DNA Methylation Research  
- My Experience in Johns Hopkins University -

KIICHI SUGIMOTO*1) 2), TOMOAKI ITO*1) 3), HAJIME ORITA*3), SAKI FUJITA*1), KAZUHIRO SAKAMOTO*2), KOICHI SATO*3), MALCOLM V BROCK*1)

*1)Department of Surgery, The Sidney Kimmel Comprehensive Cancer Center at the Johns Hopkins University School of Medicine, Maryland, USA, *2)Department of Coloproctological Surgery, Juntendo University Faculty of Medicine, Tokyo, Japan, *3)Department of Surgery, Juntendo University Shizuoka Hospital, Shizuoka, Japan

Background: I (the first author) studied DNA methylation in cancer at Johns Hopkins University from December 2013 to July 2016. In this review, we introduce our DNA methylation study in gastric cancer. Across a diverse spectrum of solid malignancies, Checkpoint with Forkhead and Ring Finger Domains (CHFR) is most frequently inactivated by promoter CpG island methylation and has shown to be a marker of poor prognosis and increased sensitivity to treatment with taxanes. We retrospectively investigated CHFR promoter methylation in gastric cancer as a method for isolating those patients who would derive the most benefit from taxane-based regimens and as an indicator of prognosis.

Materials and Methods: One hundred thirty-six formalin-fixed paraffin-embedded (FFPE) primary tumor samples were collected from patients with gastric cancer who underwent surgery with curative intent at the Juntendo University Shizuoka Hospital between 2005 and 2012. We employed Quantitative Methylation-Specific PCR (qMSP) with bisulfite-modified DNA as a template for fluorescence-based real time PCR. We categorized patients into two groups: the low group (CHFR-relative methylation value (RMV) <10.3%) and the high group (CHFR-RMV ≥10.3%) based on Akaike’s information criteria (AIC).

Results: CHFR-RMVs in cancer tissues were significantly higher than those in normal-appearing adjacent non-cancerous tissues (p=0.0001). The cancer-specific survival among the patients in the high group was significantly worse than that among the patients in the low group (p=0.002).

Conclusions: CHFR promoter methylation is an independent prognostic marker of poor prognosis in gastric cancer.

Key words: Checkpoint with Forkhead and Ring Finger Domains (CHFR), promoter CpG island methylation, gastric cancer, taxane, quantitative methylation-specific PCR (qMSP)

Introduction

I (the first author) studied DNA methylation in cancer at Johns Hopkins University in Maryland in the United States of America from December 2013 to July 2016. I belonged to the Department of Thoracic Surgery, and studied under Professor Malcolm V Brock (Figure 1). He is a thoracic surgeon who also conducts cancer research at the Johns Hopkins Kimmel Cancer Center where he has his own laboratory and oversees about ten postdoctoral fellows. The subject of this translational...
Figure 1  Johns Hopkins University
Left: The appearance of the main building of Johns Hopkins University Hospital.
Right: The photo with Professor Malcolm, Professor Sakamoto and Dr. Ito.

Figure 2  Lab members

Figure 3  What is Epigenetics?

What is Epigenetics?

Genetics
- With changes in DNA sequence
  - Mutation
  - RAS, EGFR, etc.
  - Chromosome Instability
    - MSI/MSS, etc.

Cross Talk

Epigenetics
- Without changes in DNA sequence
  - DNA Methylation
  - Histone Methylation

Cancer
  - Development
  - Progression
research is ‘Epigenetics’, specifically DNA methylation in lung and esophageal cancer (Figure-2). Epigenetics is the study of potentially heritable changes in gene expression that does not involve changes to the underlying DNA sequence, while genetics involves changes to the underlying DNA sequence\(^1\) (Figure-3). Genetic and epigenetic abnormalities are linked with each other, which can lead to cancer development and progression\(^2\). In epigenetic alterations, methyl marks added to certain bases repress gene activity by tightly packing the chromatin\(^3\). Recently, many researchers have been trying to clinically apply epigenetics to diagnosis, treatment and prevention\(^4\)\(^5\) (Figure-4). In this review, we introduce our DNA methylation study in gastric cancer.

**Backgrounds**

Gastric cancer is one of the most prevalent cancers worldwide and the leading cause of cancer-related death in East Asia\(^6\)\(^7\). The availability of both a prognostic and predictive biomarker in gastric cancer may alleviate some of these tumor-related deaths. We especially are in need of a predictive biomarker which can serve to inform us about the sensitivity of patients to treatments such as chemotherapy.

