

COMMEMORATIVE LECTURE

Isolation of disease-associated genes through genome analysis and their clinical application

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Thank you very much. Since I'm not the president of a university like Dr. Levine, I cannot talk for 30 minutes without any slides. Today, I am going to talk about what I have done so far, what is the goal of my research, and what I would like to achieve. In the latter half of my speech I would like to present you some specific research topics I am working on.

As some of you may know, I had worked as an abdominal surgeon for four years after my graduation from Osaka University, Medical School. I was wondering at that time, what makes cancer cells more progressive? What makes the differences in individual responses with regard to anti-cancer agents? Why are they effective to some patients, but not to others, and why they cause very severe side effects and kill some patients?

What I remember more when I worked as an abdominal surgeon are the patients who died of cancer. Figure 1 shows a little poem written in the diary of one of such patients, saying her appreciation to my assistance for her walking in the hallway. This patient who had cancer had been my patient for two years. The chemotherapy of a combination of anti-cancer drugs worked effectively in the beginning, but later became resistant to the therapy, and finally the patient died. I still now regret whether I was able to provide sufficient psychological support to this patient as well as other patients who died of cancer. What I learned from these patients is that cancer is a very serious disease, not only for the patient but for the family members as well. The questions and frustrations that I had during the four years of clinical practice are my motivation to work hard to control the deleterious disease, cancer.

As Dr. Levine said, dozens of genes involved in cancer were found, and it is proven that cancer is a disease caused by genetic alterations. I had the good opportunity to study genetics at Dr. Ray White's laboratory, Howard Hughes Medical Institute, University of Utah, where I worked on construction of human chromosomal genetic map as well as a gene responsible for FAP, Familial Adenomatous Polyposis. Before going to the USA, I read Dr. White's paper describing the method to isolate a gene responsible for genetic diseases and felt that it was very simple and easy. However, after moving to Utah, I realized that the number of polymorphic genetic markers was not sufficient enough to perform such studies. Hence, I was involved in the project to isolate polymorphic markers to construct genetic linkage maps. Figure 2 shows the result of Southern analysis using one of polymorphic variable number of tandem repeats (VNTR) markers.¹ This marker is also useful in forensic science, and paternity or maternity test as you can see how the chromosomes are transferred from parents to children and to grandchildren. Using hundreds of these markers, we were able to construct genetic maps for all human chromosomes that contributed enormously to the progress of studies on genetic diseases.²⁻⁵ Figure 3 shows the genetic linkage map of human chromosome 10 consisting of 30 markers that I reported. This map was the best genetic map at that time and all but three markers used in this map were isolated by my group.

In June this year, Cerela and the international collaborative groups announced that over 90% of DNA sequences of the human genome were determined. Ten years ago, scientists had the chromosomal maps and

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Fig. 1 A little poem written in the diary of one of my patients.

thought that it was very far and would take dozens of years to determine the entire DNA sequences of our genome. However, the technologies have so rapidly progressed and we now have most of the genomic sequences although it takes several years to obtain 100% of the sequences with 100% accuracy. During my research in Utah, we found in collaboration with Dr. Vogelstein that *p53* was mutated in colorectal cancers and had an important role in colorectal carcinogenesis. The picture shown in this slide was taken in 1989, when I first met with Dr. Vogelstein in Japan although I often talked with him on the phone when I was in the USA. You can see one man behind Dr. Vogelstein and me in this picture. This man is Dr. Yoshio Miki who first discovered the *BRCA1* gene, responsible for familial breast and ovarian cancer.

