Development of DNA microarray system - From Bench to Bed in Esophageal Cancers-

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Introduction

Gap junctions are plasma membrane channels formed by proteins known as connexins and which link the interiors of adjacent cells1). Gap junction channels enable small molecules and ions to diffuse directly between cells2). This cell-to-cell exchange is known as gap junctional intercellular communication (GJIC) and is involved in the regulation of cellular homeostasis, differentiation, and proliferation1, -5).

Previous studies have found many tumorigenic cell lines and primary tumors to be deficient in GJIC or to have fewer gap junctions than normal cells6). This defect in GJIC may contribute to the abnormal growth and differentiation characteristic of neoplastically transformed cells and may be due to a lower cell-to-cell permeability of gap junction channels, reduced formation of junctions, or a decreased expression of connexins in esophageal, lung and breast tissues6, 8).

Transfecting connexin cDNAs into GJIC deficient or weakly communicating tumor cells has resulted in the restoration of successful GJIC6). The transfected cells exhibited slower proliferation rates, more normal patterns of differentiation, and were less tumorigenic than their nontransfected counterparts9). In agreement with this, the
inhibition of connexin expression using antisense techniques has resulted in abnormal growth of nontransformed cells\(^{10}\). Both normal and neoplastic rat esophageal epithelial cells expressed connexin 43 gene and had high levels of homologous GJIC and heterologous GJIC between these cells expressing connexin 43 gene is remarkably reduced\(^6\). The expression of connexin 43 gene at gap junctions in breast cancer tissues at various stages of progression as well as breast cancer cell lines decreased\(^3\). Here we have showed the connexin 43 characteristic in the neoplastic tumor using DNA microarray.

**Gene expression in esophageal cancer cell lines**

1. Connexin 43 gene mRNA and protein expression in esophageal cancer cell lines

We have maintained three esophageal cancer cell lines under the DMEM medium with 10% FBS, which were TE-1, TE-2 and TE-3. Total RNA from the esophageal cancer cell lines was isolated using RNA extraction kit (QIAGEN, Inc., USA). cDNA was generated from total RNA with Superscript II (GIBCO-BRL) and used as the template for the PCR after quantity and quality of RNA from all samples were certified by RT-PCR amplification of the GAPDH gene. Each PCR reaction was carried out in a 20.0 \(\mu\)l reaction using cDNA generated from 4.0 \(\mu\)g total RNA. Amplification of connexin 43 transcripts (400-bp) was done using the Perkin-Elmer GeneAmp PCR system 9700 (Norwalk, CT, USA). The PCR solutions have been described\(^{11}\); all reactions involved at 94°C, 4 minutes initial denaturation step followed by 35 cycles for connexin 43 and GAPDH gene at 94°C for 30 seconds, 54°C for 30 seconds, and 72°C for 30 seconds, on the GeneAmp PCR system 9700 Perkin Elmer. Primer sequences were as follows: CNX43S; 5’-GCAGCTGGATTGGAAGCT-3’, CNX43A; 5’-GAATTCCTAGATCTCCAGTTGATCGG-3’. Amplified DNA fragment was electrophoresed on 1.2% agarose gels containing ethidium bromide with a DNA molecular weight marker for comparison.

We have examined the expression of connexin 43 gene in all TE cell lines using RT-PCR. GAPDH mRNA expression in all cell lines as an internal control was equally detected. Connexin 43 mRNA expression in TE-3 cells was remarkably recognized, but that in TE-1 and TE-2 was diminished (Figure 1).

Cell extracts were prepared as described previously\(^{11}\),\(^{12}\). We prepared the whole cell extracts from the TE series cells. The whole cell extracts were loaded in 7% SDS-PAGE and transferred to the PVDB membrane. This membrane was immunoblotted with anti-Connexin 43 antibody or anti-p21 antibody. There was recognized the p21 protein expression in all TE cell lines. And then connexin 43 protein expression level has been examined. Like the result of RT-PCR, connexin 43 protein expression in TE-1 and TE-2 was not almost detected. TE-3 cells overexpressed the Connexin 43 protein (Figure 2).

![Figure 1](image1.png)

**Figure 1** Expression of Connexin 43 mRNA in the TE cell lines, esophageal cancer cell lines. Upper panel shows the expression of Connexin 43 gene and lower panel shows the expression of GAPDH gene.

