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ORIGINAL ARTICLE

MNNG-Regulated Differentially Expressed Genes that Contribute to Cancer Development in Stomach Cells

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ABSTRACT

Cancer is a global health problem. There are diverse types of cancers, but there are several common pathways which lead to the development of cancer. Changes in gene expression might be the most common similarity found in almost all cancers. An understanding of the underlying changes in gene expression during cancer progression could lay a valuable foundation for the development of cancer therapeutics and even cancer vaccines. In this study, a well-known carcinogen, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), was employed to induce changes in gene expression in normal stomach cells. MNNG is known to cause cancer by inducing damage to DNA in MNNG-treated mammalian cells and animals fed with this carcinogen. An analysis was performed by comparing the differentially expressed genes (DEGs) caused by MNNG treatment with DEGs in stomach cancer cell lines. To this end, methods of analysis for functional categorization and protein–protein interaction networks, such as gene ontology (GO), the database for annotation, visualization, and integrated discovery (DAVID), Kyoto encyclopedia of genes and genomics (KEGG) and search tool for the retrieval of interacting genes/proteins (STRING), were used. As a result of these analyses, MNNG-regulated specific genes and interaction networks of their protein products that contributed to stomach cancer were identified.

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Key words
Differentially expressed genes
Gene ontology
Neoplasm
N-methyl-N’-nitro-N-nitrosoguanidine

INTRODUCTION

RNA sequencing has become the method of choice for comprehensive identification and quantification of gene transcripts. Development of next generation sequencing (NGS) technology probably enables large scale RNA sequencing, thereby analysis of the change in gene expression profile becomes easier for researchers. In most cases, when cells encounter physical, chemical, or biological stimuli, the cells respond by changing expression of the genes encoded in their genome. Gene transcripts are the resulting products of the cell’s response. Therefore, identification of transcripts that are increased or decreased might be an invaluable resource for analysis of the cell’s responses to the stimulus. Statistically significant change in expression of a gene in the cells between two experimental conditions is regarded as a differentially expressed genes (DEGs). Through analysis of readouts and changing amount of DEGs, the signaling networks...
working in the cells in response to the stimulus can be determined.

According to the World Health Organization (WHO), cancer has long been a highly ranked disease, leading deaths worldwide, accounting for nearly 10 million deaths in 2020. In Korea, gastric cancer was the most common cancer and it remained the second most common cancer in 2020 [1]. Discovery of DEGs that might contribute to formation of cancer must be a valuable foundation for development of cancer therapeutics and even cancer vaccines. Because development of cancer takes time, treatment of normal cells with a strong cancer inducer is a good alternative method for discovery of the cancer-promoting DEGs in a short period of time. A well-known carcinogen, N-methyl-N’-nitro-N-nitrosoguanidine (MNNG), has been broadly used to induce diverse cancer formation in normal cells and animals since its successful induction of stomach cancer formation in treated rats [2]. MNNG-induced cancer models have been used in determining the effects of Helicobacter pylori infection during cancer formation [3], screening of tumor suppressors [4], and early detection of cancer [5]. In addition, many scientific reports support that MNNG-induced cancer formation is a good mimetic model of animal cancer including humans [6, 7].

In the current study, an analysis of MNNG-induced DEGs in normal stomach cells was performed by comparison with DEGs from established stomach cancer cell lines at approximately change level, protein-protein interactions (PPIs) in the pathway for cancer formation. These analyses resulted in discovery of new DEGs and their PPIs in the pathways for cancer formation.

**MATERIALS AND METHODS**

1. **Cell culture, RNA preparation and NGS sequencing**

Normal human stomach cell line, HS738, and human stomach cancer cell lines, AGS and MKN45, were cultured under DMEM containing 10% fetal bovine serum (FBS). DMSO or MNNG dissolved in DMSO was added to the culture medium to be 50 μM. Two incubation periods, 6 hours or 24 hours, were adopted for monitoring direct induction or indirect induction included, respectively. An RNeasy kit from Qiagen was used for purification of total RNA from the treated cells. Sequencing of total mRNA was performed at E-Biogen (Seoul, South Korea) using NGS.

2. **Analyses of differentially expressed genes**

An analysis of the sequenced mRNA information provided in Excel format was performed using Excel based DEG analysis (ExDEGA, ver. 3.0.1) provided by E-Biogen. Collection of DEGs from the MNNG-treated cells was based on P value (<0.05) and induction folds (≥3.0) compared with DEGs from the DMSO-treated cells. The collected genes were transformed into a heat map using WebMeV (a cloud-based application supporting analysis, visualization, and stratification of large genomic data) then listed up via hierarchical clustering. Two groups of clustered genes, up-regulated and down-regulated, from AGS and MKN45 were listed next to the DEGs from normal stomach cells treated with MNNG.

