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Differentiation and adaptation epigenetic networks: Translational research in gastric carcinogenesis

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There are several kinds of epigenetic networks in the human body including the cell differentiation epigenetic network (DiEN) and the host adaptation epigenetic network (AdEN). DiEN networks are static and cell/tissue-specific. AdEN networks are variable and dependent upon environmental factors. DiEN and AdEN alterations can respectively serve as biomarkers for different kinds of diseases. Cancer is a consequence of accumulated pathophysiological adaptations of tissue stem cells to exposure of environmental carcinogens. Cancer cells are de-differentiated cells that obtain the capacity of unrestricted proliferation, local invasion, and distant migration/metastasis. Both DiEN and AdEN changes can be observed in cancer tissues. Alterations of DNA methylation are the most stable epigenetic modifications and can be sensitively detected in a small cell population. These advantages make DNA methylation the optimal biomarkers for detection of initiated cells in precancerous lesions and metastasis stem cells in cancer tissues. It has been proven that *p16* methylation can be used as a diagnostic biomarker to determine malignant potential of epithelium dysplasia in many organs including the stomach. In a large-scale validation study on the DNA methylome of gastric carcinomas (GC), the methylation status of more than 90 CpG islands has been analyzed by DHPLC. Furthermore, *GFRA1* demethylation and methylation of *SRF* and *ZNF382* are frequent events during gastric carcinogenesis and consistently correlate to GC metastasis and overall survival of GC patients from China, Japan, and Korea, respectively. In a population study, it has been demonstrated that gradual increasing of plasma *miR-211* and other *miRNA* levels may be an early risk predictor for GC development.

epigenetic, cell differentiation, adaptation, gastric carcinoma, DNA methylation, CpG islands, miRNA, biomarkers

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1 Differentiation and adaptation epigenetic modifications

There are several kinds of epigenetic networks in the human body, including the cell differentiation epigenetic network (DiEN) and the host adaptation epigenetic network (AdEN). DiEN networks are stable and cell/tissue-specific. AdEN networks are variable and dependent upon environmental factors. DiEN and AdEN alterations can respectively serve as biomarkers for different kinds of diseases. Epigenetic modifications and their composition patterns are diverse and complex in the body. It is the diversity that provides the necessary machinery needed to drive embryo stem cells to differentiate into about 210 distinct cell types in the body and enables the body to adapt efficiently to different environmental factors [1–3]. The complexity of epigenetic networks also becomes one of the main obstacles for basic and translational research on epigenetics. On one hand, the complexity and diversity of epigenetic networks constitute endless research points. On the other hand, there are numerous pathways/molecules driving the mechanisms of carcinogenesis and other pathogenesis that could be used to

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develop novel molecular stratification systems for various diseases, therapeutic strategies, and drug design.

Cancer is a consequence of accumulated pathophysiological adaptations of tissue stem cells to exposure of environmental carcinogens. Cancer cells are reprogrammed cells with the capacity of unrestricted proliferation, invasion, and metastasis. Therefore, both types of epigenetic changes can be observed in cancer tissues. Although modern epigenetic research began only 15 years ago, several clinical applications have already originated from epigenetic studies. For example, epigenetic modulators, including DNA methylation inhibitor 5-aza-deoxycytosine and histone deacetylase inhibitors vorinostat or romidepsin, have recently been approved by the FDA for patients with myelodysplasia syndrome and cutaneous or peripheral T-cell lymphoma, respectively. Developments of DNA methylation biomarkers (*Septin9*, *p16* [*cdkn2a*], *MGMT*) for cancer diagnosis and prediction of chemotherapy sensitivity are currently under clinical trials [4]. More application products will originate from epigenetic research if we can screen out and sub-classify the disease-related epigenetic modifications from the complex epigenetic networks for translational practices.