![Figure 2](image2.png)

**Figure 2** The profile of Connexin 43 protein expression in TE cell lines, esophageal cancer cell line. Immunoblot analysis for the Connexin 43 protein shows specific band as the molecular weight of 43 kDa (Upper panel) and p21 protein expression were used as an internal control (Lower panel).
2. DNA microarray analysis between the established connexin 43 transfectant cells and parental cells

The full length connexin 43 gene was isolated from the normal esophageal tissue using RT-PCR. The Connexin 43 gene was subcloned into the pTOPO TA cloning vector (Invitrogen) and was confirmed by a capillary sequencer. The connexin 43 gene was inserted into the pIRE-2 vector with a hygromycin selection. The transfection of connexin 43 gene expression vector was done to TE-2 cells using a Lipofectamine method. The transfectant cells were selected with 50 µg/ml Hygromycin. Next, we established the connexin 43 gene transfectant cells compared to the parent cell line, TE-2. The connexin 43 gene transfectant cells overexpressed the connexin 43 protein more than TE-2 cells with an immunoblot assay using anti-Connexin 43 antibody (data not shown).

For cDNA probe synthesis, 20 µg of DNase treated total RNA together with 1 µl of CDS primer mix (Clontech) in a total volume of 10 µl were heated to 65 °C for 3 minutes. A mixture consisting of 4 µl of 5 X first-strand cDNA buffer (Gibuco, Inc.), 1 µl of 100mM DTT, dNTPs, RNase inhibitor (units/ml; Stratagene, La Jolla, CA) and 5 µl of fluoro-Cy3 and fluoro-Cy5 was added into the tube and heated at 42 °C for 60 minutes after 1 µl of superscript II RNase H reverse transcriptase (200units/ml; Life Technologies, Inc.) was added. The reaction was continued at the same temperature for 60 minutes, following the addition of 1 µl of superscript II RNase H reverse transcriptase at the same temperature for next 60 minutes. The tube was heated to 70 °C for 60 minutes for enzyme inactivation. The cDNA probe labelling with fluoro-Cy3 and fluoro-Cy5 was washed and purified with 10mM Tris-HCl twice using a Millipore Spin Column. The Cancer Chip Array was hybridized for 16 hours at 65 °C after the each final solution was mixed. Array was subsequently washed once in 20 ml of wash solution 1 (2X SSC, 1% SDS) at room temperature for 10 minutes with gentle agitation and then washed once in 20 ml of wash solution 2 (0.1X SSC, 0.5% SDS) at room temperature for 10 minutes with a gentle agitation. Finally, a wash with 20 ml of 2X SSC was done for 10 minutes at room temperature. A glass array was dried up a little and soon was analyzed.

The difference of gene expression between the parental esophageal cancer cell line and the connexin 43 transfectant cell line was checked with cDNA microarray system using a Cancer Chip Array. Some kinds of genes were overexpressed more in the connexin 43 transfectants than in the parental cell lines and others kinds of genes were expressing less in the connexin 43 transfectants than in the parental cell lines. Topoisomerase II alpha (Topo IIα) and tissue inhibitor metalloproteinase (TIMP)-2 gene were overexpressed in the connexin 43 transfectant cell line (Table 1). On the other hand, the expression of multidrug resistance 1 (MDR1), MDR related protein (MRP) and matrix metalloproteinase (MMP) gene were decreased (Table 1).

These microarray data may suggest the correlation between the connexin 43 gene expression and the anti-

<table>
<thead>
<tr>
<th>High expression gene group (Ratio ≥ 2.0)</th>
<th>Low expression gene group (Ratio ≤ 0.5)</th>
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<tbody>
<tr>
<td>Keratin 7</td>
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</tr>
<tr>
<td>Keratin 18</td>
<td>IL-6</td>
</tr>
<tr>
<td>Tubulin-β</td>
<td>VEGF-B</td>
</tr>
<tr>
<td>Tissue inhibitor metalloproteinase-2</td>
<td>Matrix Metalloproteinase -2</td>
</tr>
<tr>
<td>Topoisomerase IIα</td>
<td>Matrix Metalloproteinase -10</td>
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<td>Ku antigen (70kDa)</td>
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<td></td>
<td>Matrix Metalloproteinase -19</td>
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<tr>
<td></td>
<td>Multidrug resistance 1</td>
</tr>
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<td>MDR related protein</td>
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*Ratio = the amount of Connexin 43 gene expression in Connexin 43 gene transfectant cells/ the amount of Connexin 43 gene expression in parental TE-2 cells
cancer drug sensitivity and the metastasis and invasion. We examined the drug sensitivities in the TE-2 cells and connexin 43 transfectants for Cisplatin and Etoposide. But, there was no significant difference of the sensitivities between the parental cell lines and connexin 43 transfectants.