3. **Analyses of gene ontology (GO), Kyoto encyclopedia of genes and genomes (KEGG), and STRING**

GO were performed using a web-based DAVID Bioinformatics Resources 6.8. The database for annotation, visualization and integrated discovery (DAVID) V6.8 comprises a full knowledgebase update and provides a comprehensive set of functional annotation tools to understand the biological meaning behind the long list of genes. An analysis of the connection between DEG and biological pathways was based on the KEGG database. Analysis of functional protein association networks of the selected genes was performed using the STRING web site, from which a biological database and web resource of known and predicted protein-protein interaction (PPI) are
available. In this study, only the known PPIs were used to draw networks, which were visualized using Cytoscape software (version 3.5.1).

RESULTS

1. Sorting of the DEGs

DEGs from normal stomach cells treated with MNNG were listed from top to bottom according to their induction or reduction folds (≥3.0 folds, log2 ≥1) and determination of significance was based on their P-value (<0.05) obtained using the Benjamini-Hochberg procedure. Equivalent genes expressed in DMSO-treated normal stomach cells were used as a control. Ribosomal protein SA (RPSA) is the gene most down-regulated by MNNG treatment, while AP000758.1 is the gene most up-regulated. The equivalent genes expressed in AGS or MKN45 were enumerated matching with the MNNG column (Figure 1). Seventeen and 33 equivalent genes from AGS and MKN45 had the same direction of increase or decrease with the MNNG-regulated genes, respectively (Figure 1). Thus, a number of MNNG-reduced DEGs almost twice as large had the same direction of expression with DEGs from cancer cells.

2. Analyses of gene ontology

Functional categorization of the MNNG-regulated DEGs was performed using DAVID analysis. Only the genes with known functions were considered allowing duplication for the analysis. Of the up-regulated DEGs, the highest number participated in “signaling transduction”, and the “cellular response to DNA damage stimulus” was the second most common GO. Given that MNNG causes damage to DNA, it was assumed that a number of up-regulated DEGs belonged to the GO categories of “cellular response to DNA damage” and “signal transduction” (Figure 2). In the case of the reduced genes, a large number of genes belonged to GO of cell proliferation, such as “cell division”, “mitotic nuclear division” or “sister chromatid cohesion” (Figure 2). Results of these analyses indicate that the MNNG-induced DEGs are responsible for signaling pathways required for cancer formation, while the reduced DEGs are involved in cell division.

3. MNNG-regulated DEGs that are commonly expressed in stomach cancer cells

Next, we determined the number of MNNG-regulated DEGs in common with the DEGs from two stomach cancer cell lines (AGS and MKN45). In spite of its well-known cancer-promoting activity, few MNNG-regulated DEGs were expressed in common with the two cancer cell lines. Of the DEGs from MNNG-treated normal cells, 10 and eight DEGs were commonly up-regulated in AGS or MKN45 cells, respectively, while

![Figure 1. Heatmaps of the differentially expressed genes (DEGs) by MNNG treatment. (A) Up-regulated DEGs are listed in red color from top to bottom according to induction folds. (B) Down-regulated DEGs are listed in blue color from top to bottom according to reduction folds. Expression of the MNNG-regulated DEGs in the cancer cell lines is indicated next to the MNNG column. The analyzed DEGs are included based on the following criteria: fold change ≥3, log2 ≥1, P value ≤0.05.](image-url)
an increase in seven DEGs was observed across all of the cells (Figure 3). In the case of the MNNG-reduced DEGs, 33 DEGs were commonly down-regulated across all of the cells, while only two DEGs were commonly down-regulated in AGS cells. No common gene was accordingly decreased between MNNG-treated stomach cells and MKN45 stomach cancer cells (Figure 3). The number of contra-regulated DEGs was also determined, where expression was opposite between the compared cell types. Of the MNNG-induced DEGs, 49 DEGs belonged to that category. The relationship of gene expression was much stronger between the two cancer cells compared with that of MNNG-treated normal cells. A large number of DEGs in the two cancer cell lines have the same direction of expression: 746 up-regulated and 1440 down-regulated DEGs were overlapped between each other (Figure 3).

