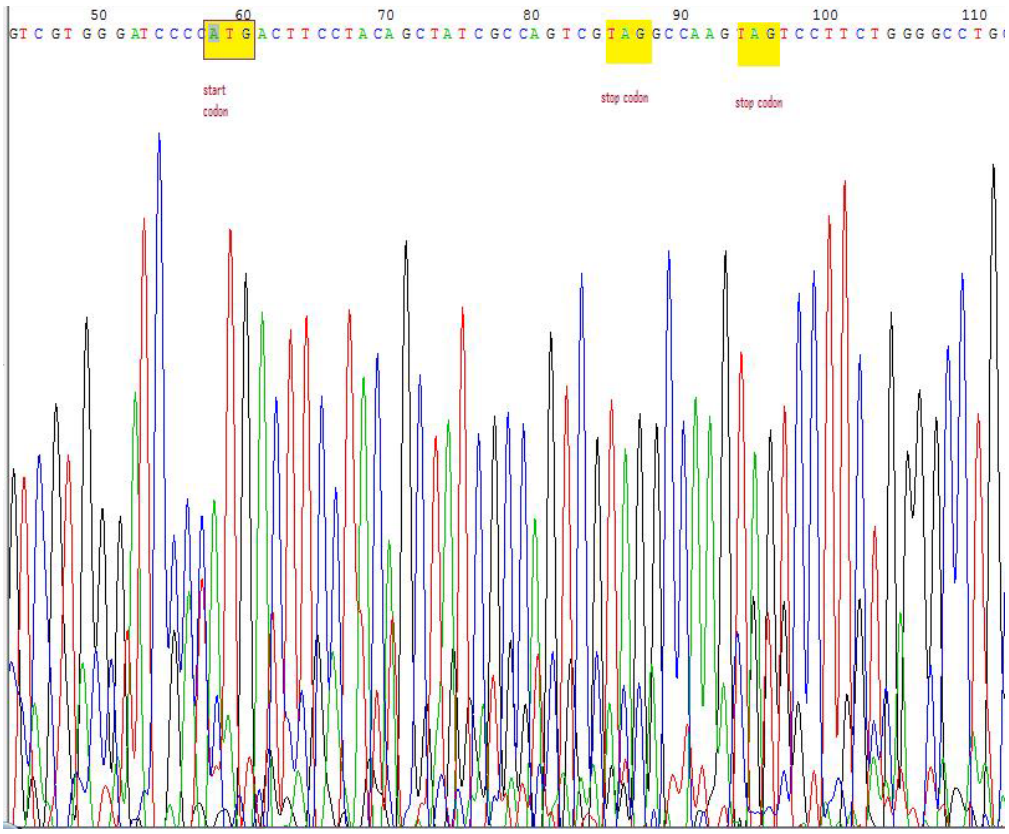

Figure 5. Chromatogram of non-coding CK19.

Discussion

Cytokeratins, also known as keratins, make the cytoplasmic intermediate filaments. These filaments are existed in normal and malignant epithelial cells. There are more than twenty different cytokeratins in human epithelia. Cytokeratin 19 is type I of human cytokeratins. CK19 is known as a tumor marker and its mRNA indicates the presence of circulating tumor cells (CTCs) in some cancer especially breast cancer (5,14). Detectable CTCs, which are used as a novel prognostic marker, have been found by RT-PCR for CK19 in 20-40% of early breast cancer patients (5,15). CK19 has been diagnosed in tumor cells and bone marrow samples from breast cancer patients using immunoassay (12). CYFRA 21-1 protein, a known CK19 fragment protein, has also been used as a tumor marker in some cancers for example lung and breast cancer (16). It is supposed that full-length mRNA can be translated to protein (17).

