Gene Expression Profiling of Esophageal Cancers Using Laser Capture Microdissected Samples

Application

Bioreagents, Gene expression, Lab-on-a-chip

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Abstract

Clinical biopsy samples of esophageal cancer were analyzed with Agilent's DNA microarrays for global gene expression studies. Specific regions of cancer lesion and surrounding normal cells were excised by laser captured microdissection. Global gene expression profiles were successfully obtained with as little as 50 ng of total RNA. Genes involved in the keratin synthesis pathway were significantly affected in these squamous cell carcinomas. Our finding provides a plausible molecular mechanism for the alteration of keratin synthesis observed in over 95% of hereditary squamous cell carcinoma in esophageal cancer.

Introduction

Esophageal cancer is the sixth leading cause of cancer deaths worldwide. Each year, in the United States alone, 13,900 new cases are diagnosed, and 13,000 patients will die from this lethal disease. However, little is known about the pathogenesis or the molecular pathways leading to the development of this cancer. Toward this objective, we carried out global gene expression profiling using microdissected cells of esophageal cancers and those from surrounding normal tissues. Gene expression profiles were compared between cancer and normal cells using microarrays containing *in situ* synthesized oligonucleotide probes representing more than 17,000 well-annotated human genes.

The DNA microarray technology is a powerful tool for monitoring expression levels of thousands of genes simultaneously. However, many DNA microarray researchers often find themselves sample limited, particularly in clinical studies where biopsy tissue are difficult to obtain. Molecular analysis on these biospy samples has been challenging. Recent developments in technologies such as laser capture microdissection (LCM) have now made these experiments much more amenable to scientists around the world. In this application note, we conducted the global gene expression analysis comparing esophageal cancer cells with normal cells.

From the first series of experiments, genes were identified whose expressions are significantly decreased or increased in cancer. Many of those genes are involved in keratin synthesis. This observation provides a plausible molecular basis for the alteration in keratin synthesis observed in a rare autosomal dominant disorder that predisposes patients to the only known familial squamous cell carcinoma [1]. Futhermore, our study demonstrates the feasibility of using a minimum amount of clinical sample to decipher complex disease and disease processes at the molecular level.

Materials and Methods

Biopsy Samples and LCM

Esophageal biopsy tissue samples (each 3 × 2 × 2 mm) were taken from four patients who were diagnosed with different stages of esophageal cancers (stages I, IV, IV, IVB). They were stored at 4 °C in saline solution before being processed, embedded, and stained in thin sections. Cancer cells were laser captured and microdissected from these biopsy samples using Laser Scissors Pro300 (Cell Robotics International, Inc., Japanese Distributor: MEIWA SHOJI Co. LTD.) following the protocol from MEIWA SHOJI (http://www.cellrobotics.com/workstation/lsws.html). LCM-extracted cells from normal esophageal tissues were used as controls.

RNA Isolation

Total RNA was isolated using Qiagen RNeasy. The quality of these RNA samples was examined using RNA 6000 Pico LabChip® Kit on Agilent 2100 Bioanalyzer Nanochip (product numbers 5065-4476, G2940BA) to ensure the integrity of RNA samples before use.

Target Amplification, Labeling, and Hybridization

Cyanine 3- or 5-labeled CTP (10.0 mM) were purchased from Perkin-Elmer/NEN Life Science (product numbers NEL 580, 581). The cRNA targets were amplified and fluorescently labeled from 50 ng, 100 ng, 250 ng of total RNA in each reaction using the Agilent Low RNA Input Fluorescent Linear Amplification Kit (product number 5184-3523) following the protocol described in the user's manual, or two-rounds of linear amplification and labeling protocol developed by Institute of Medical Science, University of Tokyo (unpublished results from Dr. Nakamura's laboratory, Japanese manual: http://www.ims.u-tokyo.ac.jp/nakamura/main/ pdf_dna2.pdf). Yields of cRNA were determined by UV spectrophotometry. For each sample pair, one was labeled with cyanine-3 and the other with cyanine-5. Hybridization was performed following Agilent's oligonucleotide microarray hybridization user's manual and Agilent's *in situ* Hybridization Plus kit (product number 5184-3568). After hybridization overnight, the slides were disassembled in 6 × SSC, 0.005% Triton X-102, washed first with 6 × SSC, 0.005% Triton X-102 for 10 min at room temperature, then with 0.1 × SSC, 0.005% Triton X-102 for 5 min on ice, and dried using a nitrogen-filled air gun.