As I mentioned, Dr. Vogelstein's and Dr. White's groups discovered *p53* mutations in human cancers 11 years ago.⁶⁻¹⁰ After returning to Japan, I continued to study familial adenomatous polyposis (FAP) and cloned the *APC* gene in collaboration with the Johns Hopkins team in 1991.¹¹⁻¹³ We also characterized frequent somatic mutations of the *APC* gene in sporadic colorectal tumors and found the mutation cluster region (MCR) in the *APC* gene.¹⁴ Colorectal cancer is often used as a model for multistep carcinogenesis as shown in this slide; Accumulation of mutations in *APC*, *K-ras*,

and *p53* transform normal epithelial cells to adenoma cells and then malignant colorectal cancer cells. However, I wondered how these discoveries contributed to clinical studies or the clinical management. Except some clinical test such as presymptomatic diagnosis for hereditary cancers, we have not been able to make great contributions.¹⁵⁻¹⁶

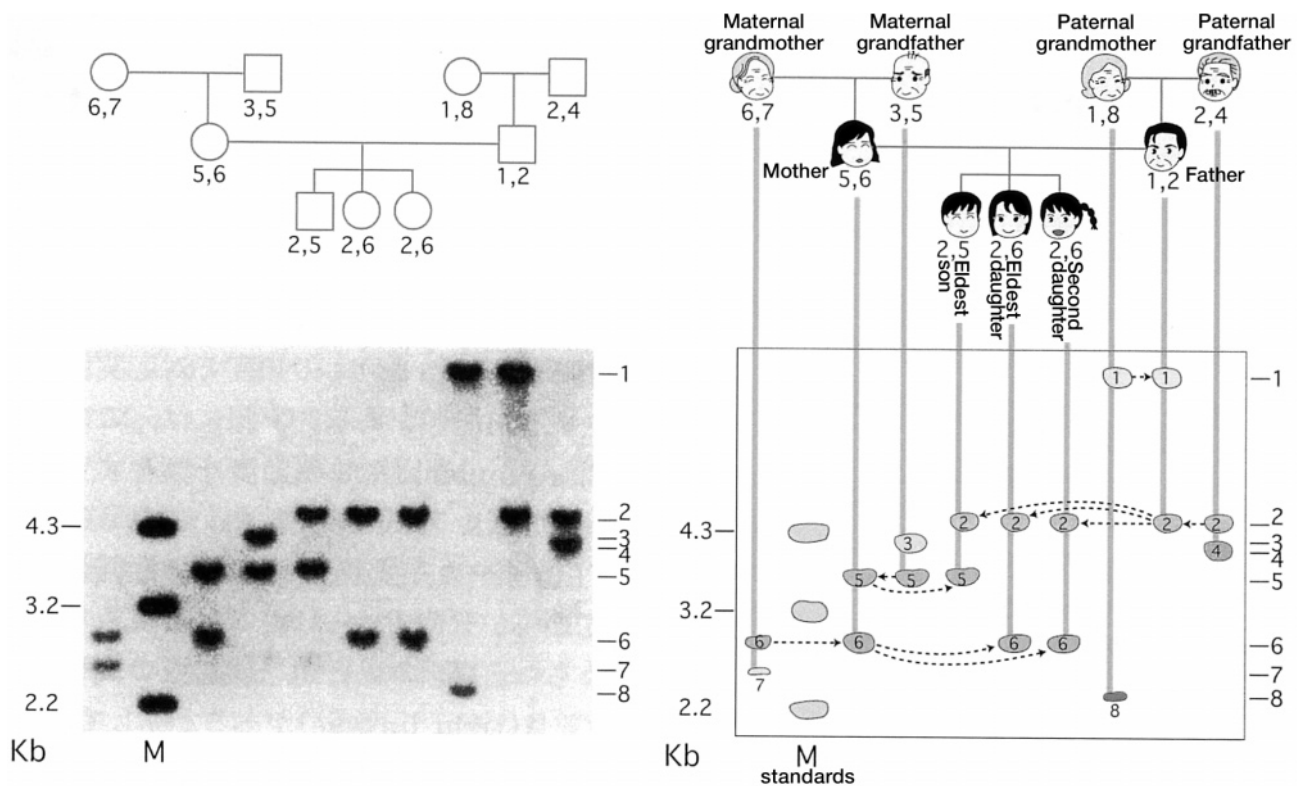
Hence, we have been working hard to isolate additional genes associated with development and progression of cancer. I would like to show two examples. One is the *Axin* gene. Dr. Yoichi Furukawa, an associate in my group, discovered that the *Axin* gene was mutated in hepatocellular carcinomas and had a tumor suppressive function in colorectal and liver cancers.¹⁷ We have isolated a dozen of the candidate genes which are involved in colorectal and hepatocellular carcinogenesis.

