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Molecular Classification and Characterization of Human Gastric Adenocarcinoma through DNA Microarray

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Abstract

Gastric adenocarcinoma (GA) is a major tumor type of gastric cancers and subdivides into several different tumors such as papillary, tubular mucinous, signet-ring cell and adenosquamous carcinoma according to histopatholigical determination. In other hand, GA is also subdivided into intestinal and diffuse type of adenocarcinoma by the Lauren's classification. In this study, we have examined differential gene expression pattern analysis of three histologically different GAs of 24 samples by using DNA microarray containing approximately 19000 genetic elements. The hierarchical clustering analysis of 24 gastric adenocarcinomas (12 of intestinal type, 7 of diffuse type and 5 of mixed type) resulted in two major subgroup on dendrogram, and two subgroups included most of intestinal and diffused type of GAs respectively. Supervised analysis of 19 intestinal and diffuse type GAs by using Wilcoxon rank T-test ($P <$ 0.01) resulted in 100 outlier genes which exactly separated intestinal and diffuse type of GA by differential gene expression. In conclusion, genome-wide analysis of gene expression of GAs suggested that GAs may subclassify as intestinal and diffused type of GA by their characteristic molecular expression. Our results also provide large-scale genetic elements which reflect molecular differences of intestinal and diffuse type of GAs, and this may facilitate to understand different molecular carcinogenesis of gastric cancer.

Keywords: Gastric adenocarcinoma, Intestinal type, Diffuse type, DNA microarray

Gastric cancer is the most frequent malignancy of the gastrointestinal tract in Korea, and is the second most frequent cause of cancer death[1]. In Korea, it accounts for an estimated 20.9% of all malignancies, with 24.4% in the male population and 16.3% in the female population, but the predominant molecular event underlying gastric carcinogenesis has been remained unknown[2].

GA can be divided into two distinct subtypes: the intestinal type (IGA) and diffuse type (DGA) that can be separated by characteristic histological features[3]. The intestinal type gastric adenocarcinoma id frequently proceeded by multifocal atrophic gastritis and is more common in elderly men. For the diffuse type, which had a less favorable prgrnosis and occurs in patients under the age of 50 and in women predominantly, the histologic precursor lesion id usually not identifiable[4]. In contrast to the Lauren classification that can be applied to small biopsies, the Ming classification requires histological examination of resection specimens. In the Ming scheme[5], the growth pattern assessed at the invasion front of the tumor as being infiltrative or expansive, with some similarity to the Lauren classification. Although histopathological diagnosis is extremely useful for the definitive as well as the supportive diagnosis of gastric cancer in clinical practice, it is limited in certain respects. Recently, DNA microarray technology has enabled to the comprehensive analysis of gene expression level, and as such yielded great insight into the molecular nature of cancer[6]. Thus, in order to identify the characteristic large-scale molecular signature of GAs, especially IGA and DGA, we have applied DNA microarray technology to examine comprehensive genetic changes by different classifications. Further, with the supervised analysis, we suggested 100 outlier genes which enable to discriminate IGA and DGA by molecular pattern.

Unsupervised Analysis of Gastric Adenocarcinomas

In order to assess different molecular profiles of gastric adenocarcinomas (GAs), 24 surgically resected tissue samples of gastric tumors were subjected to oligonucleotide microarray containing approximately 19,000 genetic elements as probe. Of 24 GAs, 11 are intestinal type, 8 diffuse type and 5 mixed type of GAs. All samples were examined by two pathologists