Expression Analysis

The arrays were scanned using the Agilent dual laser DNA microarray scanner (product number G2565AA) with SureScan technology. The information was then extracted from images by Agilent's Feature Extraction software 6.1.1 (product number G2566AA). The differential gene expression data were analyzed using Resolver® software.

Results

Thin sections of biopsy samples were stained with toluidine blue, as shown in Figure 1, and groups of cancer cells were dissected using LCM technology. Total RNA samples were then isolated from the extracted cells. The RNA samples from four patients were then pooled and labeled with fluorescent dyes of cyanine-3 or cyanine-5 using the Low RNA Input Fluorescent Linear Amplification Kit (5184-3523), Fluorescent Linear Amplification Kit (G2554A), or a two-round amplification protocol from IMS (Institute of Medical Sciences at the University of Tokyo). The labeled cRNA samples were further analyzed by the Agilent 2100 bioanalyzer before the microarray experiments were carried out.

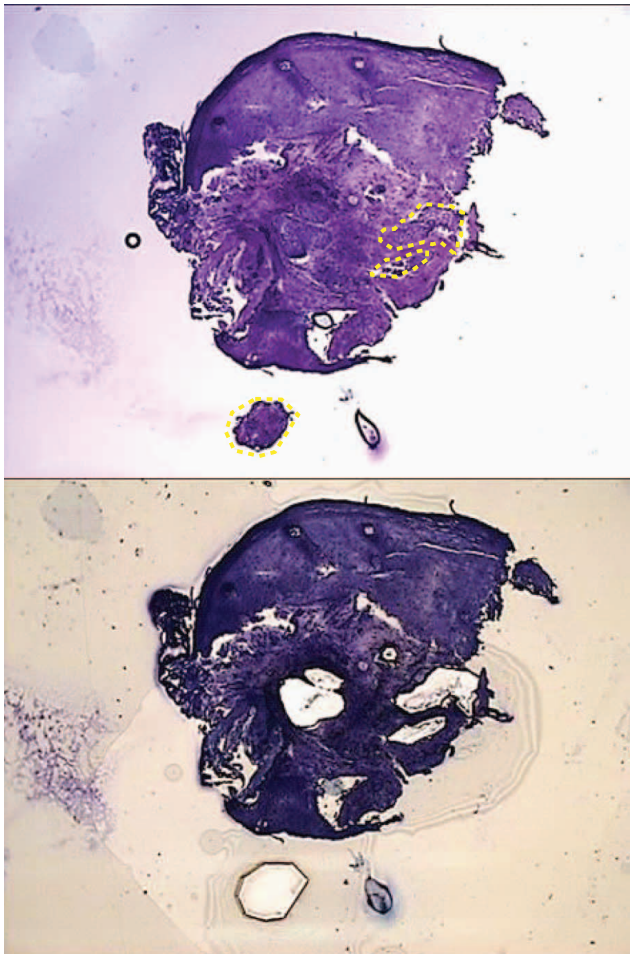

Figure 1. LCM of esophageal cancer tissue. Biopsy samples were thin sectioned and stained. Top panel shows a biopsy sample prior to microdissection and the lower panel shows those regions of cells were removed. Dotted yellow lines indicate the regions where cancerous cells were removed.

Total RNA samples from both normal and tumor cells were isolated with Qiagen's RNAeasy kit. The isolated total RNA had a concentration between 95–120 ng/µL and an OD260/280 value between 1.87–1.96. The quality of the RNA was further accessed with an Agilent 2100 bioanalyzer using RNA 6000 Pico kits. The results are shown in Figure 2.