The other subject we have focused on is *p53* that is mutated most frequently in human cancers. Although a large numbers of papers related to *p53* have been reported, it is still not fully understood how it prevents cancer, and how it kills cells. Dr. Hirofumi Arakawa and his colleagues as well as Dr. Takashi Tokino, professor at Sapporo Medical College now, have been working on genes regulated by the wild-type *p53*.¹⁸⁻²⁵ The *BAl1* and *p53AIP1* genes, which we recently reported, seem to have a potential to apply for treating cancer.

The *BAl1* gene has a function to inhibit angiogenesis of cancer cells. We inoculated glioblastoma cells subcutaneously into mice. Twelve days after the inoculation, we could see massive angiogenesis around the cancer cells. However, we did not see any angiogenesis in the mice for which the cancer cells designed to express the *BAl1* gene were inoculated (Fig. 4).

The other gene, *p53AIP1* (*p53*-dependent apoptosis inducing gene) that we published in September, may lead cancer cells to die.¹⁹ We inoculated human colorectal cancer cells at four regions in the back of a mouse and began treatment with *p53AIP1* using the adenovirus vector after the cancer cells began to make a mass of 3 mm diameter. The tumors without any treatment, or treated with saline or with adenovirus designed to express *LacZ* became very large. However, the tumor treated with *p53AIP1* did not grow and completely disappeared three weeks later. We have so far tested this therapy on nine mice, and in six of the nine mice the cancer cells have disappeared. Since the *p53AIP1* gene could kill some of the cancer cells which even *p53* cannot kill, perhaps this treatment can be applied to a very wide range of cancer cells. We are now planning to have studies leading this gene into clinical studies in the future.

I have been working in the Human Genome Center, The University of Tokyo and have been the Director of the Center since 1995. From the human genome per-



An example of genomic fingerprinting showing the genetic relationship of parents and children. The transfer of specific genes from grandparents to parents, to children is clearly indicated.

Fig. 2 Southern analysis using one of the polymorphic VNTR (variable number of tandem repeats) markers, YNH24, and a schematic presentation of its inheritance pattern in one family.

spective, we have a responsibility to train young researchers who want to study in the human genome field. I accepted many doctors who were interested in this field, and some of them successfully isolated genes responsible for genetic diseases or found the important evidence that may be useful for clinical management of patients.²⁶⁻³⁰ Dr. Shiro Ikegawa, an orthopedic surgeon, was interested in the ectopic calcification and studied on the mouse model called *ttw* (tip to walk). Using the model showing a recessive trait, he successfully discovered that this abnormality was caused by the abnormal metabolism of phosphate. He interestingly found that when these *ttw* mice were fed with food containing a high concentration of phosphate, they showed an abnormal shape of ear due to abnormal calcification of ear cartilage while no abnormality was observed when they were fed with low phosphate/low calcium food or high calcium/low phosphate food. In Japan, there are a large number of patients who have ossification in spinal ligament (OPLL), our results could lead to find the molecular pathogenesis underlying this disease and may contribute to develop the novel treatment or chemoprevention for it.

I'm very sorry to show you a shocking picture (Fig. 5) of an eye affected by gelatinous drop-like corneal dystrophy. This disease is an autosomal recessive hereditary disorder and its prevalence is 1 in 300,000 people. The only one method to treat this disease is transplantation of cornea, but several years later the patients' eyes usually show the same symptom. In order to maintain their vision, they have to continuously search for donations of cornea. Since this condition is very miserable, it is essential to find out the cause of this deleterious condition and develop a novel treatment. By cooperation with the patients and their family members, we successfully identified a gene responsible for this disease and characterized their mutations. I believe that the discovery of the causative gene should be the first step toward the development of a novel treatment.