Gene expression in esophageal cancer patients

1. Connexin 43 gene mRNA and protein expression in esophageal cancer patients

Japanese men and women with esophageal cancer underwent esophagectomy without an neoadjuvant therapy at the Department of Surgery and Science, Kyushu University Hospital (Fukuoka, Japan). Tumor and normal tissue samples were obtained after subjects provided an informed consent, frozen in liquid nitrogen, and stored at −80°C. Total RNA from the surgical resected samples was isolated using RNA extraction kit (QIAGEN, Inc., USA). cDNA was generated from total RNA with SuperScript II (GIBCO-BRL) and used as the template for the PCR after quantity and quality of total RNA from all samples were certified by RT-PCR amplification of the GAPDH gene. Each PCR procedures for the surgical resected samples were same as the PCR experiment for the cell line samples. We demonstrated the expression of connexin 43 gene with RT-PCR for the clinical esophageal cancer samples. Connexin 43 mRNA expression in all clinical normal tissues and most cancer tissues was detected. But, there was no connexin 43 mRNA expression in some esophageal cancer tissues (Figure 3). There was the difference of connexin 43 gene expression between the normal tissues and cancer tissues. In all normal tissues, the expression of connexin 43 gene was recognized, but in some of cancer tissues there was no expression of connexin 43 gene. Most of these esophageal cancers clinically were advanced types and progressed without statistically significance. In addition, the immunostaining of connexin 43 protein was done. The connexin 43 protein in normal tissues remarkably expressed but not in cancer tissues (data not shown).

2. DNA microarray analysis between the esophageal cancer tissues and normal tissues

We picked up some patients that connexin 43 gene expression in esophageal cancer tissues was not detected with RT-PCR. 20 μg of DNase treated total RNA was mixed from total RNA of some esophageal cancers and was used. DNA microarray procedures for the surgical resected samples were done as the same experiment for the cell line samples.

The difference of gene expression between the esophageal cancer tissues and normal tissues was checked with cDNA microarray system. Some kinds of genes were overexpressed more in the esophageal cancer tissues than in the normal tissues and others kinds of genes were expressing less in the esophageal cancer tissues than in the normal tissues. Among them, Connexin 26, 30 and human squamous cell carcinoma antigen (SCCA) gene expression with connexin 43 gene expression negative cancer tissues was decreasing (Table 2). The expression of connexin compartments in clinical tissues may change at the same manner.

![Figure 3](image)

Figure 3 Expression of Connexin 43 mRNA in the esophageal cancer tissue (T) and esophageal normal tissue (N). Upper panel shows the expression of Connexin 43 gene and lower panel shows the expression of GAPDH gene.
Table 2: Profile of gene expression in esophageal cancer tissues compared to esophageal normal tissues

<table>
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<th>Low expression gene group (Ratio ≤ 0.5)</th>
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<tr>
<td>Keratin 8</td>
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<td></td>
<td>Thioredoxin</td>
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<td></td>
<td>Human squamous cell carcinoma antigen</td>
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<td>(SCCA)</td>
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*Ratio = the amount of Connexin 43 gene expression in esophageal cancer tissues/the amount of Connexin 43 gene expression in esophageal normal tissues

Discussion

Gap junction plays an important role in the cellular society. Cancer cells expressing the itself special factors have some characteristics including rapid proliferation, invasion and metastasis. Stacey et al. have reported the connexin 43 expression made the rat esophageal epithelium the malignant transformation. In all esophageal normal tissues, the expression of connexin 43 gene was recognized, but in some esophageal cancer tissues and esophageal cancer cell lines, there was no expression of connexin 43 gene. Thus, in the any step of esophageal carcinogenesis, the expression of connexin 43 gene may be depressed. This evidence may be induced the malignant transformation or be one of many gene expressions after the esophageal carcinogenesis.

Next, the transfectant of connexin 43 gene suppressed the lung tumorigenicity. The growth rate in the connexin 43 esophageal transfectant cells was a little slower than the parental cell lines, TE-2. Our microarray data in the connexin 43 transfectants indicated a expression difference of gene group such as the anticancer drug sensitivity related factors and the metastasis and invasion. The expression of certain cell adhesion molecules such as cadherins, integrin may appear to be involved in the ability of cells to form permeable gap junctions.

On the other hand, the microarray data for the clinical samples indicated the decrease of connexin 26 and 30 gene expression in esophageal cancer tissues without connexin 43 gene expression. Few mutation of connexin in malignant cells have been identified and most appear to be silent. The connexin 43 gene expressed in both normal and malignant cells and no difference of gene expression between in the normal and malignant tissues may indicate that other molecular and/or structural components are involved. The gap junction channel is formed by the alignment of two hemicannels or connexons. Each connexon is comprised of six connexins. Alterations of the connexins themselves or differences in the plasma membranes may also be involved in the poor heterologous GJIC of the esophageal cells.

This microarray system could analysis totally the gene expression and supported the macroscopical aspects. The analysis system using a microarray will be able to open the new characteristic in spite of the necessity of a further development. This system in vitro could evaluate the gene expression and will enable to evaluate the biology of cancer cells in vivo.

References

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