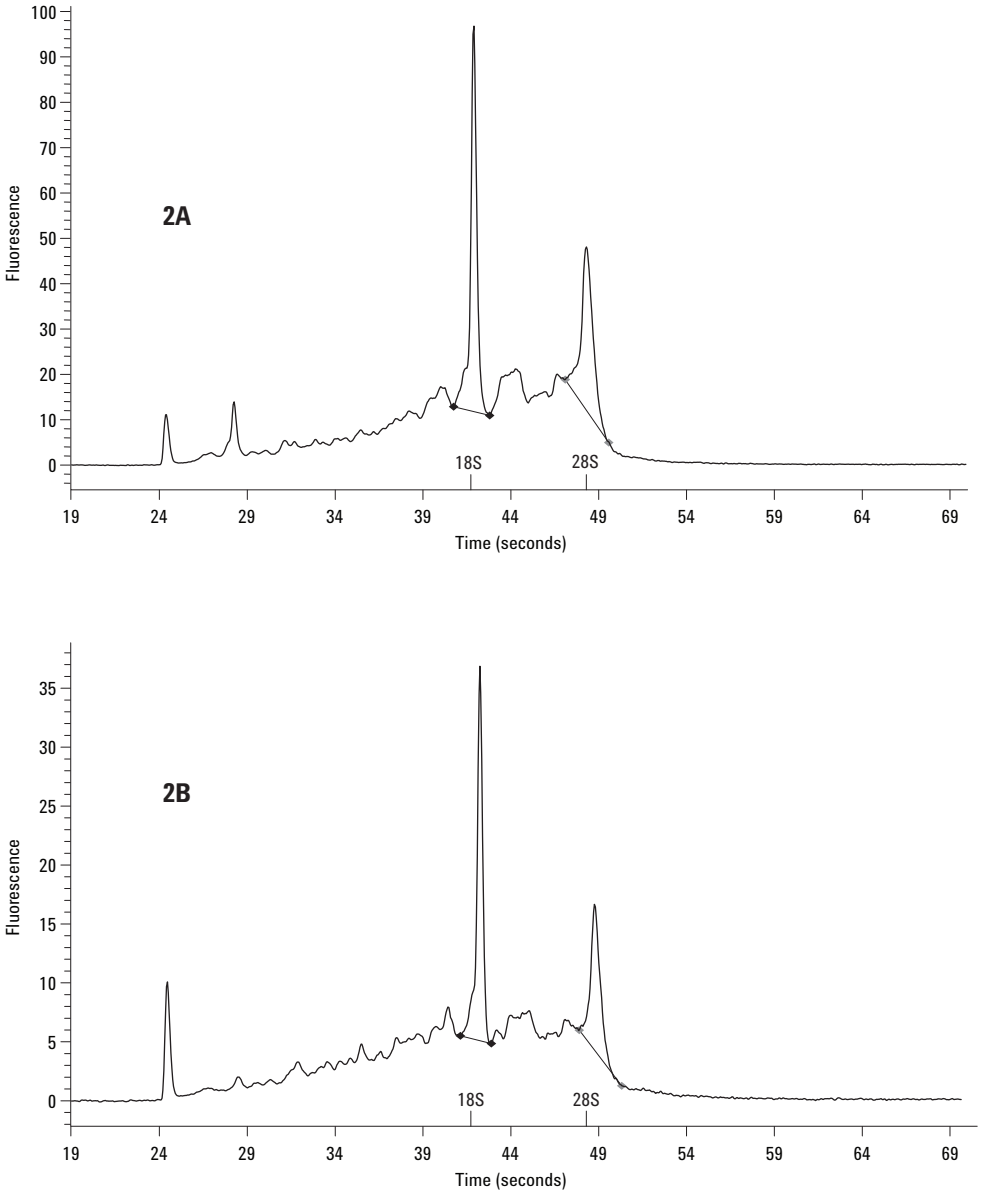

Figure 2. Bioanalyzer profiling of total RNA input and cRNA targets. Electropherograms represent total RNA from normal (2A) and tumor (2B) cells after dilution and analysis with the Agilent 2100 bioanalyzer using the RNA 6000 Pico kit. Cyanine 3-labeled cRNA targets from normal cells and cyanine 5-labeled cRNA targets from tumor cells were shown in 2C and 2D, respectively.

