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Gene expression of *cytokeratin* 19 and its molecular detection in human breast cancer cell lines

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ABSTRACT

Cytokeratins have been identified as useful tools in oncology diagnostics. In this study, *cytokeratin19* (*CK19*) expression was studied in three human breast cancer cell lines, SKBR3, BT549, and BT474 using RT-PCR. *CK19* was expressed in tumor cell of different origin, showing higher expression in invasive breast cancer with ER^+ (BT474) than invasive breast cancer with ER^- (BT549) and breast adenocarcinoma with ER^- (SKBR3). Two primer sets were used to evaluate *CK19* expression. Primer set I (hCK19/1) and primer set II (hCK19/2) were used to amplify the *CK19* human gene at a 215 bp and 384 bp, respectively, whereas PBMC and RAW264.7 (mouse macrophage) no detectable PCR products were obtained. The sensitivity for detection was determined by two methods, i.e., cDNA dilution (the dilution of cDNA from RNA of breast cancer cells) and cell dilution (the dilution of breast cancer cells in PBMC). hCK19/2 was more sensitive than hCK19/1. In cDNA dilution, the lower limits of primer set II for detection were 400, 40 and 40 cells for SKBR3, BT549 and BT474 cells, respectively. While in cell dilution all of the 3 breast cancer cells could be detected at 1 cancer cell in 10^4, 10^6 and 10^5 PBMC, respectively. The data supported the possibility that *CK19* could be detected and be the marker for breast cancer in patient blood.

1. Introduction

Identified markers that have the potential to predict tumor behavior is important in cancer diagnosis because of the variability in clinical disease progression. RT-PCR is an approach used to detect circulating tumor cells [1]. PCR amplification for tumor-specific DNA sequence abnormalities has been generally used in hematological malignancies and colorectal cancer. The main disadvantage of PCR assay for DNA sequence abnormalities in tumor is that it may detect not only the DNA of viable tumor cells, but also the DNA fragments of non-viable carcinoma cells [2]. For breast cancer, no similar specific DNA abnormality is known. Therefore, the detection of circulating breast cancer cells has been focused on tissue specific mRNAs, since the normal blood cells do not express these genes. This method has been proven to be more sensitive than immunohistochemistry. The detection of RNA transcripts using reverse-transcriptase polymerase chain reaction (RT-PCR) has proven to be fast, sensitive, cost effective and semi-quantitative assay for gene expression [3].

The known human *cytokeratin* gene family, *cytokeratin19* (*CK19*), a cytoskeletal component present in normal and cancerous epithelial cells, has been extensively used for the detection of breast cancer cells in mesenchymal tissues and seems to be the most sensitive and reliable tumor marker in both patients with early-stage and metastatic breast cancer [4–7]. Interestingly, its overexpression in carcinoma cells made this gene as a potential marker for the early detection of breast cancer. *CK19*, at molecular weight of 40 kDa, is the specific cytoskeletal structure of simple epithelia. *CK19* was first detected in the squamous carcinoma cell line and was then identified in meso-epithelial cells and non-keratinized epithelial cells. Normally, *CK19* is not expressed in keratinized stratified squamous epithelial tissue, such as in the epidermis, gingiva and hard palate. However, *CK19* is expressed in the basal cell layer of non-keratinized oral epithelial tissue [8]. The skeleton represents the most frequent site of metastases and the first metastatic site to be detected in breast cancer. These occur in more than 80% of the patients diagnosed with metastatic breast cancer [9]. *CK19* has been used as a marker of circulating epithelial cells to diagnose cancer metastasis since it is probably one of the most suitable

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markers for cancer. Expression of *CK19* was uniform in both primary and metastatic breast carcinomas [10], adenocarcinomas of the endometrium and ovary [11] and squamous cell carcinomas of the cervix and vagina [12,13]. One of the fragments of *CK19*, *CYFRA 21-1*, a degraded product of *CK19*, is soluble in serum and can be detected by 2 kinds of monoclonal antibodies [14]. CYFRA 21-1 has been introduced as a tumor marker for primary lung cancer and has been proven suitable for the diagnosis of squamous cell carcinoma of the lung [15].

In addition, ovarian cancer cell lines: CAOV3, OVCAR3, SKOV3 and could be detected *CK19* mRNA expression [16]. In breast cancer, several workers agreed that the detection of *CK19* mRNA by RT-PCR is a useful test in order to find a few disseminated tumor cells in the bone marrow of patient with breast cancer [17]. The developed RT-PCR design the specific primers for reduces the risk of false positives from amplification of any contaminating genomic DNA, since the intron-containing genomic DNA sequence would be amplified in different product sizes for easy to observe by agarose gel electrophoresis. Moongkarndi et al. have reported the sensitive detection of occult carcinoma in bone marrow and peripheral blood of patients with breast cancer using L19 and *CK19* gene by RT-PCR [18]. In this study, *CK19* expression was studied in three human breast cancer cell lines, SKBR3, BT549, and BT474 using RT-PCR. Two primer sets were used to evaluate *CK19* expression.