The aim of this study was establishment of full-length CK19 mRNA as a tumor marker in blood and tissue of breast cancer. In this regard, patients were assessed for CYFRA 21-1 protein detection in the sera. The mechanism of the release of CK19 protein fragment (CYFRA 21-1) is a proteolytic process. During apoptosis, CK19 protein is cleaved by caspase 3 and produces soluble CYFRA21-1 fragment. This fragment could be measured by immunoassay method using two monoclonal antibodies (12, 18). Statistical analysis of our data indicates that there is no significant difference between CYFRA 21-1 fragment in patient and healthy serum samples. This means that CYFRA 21-1 detection is not important alone, in breast cancer. Probably, it is important when other markers and clinical history are evaluated (19). On the other hand, statistical analysis of RT-PCR results indicates that there are significant difference for CK19 fragment in blood of breast cancer patients and healthy subjects. However, other studies showed variable results for CK19 RT-PCR assays. In this study, there is no correlation between the level of CK19 mRNA fragment and CYFRA21-1 protein. That is similar to the results of Marrakchi *et al.* (20) because of post-transcriptional and post-translational mechanisms, mRNA level dose not always show the exact level of protein (21). On the contrary, Fujita and colleagues (22) found that there are very close relationship between the rate of CK19 mRNA and the amount of CYFRA 21-1 protein in lung cancer. However, at mRNA level, there is an incomplete mRNA unable to produce CYFRA21-1 protein. Incomplete RNA may interfere with RT-PCR results; underestimate the level of circulating tumor cells.

In addition, it has been shown that some mutations in promoter region of CK19 gene can cause down regulation of mRNA CK19 expression (17,22). Multiple alignment analysis of sequences shows there are mutations in full-length CK19 in comparison with mRNA CK19 gene (Homo sapiens keratin 19 [KRT19], NCBI Reference Sequence: NM-002276.4). It has also been observed that there are mutations in CK19 gene in lung cancer (17). It is likely that some mutations in CK19 gene at genomic level, leading to the production of defective keratin filaments (17). These sequence analysis also indicates that obtained sequences in this research are very identical to sequences of known CK19 pseudogenes (CK19a: accession No. M33101, CK19b: accession No. U85961). The presence of pseudogene has been reported previously by Ruud *et al.*; Submitted CK19 pseudogens have high homology with CK19 mRNA and therefore it is supposed that they may interfere with RT-PCR leading to cause false-positive results (23). It is speculated that the source of these pseudogenes may be DNA contamination. The false positive signals had been remained even after using DNase in RNA extraction (23,24).

Moreover, some part of CK19 mutated gene in this research, has 88% identity with a known microRNA (Homo sapiens microRNA-492 (MIR492), NCBI Reference Sequence: NR-030171.1). It has been demonstrated that many of pseudogenes are transcribed to RNA as processed pseudogenes. The syntheses of complete genes could regulate by these incomplete RNAs. On the other hand, pseudogenes may also adjust tumor suppressors and oncogenes (25). Recently, it has been reported that microRNA-492 can derive from CK19 gene and co-express with CK19 in metastatic hepatoblastoma tumor (26). Moreover, exRNAs is also identified as free nucleic acids, released spontaneously from tumor cells and the exact mechanism remains to be fully elucidated (27). It has also been proved that CK19 positive cells may have stem cell-like characteristics and CK19 is categorized as a putative stem cell marker (12,28,29). Stem cells have been considered as a model to study the role of microRNA (30). More additional tests are needed to prove the existence of CK19 microRNAs in breast cancer and to explain the mechanism of their action from converting healthy cells to tumor cells. In conclusion, CK19 biomarker increases significantly in breast cancer patients. This marker is detectable by RT-PCR assay in peripheral blood or tissue samples. Also, there is not any correlation

between CYFRA21-1 protein and CK19 mRNA in women with breast cancer. The presence of newly non-coding CK19 is reported for the first time in this study. It might play a regulatory role in CK19 expression of the complete gene. However, it has already been suggested that the expressed processed pseudogenes could regulate coding gene expression as a non-coding function for mRNAs (31). Finally, regulation role of non-coding mRNAs in tumor biology is not clarified yet and needs to be more elucidated.

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