4. STRING analyses of the DEGs related to cancer formation

Individual genes in the MNNG-regulated DEGs were mapped for retrieval of a PPI network using the STRING database [8]. The STRING analysis was performed using genes that could be searched from “KEGG pathways in cancer”. Species was limited to “Homo Sapiens” and an interaction score >0.4 was applied in order to draw the PPIs. First, an analysis of MNNG-induced DEGs that overlapped with the two cancer cells was performed. Among the up-regulated DEGs, it was discovered that three proteins have roles in the cancer pathways: PIK3R2, MDM2 and LAMA5. The expression level of PIK3R2 in MKN45 was not significant. Common pathways for the three proteins were “small lung cancer”, “PI3K-AKT signaling pathway”, and “human papillomavirus infection”. PIK3R2 and MDM2 shared more pathways, however no known PPI was discovered between them (Figure 4A). Among the down-regulated DEGs, two PPIs were identified between EGF and FGF5, and between COL4A1 and COL4A2. These four proteins were commonly involved in the pathways of the “PI3K-AKT signaling pathway”. A larger number of proteins belonging to the down-regulated DEGs were found to have involvement in the cancer pathways (Figure 4B). Second, an analysis of DEGs that were exclusively common to the two cancer cells was performed. Similarly, a larger number of PPIs between protein products of the down-regulated DEGs were identified. Among the up-regulated DEGs, two PPIs between LAMA5 and LAMA3, and between FGFR4 and FGFR2 were identified (Figure 4C). PPIs from the down-regulated DEGs included two groups composed of several proteins: one group centered on several
proteins including FGFR1, EGF, GNA11, and GNB4, and the other group centered on ITGB1. The "PI3K–AKT signaling pathway" and the "Rap1 signaling pathway" were the most common pathways for the proteins involved in the PPIs (Figure 4D). These results indicate that the established cancer cells have a larger number of PPIs from the DEGs belonging to decreased expression.

5. STRING analyses of MNNG-regulated genes that are not commonly expressed in cancer cells

Some of the MNNG-regulated DEGs were not in common with the DEGs from the cancer cells. An analysis of PPIs of DEGs that belonged exclusively to MNNG-treated normal cells was performed using STRING, and their roles in possible pathways were examined. PPI between TUBA1C and TUBA1B was identified from the DEGs which showed less expression...
Figure 4. Protein–protein interaction (PPI) network analyses using the STRING database for the DEGs expressed in the cancer cells. (A) PPI between the DEGs up-regulated by MNNG-treatment, of which expression also increased in the cancer cells. (B) PPIs between the DEGs down-regulated by MNNG-treatment, of which expression also decreased in the cancer cells. (C) PPIs between the DEGs, of which expression increased exclusively in the AGS and MKN45 cancer cell lines. (D) PPIs between the DEGs, of which expression decreased exclusively in the AGS and MKN45 cancer cell lines. Each circle indicates a protein and the color inside each circle indicates a pathway from the KEGG pathway database wherein functional roles of the protein are found. The color of the strings connecting each circle indicates known interactions between two proteins (Blue: from curated databases, Red: experimentally determined).

in 24 hours than in 6 hours since MNNG treatment. These two proteins have been found to have signal roles in degenerative neuronal diseases, including “Parkinson disease”, “Amyotrophic lateral sclerosis”, and “Alzheimer disease” (Figure 5A). Next, an analysis of MNNG-regulated DEGs in normal stomach cells at 6 hours after treatment was performed for identification of PPIs. Among the up-regulated DEGs, four groups of PPIs
Figure 5. Protein-protein interaction (PPI) network analyses using the STRING database for the DEGs exclusively regulated by MNNG. (A) PPI between the DEGs, of which expression decreased in normal stomach cells after treatment with MNNG for 24 hours. (B) PPIs between the DEGs, of which expression increased in normal stomach cells after treatment with MNNG for 6 hours. (C) PPIs between the DEGs, of which expression decreased in normal stomach cells after treatment with MNNG for 6 hours.

were identified, however, the number of PPIs with identified roles in the KEGG pathway was limited. PPIs between LAMB4 and LAMB1 and between HIST1H2BH and HIST1H2BM may have roles in cytoskeleton rearrangement (Figure 5B). Three groups of PPIs were identified from the down-regulated DEGs at 6 hours after MNNG treatment. Most of the proteins involved in the identified PPIs had roles in the "PI3K-Akt signaling pathway". In particular, the "PI3K-AKT signaling pathway" was the only known pathway for the PPI between PIAPH2 and PIAPH3 (Figure 5C). These results indicate that MNNG-induced gene expression at early phase shows an increase of genes for cytoskeleton proteins and a decrease of genes for the PI3K-AKT signaling pathway.