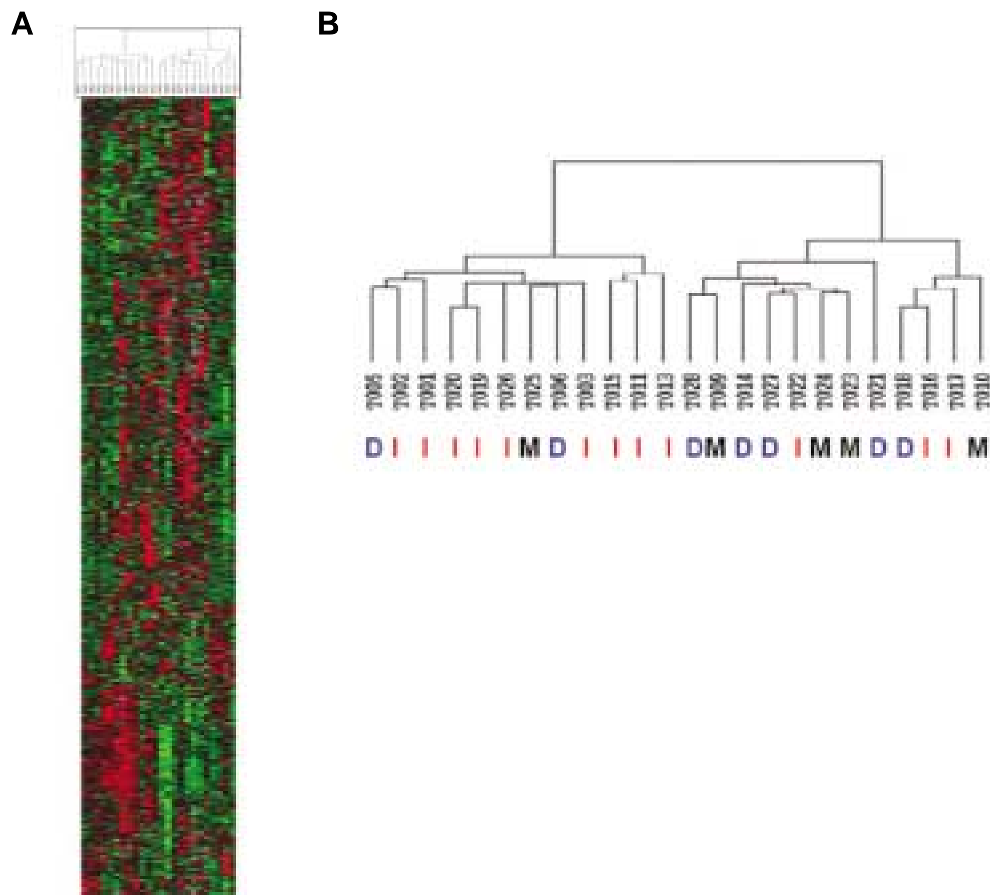

Figure 1. Unsupervised hierarchical clustering analysis of human gastric adenocarcinomas. A, Two-dimensional clustergram of the 10,313 genes selected with minimal filtering criteria. Each row represents a tumor profile; each column represents a probe's measurement. The color saturation reflects the difference in expression between the tissue specimen and the common reference RNA. B, Tissue dendrogram derived from clustering using 10,313 genes set. D, diffuse type; I, Intestinal type; M, Mixed type.

and determined by Lauren's classification of GA. Two-dimensional clustergram of the 10,313 genes selected with minimal filtering criteria resulted in two distinct molecular signatures on dendrogram of hierarchical analysis (Fig. 1A, B). The most of tissue samples in left side of subcluster are intestinal typed of GAs with a few exception and right side of subcluster are diffuse types of GAs. Mixed type of GAs are scattered in both intestinal and diffuse type subcluster.

Molecular Classification of Gastric Adenocarcinomas through Large-Scale Gene Expression Analysis