The mitotic checkpoint and tumor suppressor gene, “Checkpoint with Forkhead and Ring Finger Domains (CHFR)” has been shown to function as both a prognostic and predictive marker in a variety of cancers\(^8\)\(^-\)\(^10\). As a prognostic marker in esophageal and lung cancers, CHFR methylation portends a poor prognosis\(^11\)\(^-\)\(^12\). Across a diverse spectrum of solid malignancies, CHFR methylation also has been shown to be a marker of poor prognosis\(^11\)\(^-\)\(^12\). But, CHFR methylation can also predict taxane sensitivity leading to good outcome if a taxane-based regimen is given\(^8\)\(^-\)\(^10\). This increased sensitivity to treatment with taxanes has been demonstrated to be correlated with promoter CpG island methylation of the mitotic checkpoint and tumor suppressor gene (CHFR)\(^8\)\(^-\)\(^10\). However, despite the high prevalence of taxane-based therapy for gastric cancer in other parts of the world, in Japan, taxane has not been administered traditionally to patients with gastric cancer. A predictive biomarker of taxane sensitivity would change that logic.

We hypothesize that CHFR promoter methylation in gastric cancer can be utilized as a tool for selecting those patients who would derive the most benefit from taxane-based regimens. We also
hypothesized that in patients who do not receive taxane therapy, CHFR methylation would serve as an indicator of poor prognosis. We performed a retrospective study to evaluate the ability of CHFR to serve as both a prognostic marker of a poor outcome as well as a predictive marker of increased taxane sensitivity leading to a favorable outcome if taxane-based therapy is used.

Materials and Methods

1. Study population

Between 2005 and 2012, 136 formalin-fixed paraffin-embedded (FFPE) primary tumor samples were collected from patients with gastric cancer who underwent surgery with curative intent at the Juntendo University Shizuoka Hospital. Utilizing the same patient cohort, adjacent non-cancerous tissues with normal appearance were also investigated for CHFR promoter methylation. Database and medical records were retrospectively reviewed. Approval for this study was obtained from the Institutional Review Board of the Juntendo University Shizuoka Hospital.

2. Pathological examination

All specimens were examined in accordance with the "Japanese classification of gastric carcinoma" issued by the Japanese Gastric Cancer Association [13]. Following resection of the stomach, the surgeon opened the excised specimen along the greater curvature. The specimen and lymph nodes were fixed in 10% buffered formalin and embedded in paraffin after routine processing, and then examined.

3. Clinicopathological analysis

This study analyzed the following clinicopathological variables: age, gender, tumor location, maximal tumor diameter as well as several factors defined by the "Japanese classification of gastric carcinoma" [13]. Within those guidelines, histology was classified as papillary, tubular, signet-ring cell, or mucinous adenocarcinoma. Tumor grade was defined as well or poorly differentiated. Invasion depth was classified as T1–T3 or T4. Lymphatic invasion was characterized as either none–mild or moderate–severe. Venous invasion was likewise classified as either none–mild or moderate–severe. Lymph node metastasis was either negative or positive. And, finally staging was determined to be either stage I, II or stage III.

4. Genomic DNA Extraction and Bisulfite Treatment

FFPE samples were cut as 10 μm tissue sections. Three to five sections were then deparaffinized in xylene followed by two 100% ethanol washes. These tissue sections subsequently were digested for four hours with proteinase K (ThermoFisher Scientific, Waltham, MA). The resulting DNA underwent bisulfite conversion using reagents from the EZ DNA Methylation Kit (Zymo Research, Irvine, CA) according to manufacturer’s instructions.

5. CHFR Quantitative Methylation Specific PCR (qMSP)

CHFR promoter methylation status and survival data were determined on each patient. Quantitative Methylation-Specific PCR (qMSP) was performed as described previously [14] [15]. The bisulfite-modified DNA served as a template for fluorescence-based real time PCR. qMSP was performed using a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) and analyzed using CFX Manager with accompanying stock software. The bisulfite-modified DNA was assessed for CHFR promoter methylation with 200 nM forward primer, 5’-GTT ATT TTC GTG ATT CGT AGG CGA C-3’, 200 nM reverse primer, 5’-CGA AAC CGA AAA TAA CCC GCG-3’, and 80 nM probe, 5’-/56-FAM CGC TCG ACC/ZEN/ATC TTT AAT CCT AAC CAA ACG ACT TC/3IABkFQ/-3’. The sequences for beta-actin (ACTB) recognizing both methylated and unmethylated templates were: 200 nM forward primer, 5’-TAG GGA GTA TAT AGG TTG GGG AAG TT-3’, 200 nM reverse primer, 5’-AAC ACA CAA TAA CAA ACA CAA ATT CAC-3’, and 80 nM probe, 5’-/56-FAM/TGT GGG GTG/ZEN/ GTG ATG GAG GAG GTT TAG/3IABkFQ/-3’. Cycling conditions were 95℃ for 5 min, followed by 60 cycles of (95℃ for 30 s, 60℃ for 30 s and 72℃ for 30 s). Master mix was constructed containing 16.6 mM (NH4)2SO4, 67 mM Tris pH 8.8, 10 mM β-mercaptoethanol, 10 nM fluorescein, 200 μM of each deoxynucleotide triphosphate (dNTP) and 0.04 U/μl of Platinum Taq polymerase (ThermoFisher Scientific, Waltham, MA). Final reaction
volumes for all assays were 25 μl. A standard curve was produced using serial dilutions of original stock plasmid (Oncomethylome Sciences, Belgium). As a positive methylation control, we utilized human male Jurkat genomic DNA treated with CpG Methylase (M.SssI) (New England Bio Labs, Ipswich, MA). We evaluated the CHFR promoter with the CHFR relative methylation value (RMV) defined by Misawa et al: CHFR-RMV = (CHFR-Sample/ACTB-Sample)/(CHFR-IVD/ACTB-IVD), where CHFR-Sample and CHFR-IVD represent the CHFR methylation levels in the sample and IVD, respectively, and ACTB-Sample and ACTB-IVD correspond to ACTB in the sample and IVD, respectively.