Although the results shown in the previous slides contributed the better understanding of the etiology of various diseases including cancer, it is still very difficult to apply the results into the clinical field. However, the results we obtained may have a great possibility to prevent sudden cardiac death of patients with long QT

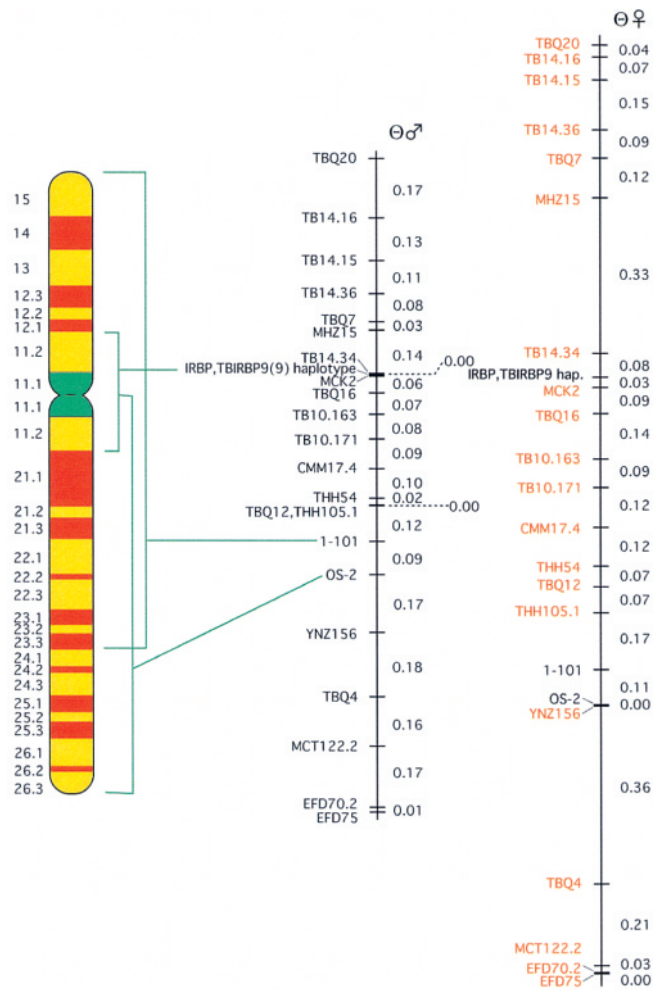
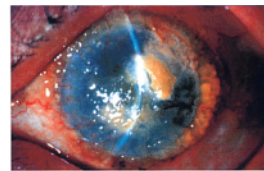


Fig. 3 A genetic linkage map of human chromosome 10 consisting of 30 polymorphic markers.



Mendelian inheritance pattern Autosomal recessive disorders (AR)
 Morbidity About 1/300,000
 The opalescent, gelatinous drop-like dystrophy of the corneal epithelium was caused by the accumulation of amyloid deposits.
 This condition is usually treated by corneal grafting; however, several reports of recurrences several years after treatment have been reported in Japan.

Mutations and Haplotypes of GDLT chromosomes

| No. | Nucleotide Change | Protein Level | Frequency | D1S2752 | D1S2648 | D1S220 | Control chromosome |
|-----|-------------------|---------------|-----------|---------|---------|--------|--------------------|
| 1 | C352T | Q118X | 86.21% | 204 | 285 | 247 | 200 |
| 2 | del632A | FS | 6.90% | 210 | 285 | 235 | 200 |
| 3 | C619T | Q207X | 3.45% | 236 | 273 | 235 | 200 |
| 4 | C509A | S170X | 3.45% | 204 | 285 | 247 | 100 |

Fig. 5 Eye affected by gelatinous drop-like corneal dystrophy and characterization of the mutations found in Japanese patients.