Since unsupervised analysis of GAs reflected that characteristic molecular profiles may discern different histopathological subtype of GAs, we next asked the characteristic molecular signature which may serve as classifier for the intestinal and diffuse type of GA. First, as from the result of unsupervised analysis, 4 of 5 mixed type GAs were belong to diffuse type subcluster, the data set to inquire molecular classifier was divided into intestinal versus diffuse and mixed type. The Wilcoxson ran T-test was used for the classifier determination and 156 genes were selected as high stringent outlier genes for these two subtypes. As shown in Figure 2, the resultant hierarchical clustering analysis was able to discern diffuse and mixed type with one exception (T22). Although this 156 classifier was successfully classifying the two distinct subtypes, all mixed types were belonging to diffuse type (Figure 2). Thus, the classification power of 156 outlier genes is not likely good classifier. We then next for further analysis of molecular classification, the mixed type of GAs were excluded for the nonparametric analysis data set. From this, 100 outlier genes were retrieved as classifier and resultant hierarchical clustering analysis was in Figure 3. As expect-

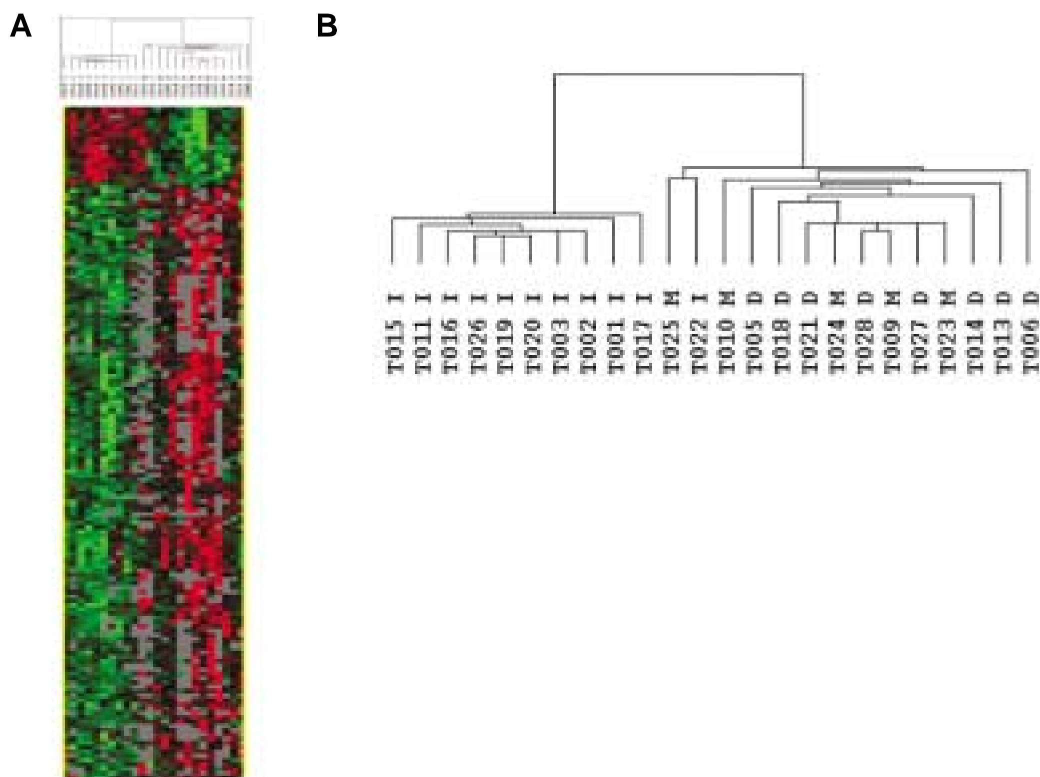

Figure 2. Supervised analysis of intestinal type and diffuse with mixed type of adenocarcinoma. Of total 24 GAs, there are 11 intestinal type, 8 diffuse type and 5 misedtype of adenocarcinoma examined by histopathological determination. To identify the outlier genes between IGA and DGA, expression data of 11 IGAs and 13 DGAa/MGAs were subjected to the The Wilcoxon rank T-test and were resulted 156 outlier genes. Two-dimensional clustergram of the 156 genes selected with minimal filtering criteria. Each row represents a tumor profile; each column represents a probe's measurement. The color saturation reflects the difference in expression between the tissue specimen and the common reference RNA. B, Tissue dendrogram derived from clustering using 156 genes set. D, diffuse type; I, Intestinal type; M, Mixed type.

ed, the molecular classifier was able to discern two distinct pathological subtype of GA with no exception on dendrogram.

