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Original Article

Identification of Differentially Expressed Genes Using Annealing Control Primer-based GeneFishing in Human Squamous Cell Cervical Carcinoma

Y.-W. Choi*, Y.-W. Kim†, S.-M. Bae†, S.-Y. Kwak†, H.-J. Chun‡, S. Y. Tong§, H. N. Lee§, J. C. Shin§, K. T. Kim‖, Y. J. Kim‖, W.-S. Ahn§

**Department of Anesthesiology, Catholic University of Korea, Seoul, Republic of Korea; †Catholic Research Institutes of Medical Science, Catholic University of Korea, Seoul, Republic of Korea; ‡Department of Biomedical Science, Catholic University of Korea, Seoul, Republic of Korea; §Department of Obstetrics and Gynecology, College of Medicine, Catholic University of Korea, Seoul, Republic of Korea; ‖Department of Obstetrics and Gynecology, College of Medicine, Hanyang University, Seoul, Republic of Korea*

ABSTRACT:

Aims: **To compare different gene expression patterns between squamous cell cervical carcinoma (SCC) and normal cervical tissue in Korean women and to identify those genes that are specifically or predominantly expressed in SCC by employing annealing control primer (ACP)-based GeneFishing polymerase chain reaction (PCR).**

Materials and methods: **Cervical cancer specimens were obtained from patients enrolled at the Department of Obstetrics and Gynecology, Kang Nam St. Mary's Hospital, Catholic University of Korea. We used a common reference that was mixed with an equal amount of RNA extracted from patients without cervical cancer. The profiles of expressed genes were compared between the SCC and normal cervix identified using GeneFishing differentially expressed gene kits, screened by a BLAST search, and confirmed by semi-quantitative reverse transcription-PCR (RT-PCR).**

Results: **Almost 100 differentially expressed genes were identified in the control and SCC samples. Using 60 arbitrary ACPs, 50 differentially expressed genes were identified, and 30 up-regulated and 20 down-regulated expressed genes were sequenced. Among 50 clones selected by ACP-based GeneFishing PCR, six genes with different expression patterns were determined and confirmed by semi-quantitative RT-PCR. The functional roles of two up-regulated genes, fibrillarin and calgranulin A, and one down-regulated gene, clusterin, were previously identified. However, the functional roles of two up-regulated genes and one down-regulated gene were not identified.**

Conclusion: **We identified distinctive gene expression profiles in Korean women with SCC using ACP-based GeneFishing PCR. Choi, Y.-W. *et al*. (2007). *Clinical Oncology* 19, 308–318**

Key words: Annealing control primer, differentially expressed gene, GeneFishing, squamous cell cervical carcinoma

Introduction

Cervical cancer is the most common cancer in Korean women and its prevalence was reported to be 15.1 of 100 000 women in 1999–2002 [1]. Cervical cancer is caused mainly by persistent infection with a high-risk group of human papilloma viruses (HPV) (notably HPV 16 and 18) that has been detected in different grades of cervical intraepithelial neoplasia (CIN) and invasive cancer [1–4]. In addition to HPV infection, two viral oncogenic proteins, E6 and E7, have also been shown to play a critical role in inducing cervical cancer by interacting with p53 and pRb and inactivating these cellular regulatory proteins [5–8].

Squamous cell cervical carcinoma (SCC) of the uterine cervix is an example of a tumour with a clinically important histological variant. SCC accounts for most tumours identified in the uterine cervix [9]. Invasive carcinoma of the cervix develops from well-defined precursor lesions, referred to as CIN, at the squamous–columnar junction. Longitudinal studies have shown that untreated high-grade CIN will develop into invasive carcinoma in 30–70% of cases over a period of 10–12 years; although in about 10% of patients, lesions can progress from *in situ* to invasive in less than a year [10,11]. Among the major factors that influence prognosis in patients with cervical cancer, the stage of the disease, the volume and grade of the tumour, histological type, lymphatic spread and vascular invasion are important [12,13]. Cytogenetic abnormalities and chromosome structural aberrations or allele loss have also been detected in these lesions [14,15].

Serial analysis of gene expression (SAGE) is based on the generation of short (9–10 bp) nucleotide sequences (tags)

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from a unique position within each species of mRNA [16]. This technique has the disadvantages of sampling error, sequencing error, non-uniqueness, and non-randomness of the tagged sequences. Differential display reverse transcription-polymerase chain reaction (RT-PCR) is based on the determination of multiple expression patterns of predetermined sequences. It is a very sensitive technique, but it is not quantitative [17]. Microarray technology is based on the scanning of large numbers of genes rapidly, with the possibility of the detection of many false-positive clones [18]. Therefore, in this study, we used annealing control primer (ACP) technology in order to provide a primer with annealing specificity to the template, and allow only targeted products to be amplified [19].