Differentiation states of somatic cells are almost lifetime-consistent. Thus, basic differentiation epigenetic networks (DiEN) should be lifetime-consistent. The stable DiEN networks are cell/tissue-specific and environmental factor-independent. In contrast, the adaptation epigenetic networks (AdEN) are local/systemic reactions and environmental factor-dependent. In fact, the epigenetic alteration profiles in pathological lesions are also disease-specific. DiEN disorders may play a driving role in the development of diseases related to disorders of cell differentiation, and thus become potential diagnosis biomarkers or therapeutic targets in these diseases. The differentiation-related diseases include cancers and precancerous lesions with cell de-differentiation, psoriasis and Alzheimer's disease with cell over-differentiation, intestinal/squamous metaplasia and endometriosis with cell trans-differentiation, and cell degenerating diseases (diabetes, cataract, vitiligo, aplastic anemia). AdEN modifications represent long-term biological responses of host cells to exposure of environmental factors. They can be employed to detect the host's susceptibility to environmental factors and predict sensitivity of disease to chemical and physical therapies. Although the DiEN-AdEN classification of epigenetic networks may significantly improve the efficiency of translational research, the concept using a corresponding class of epigenetic modifications as biomarkers for disease has not been well recognized.

In addition, due to the cell/tissue-specificity of DiEN networks, tissue/organ samples have to be used in determination of DiEN biomarkers for the corresponding diseases. Surgically resected tumor tissues or tissue biopsies are optimal biological samples for analysis of epigenetic biomarkers. It is well recognized that cancer stem cells in precancerous/cancer tissues should contribute to cancer development, metastasis, recurrence, and formation of drug resistance. However, because the changes of gene expression occur in a limited number of stem cells, their visibility would be greatly reduced by the co-existent main cell populations in which the gene expression has not changed. Therefore, detection of alterations of gene expression in these stem cells is very difficult with regular gene expression assays such as immunostaining, western blot, RT-PCR, and northern blot. The methylation status of CpG islands around transcription start sites represents the possibility for a particular gene to undergo transcription. Notably, methylated and demethylated CpG islands can be analyzed with methylation- and demethylation-specific assays, respectively. This makes the detection of CpG island methylation status so sensitive that methylation alterations that occur in a few cells from a particular tissue (about 0.1% of cell population) can be clearly displayed. Furthermore, DNA methylation can be preserved in various samples stored in a wide-range of conditions (fresh/frozen or paraffin-embedded tissue blocks, free DNA in plasma, detached cells in gastric juice, sputum, urine, stool samples, and other body fluids). These advantages make DNA methylation an optimal biomarker for cancer prevention, diagnosis, and tailored treatment [4]. Epithelial dysplasia is the main precancerous lesion in many organs in the body. We have proven that methylation of *p16*, the G1→S conversion checkpoint gene in the cell cycle, is a powerful biomarker for predicting malignant transformation of epithelial dysplasia [5,6]. This has been consistently confirmed by a number of independent studies in different countries [7–9]. Recently, we have developed a *p16*-specific probe-based quantitative MethyLight assay for the development of a *p16* methylation diagnosis kit [10].

2 Current status of DiEN and AdEN network research

Various high-throughput platforms used in 'omics' analysis are revolutionary tools for biological and medical research. Combined with bioinformatic analysis software and concepts on systemic biology, the high-throughput data enables us for the first time to see biological activities in the body from a 4D (3 dimensions and time) perspective [3]. However, because of the limited human and financial resources available for research, it is impossible to validate the huge amount of raw data that flows out daily from these platforms. The most frequent method of analysis for these omics data for scientists is to carry out bioinformatic analysis, validate a few genes, and write a manuscript for publication. About 2% of DNA sequences in the human genome encode proteins. Protein-encoding genes include housekeeping genes, tissue-specific genes, and biotransformation/adaptation genes. The ENCODE project has provided huge amount of valuable data on various epigenomes [3]. At present, it is not well

defined what gene set corresponds to cell differentiations and what gene set corresponds to adaptations. It is not clear what gene expression changes relate to pathogenesis and can be used as biomarkers either. Although there is still much omics research work that needs to be done, we have begun to dissect various epigenetics activities from the networks' point of view.