syndrome. The patients are characterized prolongation of the QT interval, leading to ventricular tachycardia and syncopal attacks, sometimes sudden death. The β -blocker is the conventional medicine used against this condition, but while roughly 70% of the patients respond well to this medicine, the remaining 30% of the patients are non-responders. The genes responsible for this condition were isolated and shown to be heterogeneous. Dr. Toshihiro Tanaka and his colleagues in my laboratory screened mutations of these responsible genes in more than 100 Japanese families carrying the long QT syndrome and found that the causative genes are associated with the efficacy of the β -blocker. His results suggested that if they have a mutation in the *LQT1* gene, the patients should be treated with the β -blocker alone, that if they have a mutation in the *LQT2* gene, the patients should have a drug to prevent arrhythmia or pacemaker implantation in addition to the β -blocker. Therefore, identification of the causative gene may enable one to explain who are responders and who are non-responders, and we may be able to apply the personalized therapy that means a very selective and the most appropriate therapy to a particular patient.

I always feel, because I was an abdominal surgeon, that I really want to provide a better treatment to cancer patients. Cancer is a very special subject for me. In 1999, nearly 270,000 Japanese people died of cancer, and 600,000 new people are found to have cancer every year. At the advanced stage, the majority of the cancer patients are treated with chemotherapy without knowing its effectiveness prior to the treatment. I'm very sorry to say that the chemotherapy is equivalent to gun shooting in the dark. In average, chemotherapy is just effective in only 20% or 30% of the patients and the remaining 70-80% of the patients suffer from the side effects without any improvements at all. It is apparent that it is not the right way to go. It is very important and essential to establish the method to predict

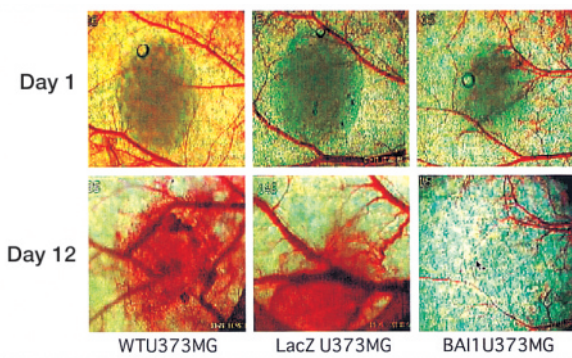


Fig. 4 Inhibition of angiogenesis induced in glioblastoma cells by means of ex vivo gene transfer of *BAI1*.

Gene expression pattern of AML patient 107

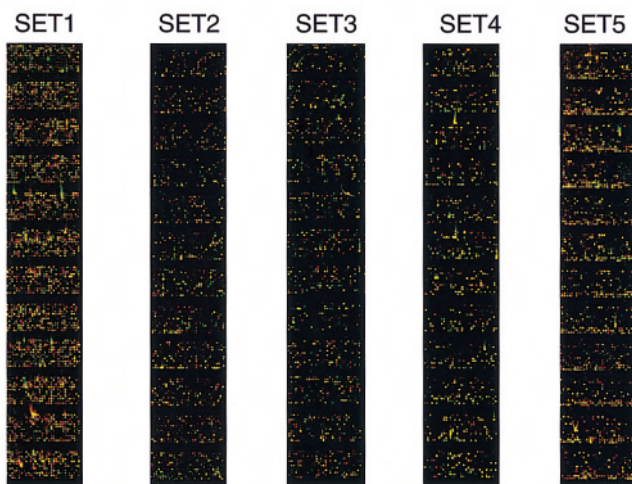


Fig. 6 A representative result of the expression profile of about 20,000 genes for AML.

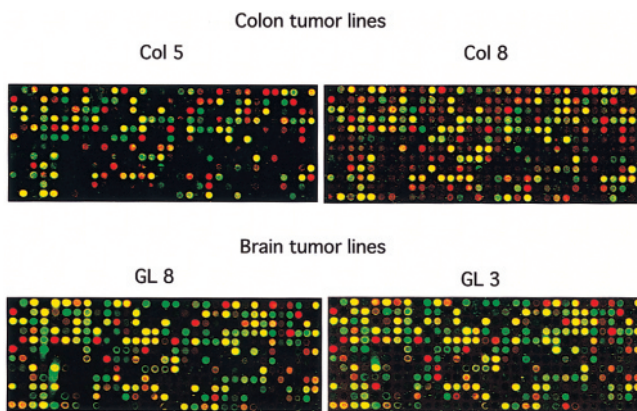


Fig. 7 Expression profiles of 384 genes, a part of our cDNA collections for cDNA microarray analysis, in two colon-cancer lines in the upper and two glioblastoma cells lines in the lower.

the sensitivity to chemotherapy prior to starting the treatment.