Discussion

In this study, we have analyzed comprehensive gene expression profiling of two histopathological different subtype of GAs by using DNA microarrays. Histopathological examination of GAs based upon Lauren's classification divides into intestinal and diffuse type of GA. However, many of cases are not clear to classify into either intestinal or diffuse type due to ambiguous morphology of cells or just mixing with two different types. In our cases, 5 samples were also found to be mixed type of GA by pathologist. When these were subject to microarray study and analyzed by their expression patterns, the mixed type of GAs were not clearly belong to any of subtypes, but also dispersed in both intestinal and diffuse type (Figure 1). This implicates that histological mixed type of GA is supposed to have heterogeneous molecular signature of gene expression. Thus, the supervised analysis asking molecular classifier for GA should be categorized by intestinal and diffuse type. The resultant molecular classifier consisting 100 genetic elements was prove to have powerful classification ability to discern two distinct subtype of GA through hierarchical clustering analysis.

There are accumulative reports to identify molecular classifiers discriminating between gastric cancer and non-cancerous tumors or prognostic biomarkers by utilizing DNA microarray or array-based CGH study[7-9]. However, no attempts have been made to analyze molecular classifier for the subtype of GA. Actually expression profiling study of GA was tried to by Knuutila group, but this have been done with xenograft tumor and one diffuse type[10]. Thus, our results are considered to suggest novel molecular

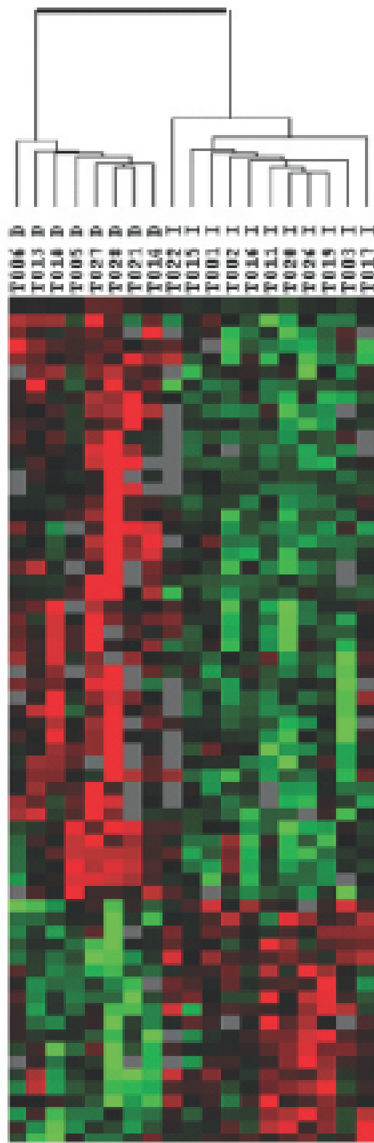

Figure 3. Molecular classification of intestinal type and diffuse type of gastric adenocarcinoma. To identify the molecular classifier between IGA and DGA, expression data of 11 IGAs and 8 DGAa were subjected to the The Wilcoxon rank T-test and were resulted 100 outlier genes. Two-dimensional clustergram of the 100 genes selected with minimal filtering criteria. Each row represents a tumor profile; each column represents a probe's measurement. The color saturation reflects the difference in expression between the tissue specimen and the common reference RNA. B, Tissue dendrogram derived from clustering using 100 genes set. D, diffuse type; I, Intestinal type.

classifier for GA classification. Among the list of 100 outlier genes, integrin related genes (ITGA1), Zink finger protein (ZNF253) and growth differentiation factor (GDF1) are up-regulated in intestinal type compared to diffuse type. Tubulin (TUBA3), fibroblast growth factor (FGF12), cadherin (PCHD20, CDH23) is down-regulated in intestinal type (data not shown). These genes that are differentially regulated in either intestinal or diffuse type GA may explain different molecular mechanisms underlying gastric cancer initiation and progression.