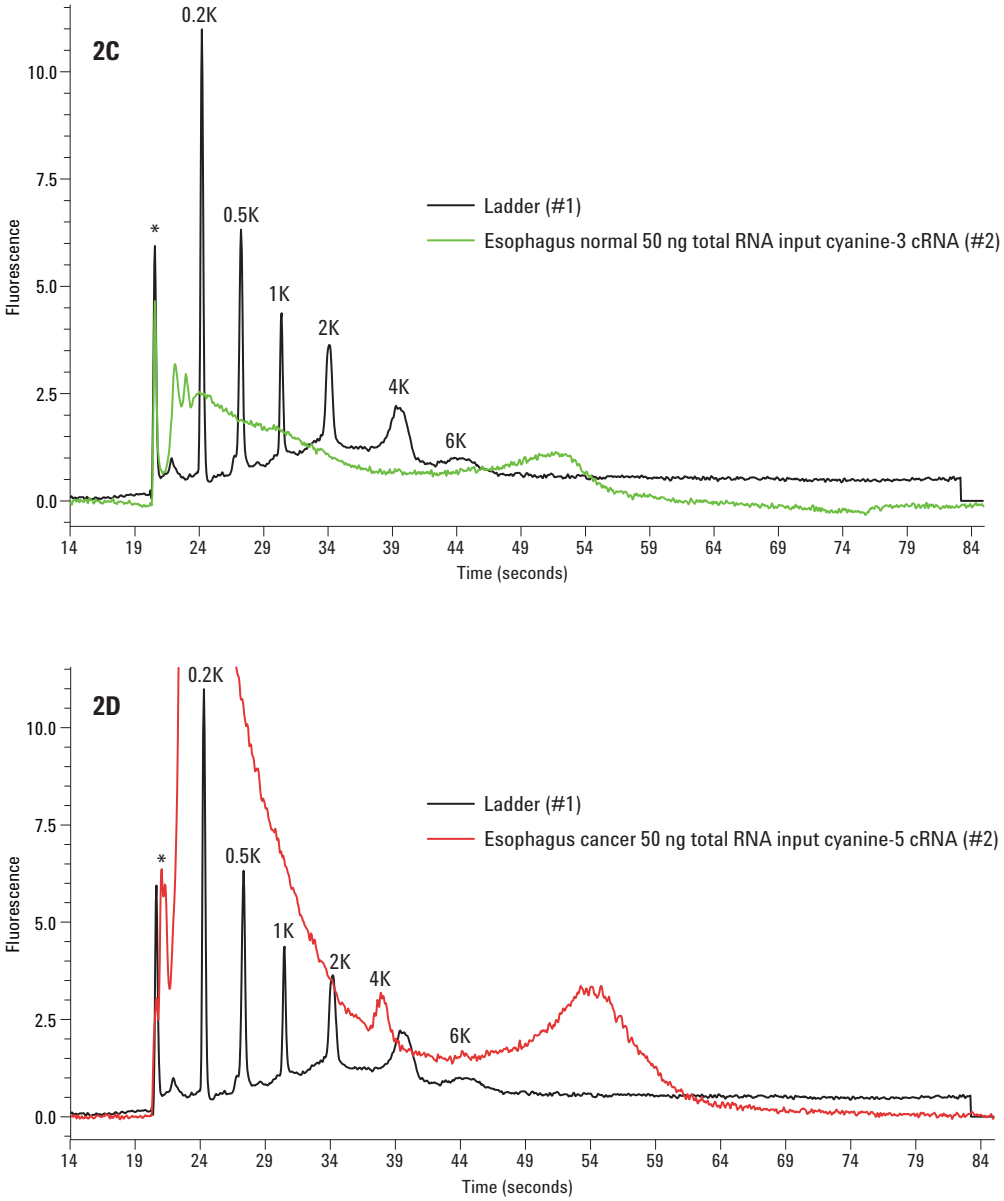

Figure 2. (cont.) **Electropherograms for cyanine-3 labeled normal (2C) and cyanine-5 labeled tumor cRNAs (2D). The labeled cRNAs were diluted and analyzed using the Agilent 2100 bioanalyzer and the RNA 6000 Pico kit. #1: RNA ladder; #2: cRNA targets (dye markers were shown as "*" on the left).**