Materials and Methods

Patient Information

All cancers were staged according to the International Federation of Gynecology and Obstetrics (FIGO) recommendations [20]. The staging evaluation routinely included a complete history and physical examination, complete blood count, pelvic examination, sigmoidoscopy, cystoscopy and intravenous pyelogram. Cervical cancer biopsies were obtained from patients in the Department of Obstetrics and Gynecology according to procedures approved by the Institutional Review Board of the Catholic University of Korea. The disease status was assigned according to FIGO. Briefly, all patients were Korean. Of the 18 patients with cervical cancer, six patients were classified as having stage IA, six patients were classified as having stage IIA, and six patients were classified as having stage IIB. In this study, we used a common reference that was mixed with an equal amount of RNA extracted from patients without cervical cancer.

Samples (50—150 mg) were excised separately from the ectocervix and the endocervix. All normal tissue has been taken from depth of 1—2 mm under the microscope to avoid the underlying stromal tissue contamination. The results were then examined with a microscope. The normal samples were immediately placed in vials containing 2 ml Trizol, stored at 4°C for up to 12 h, and then frozen at −80°C.

GeneFishing Reverse Transcription-Polymerase Chain Reaction

Total RNAs from the cervical tissues of SCC and normal cervix were isolated by gentle homogenisation using Trizol. RT was carried out using GeneFishing differentially expressed gene kits as follows: 3 μg of the extracted RNAs was put into a tube containing RNase-free water and 10 μM dT-ACP1 with a final volume of 9.5 μl using a Perkin Elmer DNA thermal cycler 9600. Equal amounts of RNA were compared to identify differentially expressed bands in the samples. The mixture was incubated at 80°C for 3 min and spun briefly after chilling on ice. Twenty microlitres of reaction solution, consisting of 5× RT buffer, 2 mM dNTP, 40 u/μl RNase inhibitor, and 200 u/μl M-MLV RT, was added to the mixture. The tube was incubated at 42°C for 90 min, heated at 94°C for 2 min, chilled on ice and spun briefly. The synthesised first-strand cDNA was diluted by adding 80 μl of RNase-free water. The cDNA samples were stored at 20°C until use. PCR amplification was conducted using the same GeneFishing differentially expressed gene kits in 50 μl of reaction solution, consisting of 10× buffer without $MgCl_2$, 25 mM $MgCl_2$, 5 μM arbitrary ACPs, 10 μM dT-ACP2, 2 mM dNTP, 2.5 U Taq DNA polymerase and 50 ng of first-strand cDNA, using a DNA thermal cycler. Each kit had 20 different arbitrary ACP. The DNA fragments produced by PCR were separated by electrophoresis on a 1.5% agarose gel. The bands were photographed using Polaroid film under ultraviolet light after ethidium bromide staining and analysed by densitometry.

Cloning and Transformation

Differentially expressed bands were extracted and cloned into a TOPO TA cloning vector, following the manufacturer's instructions. To confirm the identities of the insert DNA, isolated plasmids were sequenced automatically by an ABI prism 3100 genetic analyser. Sequencing of the DNA was carried out by Macrogen Co. (Seoul, Korea). The DNA sequence of each gene was analysed by searching for similarities using the BLASTX program at the National Center for Biotechnology Information GenBank.

Primer Sequence

The DNA sequences of each gene were compared with and confirmed by comparison with sequences in GenBank. The primer sequence of each gene was designed using the PRIMER 3 program. Synthesis of the primer was carried out by Bioneer (Seoul, Korea). The primer sequences of six confirmed genes are shown in Table 1.

Table 1 — Primer sets used for semi-quantitative reverse transcription-polymerase chain reaction

Clone	Primer sequence	Product size (bp)
C-8	Forward: UP: 5′-CAG TCA GAG TGT TAG CAG CA-3′	
	Reverse: 5′-ACA GTT CGT TTG ATT TCC AC-3′	255
C-16	Forward: 5′-CTT CCA TTC TGG TGG CAA-3′	
	Reverse: 5′-GCA GTT GGC CTT AAT GGA-3′	615
C-19	Forward: 5′-GCC TTG AAC TCT ATC ATC GA-3′	
	Reverse: 5′-GGC CCA GTA ACT CAG CTA-3′	275
C-24	Forward: 5′-GTG GGC AGA AGT ATG GAA TA-3′	
	Reverse: 5′-GAA ATT TTC AGC CTT GCT AA-3′	153
N-4	Forward: 5′-CTG CTC TTT GCC TCC TAT AA-3′	
	Reverse: 5′-CAT AGG CAC TGT GCA AGT TA-3′	259
N-11	Forward: 5′-CGC CCT TCT ACT TCT GGA-3′	
	Reverse: 5′-TGA GCA TCT TCC ACT GGT A-3′	617