In conclusion, the large-scale molecular signature presenting in this study define the molecular classifier of GA and further suggest possible genetic elements responsible for the differentiation of GA, intestinal and diffuse type respectively. Our results may also provide useful information to explain the diversity of intracellular mechanism of gastric carcinogenesis.

Methods

Patients and Tissue Samples

Twenty four GAs of the stomach were included in this study. All cases were identified prospectively and consecutively in the Department of Pathology at Yonsei University Medical Center between September 1995 and November 2002 for molecular marker studies. Among these 24 cases 4 cases are mixed type of GA. Conventional pathologic parameters (tumor size, number, and grade) were examined prospectively without prior knowledge of the molecular data. For RNA extraction, fresh tumors and adjacent nontumorous smooth muscle or mucosal tissues were obtained immediately after surgical excision, and stored at −70°C before use. To enrich the tumor cell population, areas with more than 90% of tumor cells were selected from hematoxylin-eosin stained slides using the cryostat microdissection technique.

Preparation of Oligonucleotide Microarray

The 60 mers of Human Oligolibrary^{TM} package representing 18,664 LEADSTM clusters plus 197 controls (GAPDH) was purchased from Compugen/Sigma-Genosys and spotted onto a glass slide at the microarray core facility of Microdissection Genomics Research Institute at College of Medicine, The Catholic University of Korea.

RNA Preparation and Quality Assay

The quantity and quality of RNA of total and amplified RNA were assessed by using a Bioanalyzer 2100 (Agilent Technologies). For the evaluation study of amplified targets, we used Universal Human Reference (UHR) RNA (Stratagene) and a combination of RNAs (MIX) from adult human liver, adult human heart, adult human skeletal muscle (Stratagene) as our standard RNA for comparison analysis, and these

were labeled with Cy-3, or Cy-5[11,12].

Targets Preparations and Hybridization using Total RNA

Each 30 μg of UHR or MIX was labeled by conventional protocol with slight modification[13]. In brief, RNA was primed with 1 μg of oligo (dT) primer (Invitrogen) at 65°C for 10 min and reverse transcription was followed by adding 21 μL of master mix solution: 8 μL of 5X first strand buffer, 4 μL of 0.1 M DTT, 2 μL of 20X dNTP (10 mM dATP, dCTP, dGTP and 4 mM dTTP), 4 μL of 1 mM Cy3-dUTP(NEN) or Cy5-dUTP (NEN) and 2 μL of Superscript™ II (200 U/μL, Invitrogen). The Cy5-and Cy3-labeled targets were then combined and purified with a Microcon® YM-30 column (Millipore). The volume of purified targets were adjusted to 16 μL, and then mixed with 40 μg of Herring Sperm DNA and 20 μL DIG Easy hybridization solutions (Roche). The targets were then hybridized onto spotted-oligoarrays at 42°C for overnight. Washed arrays were scanned by using GenePix 4000B (Axon Instrument) and Cy3/Cy5-signals were measured by using a GenePix Pro 4.1 (Axon Instrument).

Scanning and Data Analysis

The arrays with hybridized targets were scanned using an Axon scanner and the scanned images were analyzed using GenePix® Pro 4.1 software (Axon Instruments) and spots of poor quality determined by visual inspection were also removed from further analysis. The resulting data collected from each array was submitted to the BioArray Software Environment (BASE) database at microarray core facility of Department of Pathology, College of Medicine at The Catholic University of Korea (http://genomics.catholic.ac.kr/). Data were normalized using the method of Linear Models for Microarray Data (LIMMA) and R-package for Statistics for Microarray Analysis (SMA). Spots of which size are less than 50 μm were eliminated for analysis unless otherwise specified. Pearson's correlation coefficient was calculated using S-PLUS program and Cluster, and TreeView programs were used for visualization of data.

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