A number of high-resolution epigenomes in the ENCODE project have been reported recently [3]. Lister et al. [11] and Laurent et al. [12] have respectively reported DNA methylomes at the single-base resolution in human embryo stem cells and the differentiated fetal or neonatal lung fibroblast cells using second-generation deep bisulfite-sequencing. Chodavarapu et al. [13] has studied the genome-wide relationship between DNA methylation and nucleosome positioning in a human embryo stem cell line and *Arabidopsis thaliana*. Using chromatin immuno-precipitation (ChIP) enrichment technologies, Barski et al. has generated high-resolution maps for the genome-wide distribution of 20 histone lysine and arginine methylations as well as histone variant H2A.Z in human blood $CD4^+$ T cells [14]. These genome-wide high-resolution data are important resources for investigations of DiEN networks. Using these databases, scientists can get preliminary information on DiEN networks in individual chromatin regions throughout the whole genome of these cell types. Multiple epigenomes and RNA transcriptomes in more normal cell types need to be sequenced to explore cross-talk between different types of epigenetic modifications in various intragenic and intergenic non-coding regions in the genome [15,16]. Differences in the epigenomes between different cell types are important clues for investigating the functions of the 98% non-coding regions in the genome. Comparison studies on epigenetic networks in normal tissues with pathological lesions are also essential for translational research.

DNA methylation is the most stable epigenetic modification. Recent studies have shown that the status of DNA methylation at some regions in the gene body may be the consequence of a dynamic balance between methylation and hydroxymethylation or demethylation [17–19]. Moreover, rather than being actively transcribed or completely silenced, many genes are transcribed at different levels in tissues. Methylation contributes to these transcriptional differences between tissues is often observed in CpG island shores (DNA sequences near CpG islands), but not in the core CpG islands around the transcription start sites [20,21]. Establishment of *de novel* DNA methylation *in vivo* involves multiple processes. DNA methylation is initiated at one side of the CpG islands and then extended into the core region [22–24]. Our studies have shown that nucleosomes in tumor suppressor genes (*p16*, *spint2*, and *hMLH1*) may be the basic units for extension of *de novel* DNA methylation and demethylation in both cell lines and gastric tissues. We suggest that most CpG sites in the same nucleosomal DNA may be methylated/demethylated simultaneously, then extended to its flanking nucleosome one by one [24,25]. This is consistent with the recent genome-wide study that DNA methylation in an embryo stem cell line correlates to nucleosome positioning [13]. In studies of alterations of DNA methylation as cancer markers, methylation of crucial CpG sites inversely correlated with inactivation of gene transcription should be characterized and used to represent inactivation status of the gene by DNA methylation. Detection of methylation in these CpG sites for more than one nucleosome DNA may be necessary, if possibk, during the development of methylation biomarker kits.

Notably, the stability of DNA methylation is gene or nucleosome-dependent. For example, *p16* inactivation by methylation in cancer cells is very stable. Methylation of *p16* CpG islands can be gradually restored within about two-weeks after demethylation induced by DNMT1 inhibitor treatment, with or without combined treatment of HDAC inhibitor [26]. After the *p16*-methylated cell line AGS is fused with the *p16*-unmethylated cell line MGC803, we find that the methylation status of *p16* CpG islands is very consistent in fusion cells. Most *p16* alleles from the AGS cells are methylated, while *p16* alleles from the MGC803 cells are not methylated in the fusion cells. This is similar to *p16* hemi-methylation in HCT116 cells in which the mutant *p16* alleles are not methylated and the wildtype alleles are methylated. In contrast, the methylation stability of *Spint2* is nucleosome-dependent. The *Spint2* CpG island is completely methylated in MGC803 cells and not methylated in AGS cells. Interestingly, all *Spint2* alleles in the fusion cells, whether from MGC803 or AGS, are fully demethylated at two 5′UTR nucleosomes, but completely methylated at the exon-1 coding nucleosome from MGC803 derived alleles (Lu Z M, et al., unpublished data). Both *p16* and *Spint2* are actively expressed in the fusion cells. Therefore, characterization of methylation stability of candidate genes in cancer cells and its associations with differentiation/adaptation is also a key issue in translational research [27].

Some association studies have been reported on the potential clinical application of using global levels of certain types of histone modifications in tissue samples. Changes of histone modifications in tissues may be used as biomarkers for the prediction of prognosis of cancers. However, it is unknown how these histone modifications affect behavior of cancer cells and the clinical outcomes of cancer patients. Combination of ChIP and genome-wide deep sequencing may be a powerful tool able to characterize their target genes/loci and related epigenetic networks in cancer tissues.

