Molecular Prediction of Anti-cancer Response to Combination Therapy with Interferon-alpha and 5-FU in Advanced Hepatocellular Carcinoma

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Hepatocellular carcinoma (HCC) is one of the major causes of death from malignancy worldwide. Although recent progress in both diagnostic and surgical techniques has resulted in considerable improvement in the morbidity and mortality rates, the overall outcome remains far from satisfactory. The prognosis is miserable, particularly in patients with tumors that have invaded the major branch of the portal vein, and survival is generally limited to a few months after diagnosis, despite multimodal therapies even in cases suitable for surgical resection.

Recently, we applied both subcutaneous administration of interferon(IFN)-alpha and intraarterial infusion of 5-FU for far advanced HCC patients with multiple lesions and tumor thrombi in the major branches of the portal vein (Vp3 or 4). Interferon-alpha (5million U) was administered on days 1, 3, and 5 of every week. Continuous infusion chemotherapy (5-FU, 300 mg/m²/day) through the proper hepatic artery was performed every 2 weeks for 2 weeks via a catheter connected to a subcutaneously implanted drug delivery system. The response rate of such treatment was approximately 45% in our patients with highly advanced HCC. The combination treatment markedly decreased tumor size and levels of tumor markers with an encouraging response rate and prolonged survival time in the responders. Furthermore, the clinical response completely reflected the survival benefits. On the other hand, almost all non-responders died within 6 months. No response to the combination therapy was seen in 55% of our patients. This chemotherapy may also have multiple adverse effects, including leukopenia, thrombocytopenia, and depression. Therefore, accurate prediction of chemosensitivity is desirable, not only so that non-responding patients do not lose a limited chance to take advantage of other possible treatments, but also to eliminate suffering of these patients due to debilitating side effects. Unfortunately, there are currently no useful indicators to distinguish between patients who are likely to respond to this combination chemotherapy and patients who are not.

In this study, genes possessing the ability to predict patient responses to 5-FU and IFN-alpha combination chemotherapy were tried to be selected by gene expression profile analysis using adaptor-tagged competitive polymerase chain reaction (ATAC-PCR) technology, a polymerase chain reaction (PCR)-based array system. The performance of this prediction method was estimated with both a complete cross-validation and an external validation dataset.

**Materials and Methods**

Between August 1998 and January 2003, 20 HCC patients with multiple tumors spreading to bilateral lobes
with tumor thrombi in the major branches of the portal vein underwent palliative surgery to reopen the portal flow and recover liver function. After obtaining informed consent, we collected HCC tissue from the main resected tumors and isolated the total RNA from these samples for PCR-based array experiments. All 20 patients had visible tumors in the remaining liver. The patients were then subjected to a treatment regimen of combination chemotherapy with 5-FU and IFN-alpha. The chemotherapeutic response was clinically evaluated according to the Eastern Cooperative Oncology Group (ECOG) criteria. In this study, responders were defined as patients with complete response (CR) or partial response (PR); non-responders were defined as patients with stable disease or progressive disease (PD). Furthermore, as an independent validation dataset, we collected liver tissue specimens and clinical data from other patients who underwent hepatic resection of all visible tumors. Although these patients had tumor(s) with major portal vein tumor thrombi, the area of spreading tumor(s) was limited to two segments of the liver. The treatment regimen of combination chemotherapy and the method of follow-up in the validation dataset were the same as the procedures used in the original dataset. To select genes expressed in liver tissues, we constructed three cDNA libraries: one from a mixture of HCC and non-tumorous livers, one from normal livers, and one from metastatic liver cancers. We designed PCR primers for ATAC-PCR reactions for a total of 2,066 genes from these expressed sequence tag collections. In total, we prepared 3,080 primers for ATAC-PCR; this total includes an additional 414 genes established in previous literature.

**Results**

According to the ECOG criteria of objective response, 8 patients (40%) were classified as responders, demonstrating either CR or PR for at least 4 weeks. The remaining 12 patients (60%) were classified as non-responders, exhibiting either stable disease or PD. The clinicopathological characteristics of responders (CR and PR) and non-responders (stable disease and PD) were compared by the t test. There were no significant differences in any factor between the two response groups.