DISCUSSION

Transformation of normal cells to cancer cells essentially relies on changes in expression of genes required during the transformation [9]. Regardless of its manner of expression, whether it is genetically induced or epigenetically induced, it is obvious that a gene expression pattern that is different from normal is prerequisite. Therefore, attaining an understanding of DEGs can provide the underlying mechanism of cancer development. Development of a rapid sequencing technique known as NGS has enabled a systemic
approach to analysis of DEGs. Due to the capacity for rapid sequencing, almost all of the mRNA identity in a cell can be identified in a short period of time. In this study, because MNNG is recognized as a cancer-inducer to a broad range of cancers in vitro and in vivo, MNNG was employed to induce changes in gene expression in normal stomach cells [9, 10]. Purification and sequencing of the whole mRNA was performed using an NGS machine. Large scale sequencing data was formatted as Excel based differentially expressed gene analysis (ExDEGA), with which further analyses including DAVID, GO, and KEGG were performed.

Previously, study of MNNG-induced gene expression was conducted by others using an oligonucleotide microarray containing ~8,000 probe sets [11]. According to the results, up-regulated genes belonged to extracellular matrix remodeling (i.e. Collagen types I, II, V, MMP3), immune response (i.e. lysozyme, complements), and ossification (i.e. Osteoblast-specific factor). The down-regulated genes belonged to hydrocarbon metabolism (i.e. aldose A, aldehyde-dehydrogenase), gastric juice (ion transporter genes), mucous production (Mucin 5), and gastric hormone (gastrin and somatostatin) [12]. The MNNG-regulated DEGs identified in our results are different from those of the previous probe-based analysis. Differences in sequencing methods might be the major reason between pre-selected probe sets and total expressed mRNA sequencing. In our results, the highest number of up-regulated DEGs belonged to the GO categories of “cellular response to DNA damage” (Figure 2). Thus, it was confirmed that the experimental processes were adequate.

Two differentiated methods of analysis were employed in this study. The first analysis was a comparison between MNNG-regulated DEGs and DEGs from the two cancer cell lines, to determine whether DEGs contributed to cancer at early stage development or later stage maintenance. The second analysis was that of protein interaction networks of the DEGs with roles in the cancer pathway. Simply indicating an increase or decrease of expression of a gene cannot determine the difference between normal cells and cancer cells. Actual functions in cells are performed by proteins that interacted with other molecules and each other, therefore analysis of PPIs of protein products of the DEGs results in a clearer understanding of the function of the gene and its contribution to cancer formation [13, 14]. Here a couple of significant MNNG-induced PPIs that might have the capacity to promote stomach cancer formation were identified. Of the MNNG up-regulated DEGs at 6 hours, PPIs between LAMB4 (laminin beta 4) and LAMB1 (laminin beta 4), and between HIST1H2BH (histone H2B type 1-H) and HIST1H2BM (histone H2B type 1-M) PPIs were exclusively identified (Figure 5B). Therefore, in MNNG-induced early phase, even when not reaching cancer status, cytoskeletal changes and histone modification could occur. Subsequently, PPIs found in cancer cells transformed from normal cells by MNNG treatment were identified by analysis of MNNG-induced DEGs overlapped with DEGs from the two cancer cell lines. From the larger number of PPIs identified, proteins including FGFR1, EGF, GNAI1, GNB4, and ITGB1 had centered roles. FGFRs 1-4, which are transmembrane proteins, have oncogenic or anti-oncogenic roles depending on the ligands and cellular environments [15]. EGF regulates important cellular processes, such as proliferation, differentiation, and survival by binding to its receptor, EGFR [16]. GNAI1 (guanine-nucleotide binding subunit I subunit alpha-1) and GNB4 (guanine-nucleotide binding subunit beta-4) are parts of heterotrimetric signal-transducing proteins [17]. ITGB1 (Integrin subunit beta 1) is a cell surface receptor that works as a collagen receptor [18]. Collectively, these identified proteins indicate that MNNG-induced disturbance at the area of cellular communication with the environment, cell surface, might be critical to inducing cancer formation. In addition to the changes in membrane, the PI3K-AKT signaling pathway was the down-stream signaling pathway that was mainly modified by MNNG treatment.
Changes in interaction of membrane proteins together with down-regulation of the PI3K-AKT signaling pathway lead to disruption of proper cellular responses. Recent research papers used MNNG as a proven cancer-inducer for examining anti-cancer effects of the molecules [6, 7]. But these studies had been conducted without considering MNNG-induced genetic changes. This study could enable more precise interpretation about function of the anti-cancer molecules and developing more effective cancer therapeutics by providing target genes or protein networks to be intervened. We expect that these results may shed light on the understanding of gene expression and their protein networks that contributes to neoplasm initiation in normal stomach cells.

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