Differential methylation hybridization using CpG island microarray and restriction landmark genome scanning (RLGS) are two first generation assays for detection of DNA methylomes of tissue or cell samples. These assays can be conveniently used to screen methylation differences between tissue samples. These array studies reveal that the alterations of DNA methylation have non-random and tissue- and tumor type-specific patterns [20,28]. These plat-

forms are also frequently used to characterize the methylation differences in the genomes related to development and prognosis of cancers [29–31]. Because of low detection sensitivity and limited cell number, alterations of methylation in CpG islands that occurred in cancer stem cells could not be detected using these array-scanning assays. It is almost impossible to validate the numerous methylation alterations with precise bisulfite-sequencing due to time/cost constraints. Because of the lack of information on the distribution of methylated CpG sites in the candidate CpG islands, the feasibility and efficiency to validate candidate methylation using CpG site-specific assays such as methylation-specific PCR (MSP), combined-bisulfite restriction assay (COBRA), MethyLight, etc are quite low. Consequently, almost all of the raw data generated from these arrays has not been confirmed yet. In the following paragraphs, we briefly introduce the progression of our large-scale validation study on the DNA methylome of gastric adenocarcinomas using a cheap, efficient, denatured high-performance liquid chromatography (DHPLC) method and investigation of epigenetic epidemiology on gastric carcinomas.

3 Alterations of DNA methylation as biomarkers for metastasis of gastric carcinomas

Cancer is a cell de-differentiation disease. It is a consequence of carcinogen-induced pathophysiological adaptations in normal cells. Malignant transformed cells are epigenetically reprogrammed with a decreased genetic stability. Cancer cells are capable of stimulating a series of epigenetic changes in the stromal cells that lead to angiogenesis and extracellular matrix degradation to favor tumor growth, invasion, and metastasis. At the same time, the human body can make a series of local and systemic epigenetic adaptations against cancer cells.

Progression of precancerous lesions, cancer invasion, metastasis, chemosensitivity, or drug resistance are biological properties of cancer cells. Thus it is possible to determine these properties using biomarkers. Increased invasion and migration abilities of cancer cells and decreased defense ability of stromal cells to restrict cancer cells are two crucial steps in the process of metastasis. It is recognized that only part of the cancer stem cells in tumor mass are able to invade, migrate, and colonize in distant organs or tissues. We hypothesize that alterations of DNA methylation in these metastatic stem cells can be sensitively detected and serve as good prediction markers for cancer metastasis.

Using the methylated CpG island amplification in combination with microarray (MCAM) [31], we analyzed the methylation status of the CpG islands in 6177 genes in the genome of 4 metastatic and 4 non-metastatic GCs and their corresponding surgical normal margin samples, respectively. Using differential data of normalized methylation signals between 8 GC and 8 normal samples, we have selected a set of candidate CpG islands based on significant differential methylation related to carcinogenesis for further validation. Similarly, using the differential data of tumor/normal signal ratios between 4 metastatic and 4 non-metastatic GCs, we have also selected a set of metastasis-related candidate CpG islands. From the list of candidate genes, CpG islands of 90 genes were bioinformatically enrolled into further validation studies, if they (or their family members) could be involved in cell proliferation or migration [32].

It is well recognized that repressive effects of methylation of CpG sites in CpG islands on gene transcription is region-dependent. These CpG sites are called crucial CpG sites when their methylation correlates to complete inactivation of the corresponding genes. Generally, the crucial CpG sites may locate within a two-nucleosome region around transcription start sites. Because the crucial CpG sites are not characterized for most CpG island-containing genes, we used the DHPLC assay to validate alterations of the methylation status of all 90 genes. DHPLC is a convenient assay for quantifying the proportion of methylated CpG islands we established previously [33,34]. Like bisulfite sequencing, PCR products (400–1000 bp) of methylated and unmethylated CpG islands amplified with CpG-free primers are analyzed with this assay. These PCR products can be directly sequenced if further confirmation and characterization of key CpG sites are necessary.