For this big challenge, we need to characterize in detail the nature of cancer cells and I thought that the systematic analysis of expression profile may provide an answer to us. Figure 6 shows the representative result of the expression profile of about 23,000 genes for AML. Green, red and yellow colors indicate the degree of expression levels of each gene that corresponded to each spot. Using the expression profiles of cancer cells, I believe that we will be able to characterize the different nature of cancer cells. For example, Figure 7 shows the expression profile of 384 genes, a part of our cDNA collections for cDNA microarray analysis, in two colon tumor lines in the upper and two glioblastoma cell lines in the lower. You can compare the expression profiles

Lung Cancer Tumor Lines

Sensitivity of NSCLC lines to anticancer drugs

| Drug | Relative tumor size rate(T/C%) | | | | | | | | | |
|------|--------------------------------|-------|-------|------|-------|-------|------|------|-------|-------|
| | Lu-116 | Lu-99 | Lu-65 | LC-6 | Lu-61 | QG-56 | LC-1 | L-27 | LC-11 | LC-17 |
| MMC | 5 | 12 | 5 | 1 | 14 | 60 | 21 | 52 | 49 | 6 |
| CPM | 8 | 10 | 4 | 21 | 61 | 64 | 31 | 57 | 71 | 51 |
| ACNU | 0 | 25 | 2 | 0 | 13 | 58 | 54 | 57 | 76 | 81 |
| DDP | 21 | 13 | 19 | 23 | 23 | 62 | 27 | 65 | 62 | 56 |
| ADR | 35 | 72 | 47 | 31 | 78 | 55 | 50 | 59 | 63 | 68 |
| VCR | 2 | 18 | 27 | 40 | 86 | 99 | 58 | 72 | 85 | 122 |
| VLB | 1 | 8 | 13 | 4 | 13 | 43 | 25 | 10 | 73 | 43 |
| S-FU | 62 | 46 | 70 | 100 | 105 | 72 | 66 | 91 | 79 | 67 |
| MTX | 85 | 85 | 83 | 38 | 100 | 104 | 97 | 105 | 92 | 103 |

(at.MTD)

Fig. 8 A summary of the efficacy of nine anti-cancer drugs for ten lung-cancer cell lines (the Central Institute for Experimental Animals). The number “100” indicates that the sizes of the tumors treated or untreated with anti-cancer drug were same and that the drug was not effective to the cancer cells. On the other hand, “zero” means that the cancer cells disappeared in this particular mouse, suggesting that this particular drug was very effective.

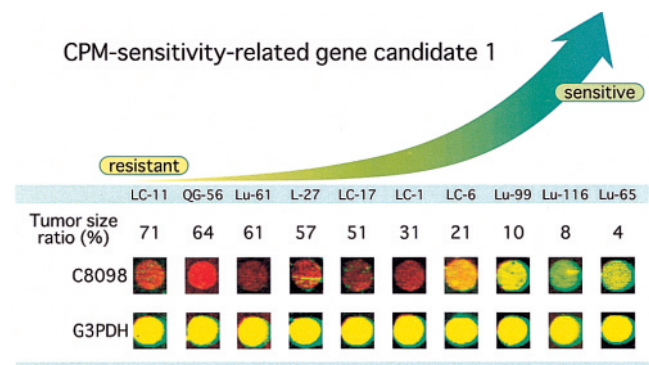


Fig. 9 A representative result showing the significant positive correlation between the expression level of C8098 and the efficacy to anti-cancer drug (CPM).

of two colon cancers and notice that the patterns of the two colon cancer cells are very similar, but very different from those of two glioblastoma cells. However, if you look very carefully and compare the expression patterns of the two colon cancer cells, you should notice differences in the colors between the two. These subtle differences may reflect the differences in the character and properties of different cancers and some of these differences may be associated with the responsiveness against anti-cancer drugs. Using the computer analysis, we may be able to distinguish cancers that respond poorly to anti-cancer drugs from those that respond well.