We performed a hierarchical cluster analysis of the samples using all 3,080 genes. When the clinical samples were sorted on the basis of similarity in gene expression, the samples could be separated according to the response to 5-FU and IFN-alpha combination chemotherapy, i.e., responders and non-responders, with only a few exceptions. Next, we applied PCA, a statistical method for reducing the number of data dimensions, to more simply present the relationships between the samples. On displaying the expression patterns of all 3,080 genes in three-dimensional space, we observed that most responders and non-responders were located separately, indicating distinct gene expression patterns.

To construct a molecular prediction system, we used a supervised approach, a WV algorithm using S2N correlation ratios. The ability of this method to predict patient responses to 5-FU and IFN-alpha combination chemotherapy was evaluated by leave-one-out cross-validation. In this first cross-validation analysis, we used all 3,080 genes to avoid any selection bias. The prediction accuracy of this WV method was 85.0%, and its 95% confidence interval ranged from 66% to 96%. The positive and negative predictive values were 100% and 80%, respectively. These results demonstrate that this method provides a valuable prediction of the chemotherapeutic response. To determine whether all 3,080 genes are necessary for prediction, we compared the prediction accuracy of a small number of genes to the prediction accuracy of all 3,080 genes. When the predictive genes were defined as genes with significant P values (P<0.01) by the random permutation tests, the
complete cross-validation without information leakage showed an identical performance with the initial cross-validation using all 3,080 genes. We therefore selected only 63 genes with significant P values between the 8 responder patients and the 12 non-responder patients. The expression patterns of these 63 genes exhibited distinct profiles between the two groups. To further evaluate our prediction system, we prepared an independent dataset, which consisted of 11 HCC patients with major portal vein tumor thrombi. Although these patients had no visible tumors after resection before combination chemotherapy, the risk of recurrence within the early postoperative period due to intrahepatic micrometastasis was very high. Moreover, in these advanced HCC cases with major portal vein tumor thrombi, the survival time was strongly correlated to the chemotherapeutic response. In fact, the median survival times of responders and non-responders in the original dataset were 28 and 7 months, respectively, and the difference was statistically significant (P< 0.002). We therefore performed overall survival analysis and disease-free survival analysis instead of estimation to determine the prediction accuracy of objective response. Our prediction method using 63 genes classified patients into either a good signature group or a poor signature group. The overall survival rates were significantly different between these two predicted groups (P<0.001). In addition, the good signature group had a distinctly better prognosis for disease-free survival than the poor signature group (P<0.002).

**Discussion**

DNA microarray technology allows parallel expression analysis of thousands of genes to address complex questions in tumor biology. Many trials predicting the prognosis of various human malignancies have been reported using DNA microarrays. We performed a high-throughput quantitative PCR based on ATACPCR to analyze the genetic differences in HCC. This assay requires smaller amounts of RNA than DNA microarray analysis.

This combination chemotherapy with 5-FU and IFN-alpha is promising for advanced HCC, but we need to consider the side effects. IFN-alpha and/or 5-FU sometimes induce severe adverse effects, including myelosuppression, fever, and depression. In particular, adverse myelosuppression is an important factor in HCC cases not only because thrombocytopenia and/or leukopenia are frequently present before chemotherapy, but also because treatment often has to be discontinued due to these side effects. Therefore, accurate prediction of sensitivity to the first line chemotherapy is necessary for advanced HCC patients so that the patients who will not respond to the therapy can be protected from debilitating side effects. These results illustrate the potential of biological technology to advance diagnostic methods, allowing physicians to plan beyond empirical results toward a more molecularly well defined, personalized therapy. Because our molecular prediction system involves only a small number of predictive genes and a simplified algorithm that do not require either statistical software or specialists, this system should easily lead to future clinical application.

In conclusion, molecular analysis of 63 genes can predict the response of patients with advanced HCC and major portal vein tumor thrombi to combination chemotherapy with 5-fluorouracil and interferon-alpha.
Present Position
Professor & Chairman Department of Surgery, Graduate School of Medicine, Osaka University.

Education and Training
1970-1973 Resident, Department of Surgery, Osaka University Hospital
1974-1979 Surgical Staff, Osaka University Hospital
1979-1981 Visiting Fellow, Department of Surgery, Sloan-Kettering Cancer Center
1981-1987 Instructor of Surgery, Osaka University Medical School
1987-1990 Assistant Professor of Surgery, Osaka University Medical School
1990-1994 Associate Professor of Surgery, Osaka University Medical School
1994-1998 Professor of Surgery, Osaka University Medical School
1999 Vice Director, Osaka University Hospital
2004 President of the Japanese Liver Transplantation Society

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