DHPLC assays are setup for the detection of methylation of all 90 CpG islands, respectively. Using these assays, we found the methylation of 15 genes (*BMP3*, *BNIP3*, *ECEL1*, *ELK1*, *GFRA1*, *HOXD10*, *KCNH1*, *p16*, *PSMD10*, *PTPRT*, *SIGIRR*, *SRF*, *TBX5*, *TFPI2*, *ZNF382*) are significantly related to gastric carcinogenesis. Of them, methylation-inactivation of *SRF* or *ZNF382* and demethylation-activation of *GFRA1* are not only related to the development of GC and other cancers, but are also consistently related to metastasis and overall survival of GC patients in these discovery, testing, and international validation cohorts. These findings show that alterations in methylation of *SRF*, *ZNF382*, and *GFRA1* CpG islands are potential biomarkers for the prediction of GC metastasis [35–37]. It is necessary to study the feasibility of their clinical applications in prospective studies among cancer patients without baseline metastasis. In addition, we find that the methylation status of 9 *miR* CpG islands (*miR-9-1*, *miR-9-3*, *miR-34b/c*, *miR-137*, *miR-193b/365a*, *miR-200b/200c/429*, *miR-210*, *miR-375*, and *miR-663*) is significantly changed during gastric carcinogenesis. *miR-9-1* and *miR-137* methylation are mainly detected in GC samples, indicating they could be used as cancer-specific biomarkers. *miR-9-1* methylation is also weakly associated with GC metastasis and patients' overall survival. Notably, we found that the methylation status of these CpG islands is inversely and consistently correlated to transcription of the corresponding mature miRNA level by quantitative RT-PCR in a panel of cell lines *in vitro*. In gastric tissue samples, such inverse methylation-repression association

could also be observed for *miR-9-1*, *miR-9-3, miR-137*, and *miR-200b*. These results suggest that transcription of *miR* genes in the genome may be regularly regulated by the methylation status of CpG islands [38].

4 Progression of epigenetic epidemiology of gastric carcinomas

H. pylori infection causes chronic gastritis, digestive ulcer, and gastric mucosa-associated lymphoid tissue (MALT) lymphoma. Because there are close relationships between chronic atrophic gastritis and GC, *H. pylori* infection is also considered as a risk factor of GCs. It has been reported that *H. pylori* infection induces alterations of methylation of many CpG islands through inflammatory factors [39,40]. The inflammatory factors may theoretically release from the local gastritis lesions to the bone marrow through circulation. It is interesting to investigate if *H. pylori* infection leads to alterations of DNA methylation in both gastric mucosa and white blood cells (WBC). Using a high-throughput platform at Dr. Yongsong Kim's Lab in Korea, we are comparing the DNA methylomes of gastric mucosa biopsies and peripheral blood WBC samples from gastritis patients before and after *H. pylori* eradication, respectively. The DHPLC assay will be used to validate the array data in more patients.

There are a number of reports on alterations of plasma mature miRNA levels in advanced cancer patients. But it is uncertain whether these alterations can be detected in early cancer patients and subjects with precancerous lesions [4]. It is hypothesized that circulating miRNAs may release locally from cancer tissues or from other tissues/organs as systemic adaptations. To clarify the feasibility of using circulating miRNA as early predictors for GC development, Song et al. have determined circulating miRNA profiles in serum samples from GC patients and gastritis controls enrolled in a prospective study [41]. Using a low-density miRNA array, they found that the average levels of 8 miRNA (*miR-221*, *miR-744*, *miR-376c*, *miR-191*, *miR-27a*, *let-7e*, *miR-27b*, and *miR-222*) are significantly different between these patients in the training cohort (n=28) and testing cohort (n=136). Further analysis showed that the average *miR-222* level is increased in gastric dysplasia patients, compared with gastritis patients without dysplasia. Notably, historical comparison of serial serum samples from 20 GC patients (collected in 1989, 1992/1994, and 1999/2003 before cancer diagnosis) shows that levels of serum *miR-221*, *miR-744*, and *miR-376c* had progressively increased in these patients before GC diagnosis. However, increasing patterns of these miRNAs were not observed in gastritis control subjects. It is well recognized that it is difficult to detect cancer risk using single sample analysis data. Dr. Song's work implies that dynamic changes of circulating miRNA levels might be one kind of useful predictor of cancer risk.

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