We have been taken various approaches to find genes associated with drug efficacy using cancer cell lines, xenografts, and also clinical cases. We have been examining the correlations between the expression profiles and the sensitivities to each of nine different anti-cancer drugs using xenograft materials in collabo-

ration with the Central Institute for Experimental Animals. Cancer cells were inoculated into nude mice and then treated with each of the nine anti-cancer drugs, MMC, CPM, ACNU, DDP, ADR, VCR, VLB, 5-FU, and MTX. Two weeks later, the sizes of the tumors were measured and the efficacy of each drug was estimated. Figure 8 presents a summary of the experiments. The number “100” indicates that the sizes of the tumors treated or untreated with anti-cancer drug were the same and that the drug was not effective to the cancer cells. On the other hand, “zero” means that the cancer cells disappeared in this particular mouse, suggesting that this particular drug was very effective. We then analyzed the expression levels of each of the 23,000 genes using the cDNA microarray. Figure 9 shows the representative result showing a significant positive correlation. As you can see, the expression levels indicated by the different colors clearly correlated with the sensitivity to this particular anti-cancer drug. Currently, we completely finished the expression analysis of the 90 cancer cell lines and picked up hundreds of genes showing some positive association with the effectiveness of each of the nine anti-cancer drugs.

I would also like to show the results using clinical materials. Expression profiles of tissues obtained from 20 patients, who had been operated on for esophageal cancer, all at stage III or stage IV advanced cancer, were analyzed. Most of the patients had a non-curative operation and were confirmed to have residual cancer cells microscopically or macroscopically. After surgical treatment, all of the patients had chemotherapy of a combination of cisplatin and 5-FU. We divided these patients into three different classes on the basis of their survival period after the surgical operation; eight patients who survived more than 30 months were classified as group 1, six who died within 12–30 months after the operation as group 2, and those who died within 12 months as group 3. Since the main tumor in the esophagus had been resected, we cannot say that the survival length reflected the efficacy of chemotherapy. However, we can speculate that this longer survival may likely be related to the efficacy of chemotherapy and for the patients who died within one year, it is very likely that chemotherapy was not effective at all.

The nature of the cancer cells is very likely to be determined by the combination of the genes expressed in the cell. We attempted to compare the expression profiles between the tumors belonging to group 1 and group 3. The expression patterns seem to be quite similar, but there were substantial differences in the expression levels of some genes. We then analyzed all the data and picked up 52 genes whose expression levels may be correlated with the prognosis of these patients. cDNA spots corresponding to some genes were commonly red in tumors belonging to group 1, but yellow or

green in those to group 3. Some showed the inverted patterns, indicating the strong correlation between the expression levels of these genes and the prognosis of the patients. Furthermore, to apply these results to clinical diagnosis, we convert these color patterns to numerical scores by means of algorithms we established. The three groups were clearly separated by the drug response score (DRS), indicating that this scoring system may have a great potential to establish the method to predict the sensitivity to anti-cancer drugs. To further clarify the scoring system, we obtained and examined DRSs of tumors of seven additional cases. The results of these seven cases were consistent with those of the original test cases without any exception. The goal of my approach is summarized in the last slide. I would like to accumulate the information of expression profiles and clinicopathological data from a large number of clinical cases and establish the database that can predict the most appropriate treatment for a particular patient. We should establish the personalized medicine using genetic or genomic information, “an appropriate dose of the right drug to the right patient”.

I thank all of my co-workers for helping my research and it is sure that I could not receive the Keio Medical Prize, the very prestigious prize, without their joining to my laboratory.

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