The cRNAs were amplified and labeled with either cyanine-3 or cyanine-5 using Agilent's Low RNA Input Linear Amplification Kit or one of the methods mentioned privously. As little as 50 ng of total RNA was used in these reactions. The cyanine-3 labeled normal cRNA and the cyanine-5 labeled cancer cRNA were analyzed using the Agilent 2100 bioanalyzer and the RNA 6000 Pico and Nano kits. These profiles showed a successful amplification for the RNA samples (Figures 2C and 2D). The fluorescence for cyanine-3 sample (Figure 2C) is lower compared to that of the cyanine-5 sample because of the fluorescence overlap between cyanine-5 and the dye used to stain the RNA.

Next, 500 ng of cRNAs from each channel were hybridized to human 1A microarray G4110B, containing *in situ* synthesized 60-mer oligonucleotide probes that represent more than 17,000 well-annotated human genes. The arrays were processed with Agilent's standard SSC protocol, dried under nitrogen, and scanned with Agilent's scanner. The data was extracted by the Feature Extraction software and imported into Resolver for further analysis. One typical profile comparing the cRNA targets generated from microdissected esophogeal cancer cells with those from normal cells is shown in Figure 3.

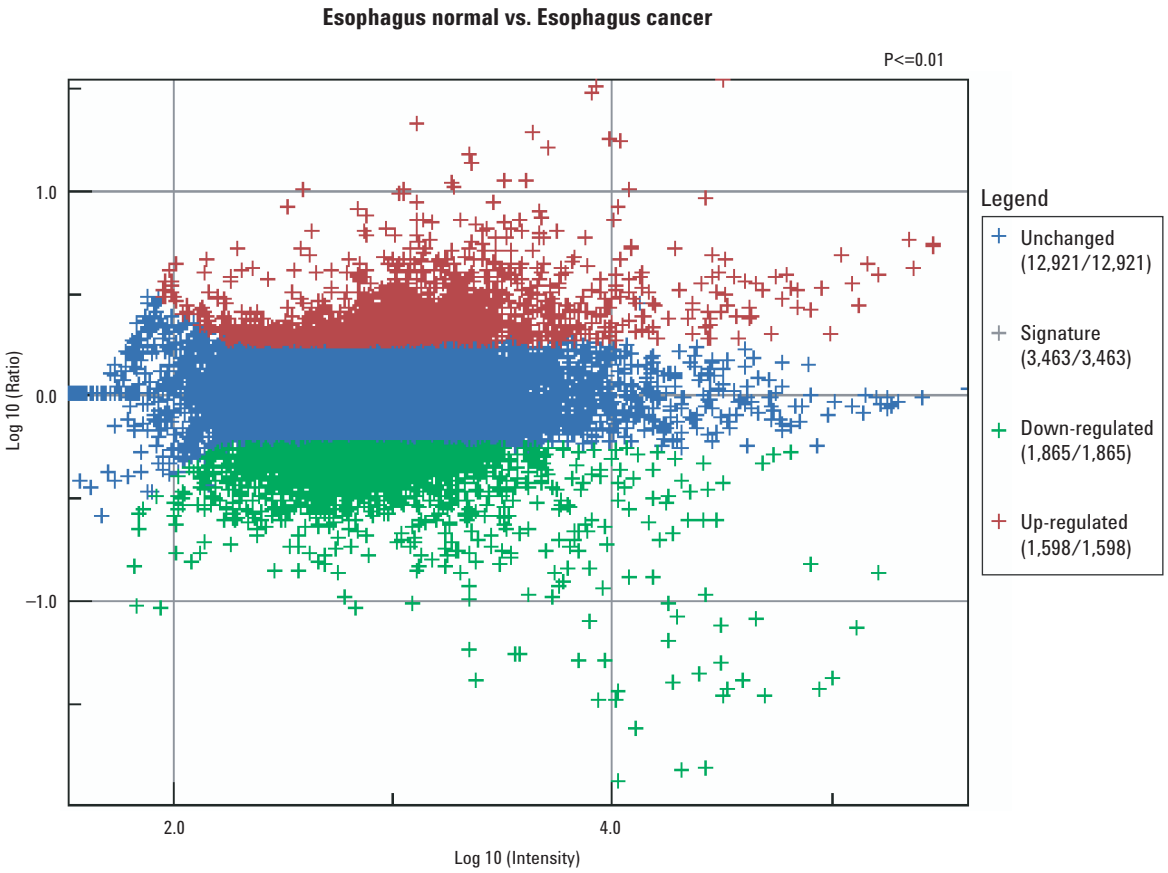

Figure 3. The detection of differentially expressed genes in esophageal cancer shown in red and green.

In this profile, a total of 3,463 signature genes were identified with 1,865 for the down-regulated and 1,598 for the up-regulated. Signature genes are those considered by the software program to be significantly above background and they are the sum of both up- and down-regulated genes. The results were highly reproducible for the three amplification and labeling methods tested (Figure 4). Here data obtained from one microarray is plotted along the y-axis while that from another microarray is plotted along the x-axis. The high correlation coefficients indicate excellent reproducibility of microarray results.

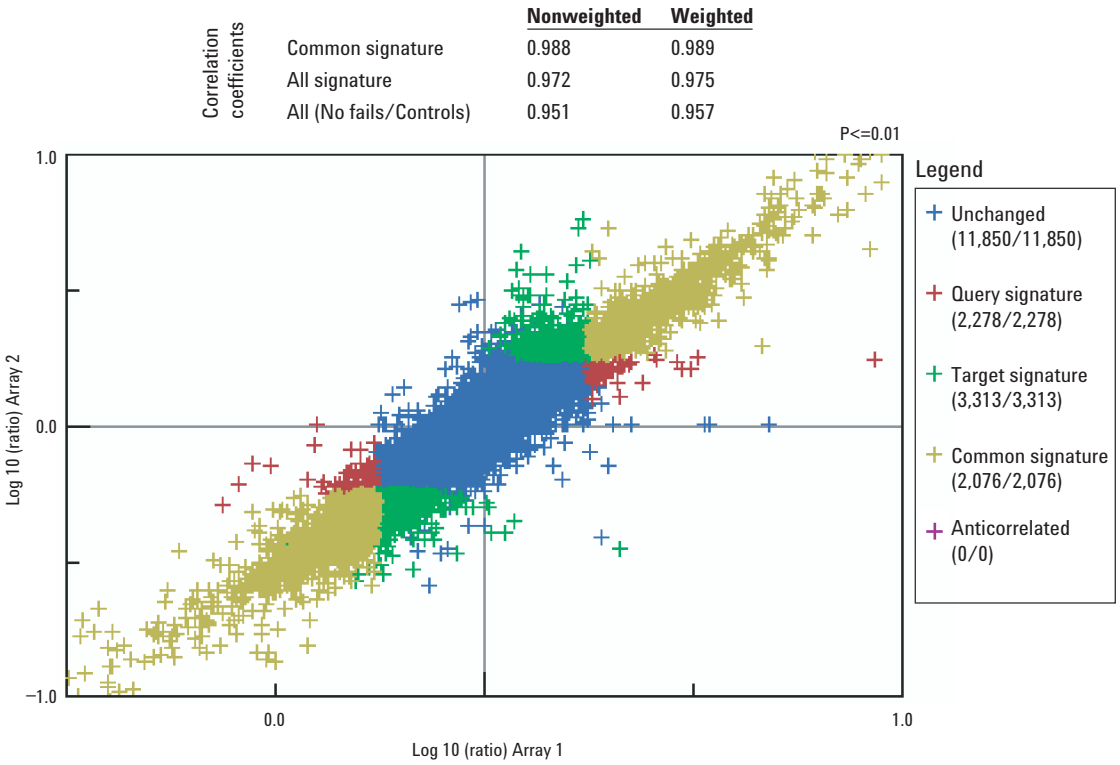

Figure 4. Log ratio comparison of two replicate arrays. Data analysis was done using Resolver.