2. Materials and methods

2.1. Nucleic acid extraction

All carcinoma cells, i.e., human breast cancer cell lines (SKBR3, BT549 and BT474), and mouse macrophage cell line (RAW264.7) as negative control, were grown into log phase in humidified atmosphere of 5% CO_2 at 37 °C. Cells were trypsinized and adjusted into total 10^6 cells in 1 ml. In the other hand, PBMC from normal population donors were obtained and also adjusted into total 10^6 cells in 1 ml. Cells were brought for genomic DNA isolation by phenol–chloroform method [19]. For quantification, the DNA was diluted in TE buffer pH 8.0 and measured the absorbance at 260 and 280 nm by Lambda 35 spectrophotometer of Perkin Elmer (Waltham, MA, USA).

With slightly modification from Donald MC, 1999 [20], total cellular RNA from cell line and PBMC were extracted with Guanidinium phenol–chloroform method. Briefly, the cells grown in monolayer cultures were washed once with ice cold PBS pH 7.4 and removed from plate by cell scraper. The suspended cells were transferred into 15 ml sterile tube and then centrifuged at 500 × *g* for 5 min. The cell pellet was resuspended with 1 ml of denaturing solution and homogenized by vortex mixing. For PBMC, the suspension cells were centrifuged at 500 × *g* for 5 min to obtain the cell pellet and treated the same way as tumor cells. After homogenization, 0.1 ml of 2 M sodium acetate (pH 4.0), 1 ml of saturated phenol (pH 5.2) and 0.2 ml of chloroform:isoamyl alcohol mixture (49:1 v/v) were added subsequently. The mixture was incubated on ice for 15 min and centrifuged at 10,000 rpm by Mikro 22R centrifuge of Hettich (Tuttlingen, Germany) for 20 min at 4 °C. The aqueous phase was collected into a fresh tube and then precipitated with isopropanol to obtain RNA. The RNA pellet was dissolved in DEPC-treated water. The extracted RNA was measured the absorbance at 260 and 280 nm by Lambda 35 spectrophotometer of Perkin Elmer and kept at −80 °C until needed.

2.2. Primer design

The sequences of human *CK19* gene and β_2-microglobulin used in this study were retrieved from the National Center for Biomedical Investigation (NCBI) database with the following GenBank accession no.: human *CK19* gene (AF202321), and human β_2-microglobulin gene (DQ217933) were used. All primers were designed manually except pairs of primers hCK19/1 (F/R) and hβ_2m (F/R) were followed by Moongkarndi (1998) and Krafft (1997) [18,21]. The primers were analyzed by using free accessed websites; Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), Oligoanalyzer 3.0 (http://www.idtdna.com/analyzer/Applications/Oligoanalyzer) and In-silico simulation of molecular biology website (http://genome.ucsc.com/PCR).

For gene amplification, the primers were designed to extend overlapping at least 1 intron in order to distinguishing between DNA and RNA template that may cause false positive. Moreover, the primers should not be specific with pseudogene position to avoid false positive detection [3]. In human *CK19* gene, the primer was designed to extend overlapping between exon 3 to exon 5. The details of nucleotide primers were shown in Table 1.

2.3. PCR and RT-PCR amplification

2.3.1. cDNA synthesis (reverse transcription)

Total RNA (2 μg) was digested with DNase I (BioBasic Inc., Amherst, NY, USA) and the cDNA was synthesized by reverse transcription reaction (RT). The cDNA was synthesized in a mixture containing total RNA: primer, 5 μl of 5X RT buffer, 1.2 μl dNTP, 0.5 μl of 40 U/μl RNasin, 1 μl of 1 μl/reaction INPROM II RT (Promega Corporation, Fitchburg, WI, USA). The mixture was incubated at 25 °C for 30 min, 42 °C for 60 min and 95 °C for 5 min using Biometra's T-Personal Thermal Cycler (Göttingen, Germany), then chilled immediately on ice for PCR analysis or stored at −20 °C until subsequent PCR.

2.3.2. DNA amplification (polymerase chain reaction)

For optimization, DNA was used as a template. Briefly, 25 μl of PCR mixtures were amplified for the reaction containing DNA tem-

Table 1
Nucleotide sequences of all primers.

Primers code	Sequences (5′–3′)	Gene and location	Amplification size (bp)	
			DNA	RNA
PM-UA1	F: GCG GGA CAA GAT TCT TGG TG R: CTT CAG GCC TTC GAT CTG CAT	hCK19: 546–565 hCK19: 759–739	215	215
PM-UA5	F: TTT GAG ACG GAA CAG GCT CT R: TAA CCT CGG ACC TGC TCA TC	hCK19: 640–660 hCK19:1024–1044	797	385
PM-UA4	F: CTT GTC TTT CAG CAA GGA CTG G R: CCT CCA TGA TGC TGC TTA CAT GTC	hβ_2M: 279–300 hβ_2M: 435–412	784	158
PM-UA6	F: TTG AGA CAG AAC ACG CCT TG R: GGA TCT TGG CTA GGT CGA CA	mCK19: 575–595 mCK19: 794–814	607	240
PM-UA7	F: CTG ACC GGC CTG TAT GCT AT R: TTT CCC GTT CTT CAG CAT TT	mβ_2M: 97–117 mβ_2M: 226–246	3218	150

h: human, m: mouse.