More than 97% of the signature genes are correlated with no anticorrelated signature genes. Similar results were obtained using three different labeling methods; Agilent's Low RNA Input Fluorescent Linear Amplification kit, Fluorescent Linear Amplification kit. and the IMS two-round protocol.

We examined the top up- and down-regulated genes in these experiments and found that many of the up- and down-regulated genes in tumor cells are related to keratin synthesis or keratinocyte differentiation. Some of the examples of the top down-regulated genes are listed in Table 1. The fold value for change is an average from nine arrays with three different labeling schemes. This finding suggests that there is a deregulation of the keratin synthesis. It provides evidence for the reported link between the esophageal squamous cell carcinoma and the alteration of keratin synthesis.

Table 1. Examples of the Highly Down-Regulated Genes in Esophageal Cancer

Gene	Description	Fold change
KRT13	Keratin 13, a type I keratin and a component of intermediate filaments. Contributes to epidermal differentiation and may be linked to tumor progression. Mutation of the corresponding gene causes the mucosal leukokeratosis, white sponge nevus.	20
SPRR2A	Small proline-rich protein 2A, a member of the SPRR family of proteins that are structural components of the cornified cell envelope, up-regulated during keratinocyte terminal differentiation, may have roles in control of cell size and shape.	21
SPRR2C	Small proline-rich protein, a member of a family of proline, cysteine, and glutamine-rich proteins that are induced during epidermal keratinocyte differentiation and may be metal-binding or structural proteins such as cornified envelope precursors.	25
SPRR1A	Small proline rich protein (cornifin) 1A, member of cornified envelope precursor protein family responsible for crosslinked cell envelope formed beneath plasma membrane, up-regulated during calcium and TPA-induced keratinocyte terminal differentiation.	19
SPRR3	Small proline-rich protein 3, member of the small proline-rich protein family that are structural components of the cornified cell envelope, up-regulated in lesions of cutaneous inflammatory and neoplastic diseases.	15
IVL	Involucrin, a structural protein and terminal differentiation marker of the cornified envelope of keratinocytes, cross-links to proteins via transglutaminase and is a substrate for ceramide attachment.	15
EMP1	Epithelial membrane protein-1 (tumor-associated membrane protein), a member of the PMP22/EMP/MP20 family of membrane glycoproteins, plays a role in squamous cell differentiation.	24
KRT6A	Keratin 6A, a type II keratin; induced during states of hyperproliferation and malignant transformation; Mutation of the corresponding gene causes Jadassohn-Lewandowsky syndrome.	11
KRT4	Keratin 4, forms intermediate filaments with keratin 13 (KRT13) in suprabasal epithelial cells, as an epithelial cell differentiation marker and may contribute to its development; mutation of the corresponding gene causes White sponge nevus.	11
KRT16	Keratin 16, a type I keratin and component of intermediate filaments, contributes to cell shape and may be involved in cell proliferation, adhesion, migration, wound healing; gene mutation causes Pachyonychia congenita and Pachyonychia keratoderma.	11

Conclusions

- Many of the top up- and down-regulated genes are related to keratin synthesis or kerotinocyte differentiation.
- This finding provides a plausible molecular mechanism for the abnormal keratin synthesis observed in over 95% of the hereditary squamous cell carcinoma in esophageal cancers.
- The feasibility of doing global gene expression profiling was demonstrated using only 50 ng of total RNA isolated from cells extracted from clinical biopsy samples by laser microdissection.

Reference

1. J. M. Risk, H. S. Mill, J. Garde et al. The tylosis esophageal cancer locus: more than just a familial cancer gene. (1999) *Dis Esophagus* **12**:173-176.

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