6. Statistical analysis

The Fisher's Exact probability test was employed to compare discrete variables. Continuous variables were compared using the Mann–Whitney U-test for individual comparisons and the Wilcoxon signed rank test for paired comparisons. The Kaplan–Meier method was used to calculate cancer–specific survival, and univariate analyses were performed with the log–rank test. When the cut-off values for age, maximal tumor diameter and CHFR–RMV — where cut-off values were not definitely defined — were analyzed, Akaike’s information criteria (AIC) were used. When the AIC was the smallest, values were fixed as the cut-off values. JMP 9 software (SAS Institute Inc., Cary, NC, USA) was used to analyze the data. Statistical significance was set at p < 0.05. Values are expressed as the median (range).

Results

1. Patient characteristics

The median age was 71 years (33–89 years). There were 97 males (71.3%) and 39 females (28.7%). Histological classification was tubular adenocarcinoma in 72 patients (52.9%), poorly differentiated adenocarcinoma in 49 patients (36.0%), signet-ring cell carcinoma in 12 patients (8.8%), papillary adenocarcinoma in two patients (1.5%), and mucinous adenocarcinoma in 1 patients (0.7%). The median time of follow-up for survivors was 48.8 months (range: 0.9–112.8 months).

2. CHFR-RMV in cancer tissues and normal–appearing adjacent non-cancerous tissues

The median CHFR–RMV in cancer tissues was 0.6% (0.0–63.6%) in all patients. The median CHFR–RMV in normal–appearing adjacent non-cancerous tissues was 0.5% (0.0–7.0%) in the 53 patients where we could obtained non-cancerous tissues while the median CHFR–RMV in cancer tissues was 1.1% (0.0–35.6%) in the same 53 patients where we could obtained non-cancerous tissues while the median CHFR–RMV in cancer tissues was 1.1% (0.0–35.6%) in the same 53 patients.
patients. CHFR-RMVs in cancer tissues were significantly higher than those in normal-appearing adjacent non-cancerous tissues \((p = 0.0001)\) (Figure-5).

3. The cut-off values for age, maximal tumor diameter and CHFR-RMV

We divided 136 patients into the groups: the patient group with \((n=7)\) and without taxane-based therapy \((n=129)\) because the prognostic significance of CHFR methylation is different between the two groups. In the patient group without taxane-based therapy \((n=129)\), the AICs were calculated in order to fix the cut-off values for age, maximal tumor diameter and CHFR-RMV in cancer tissues relative to cancer-specific survival. Consequently, the AIC was the smallest when the cut-off value for age was 82 years as well as when the cut-off values for both maximal tumor diameter and CHFR methylation level were 55 mm and 10.3%, respectively. Therefore, we fixed the cut-off values as patient age of 82 years, maximal tumor diameter of 55 mm and CHFR-RMV of 10.3%. With respect to CHFR-RMV, patients were categorized into two groups: 112 patients in the low group \((\text{CHFR-RMV} < 10.3\%)\) and 17 patients in the high group \((\text{CHFR-RMV} \geq 10.3\%)\).

4. Prognostic factors related to cancer-specific survival

Comparisons of the cancer-specific survival rates according to the clinicopathological factors and CHFR-RMV in the patient group without taxane-

| Table-1 Comparisons of the cancer-specific survival rates according to clinicopathological factors and CHFR-RMV |
|-------------------------------------------------|-----------------|-----------------|
| Total                                           | 136             |                 |
| Age                                             | 71 years \((33-89)\) \(^a\) |                 |
| Gender                                          |                 |                 |
| Male                                            | 97 \((71.3\%)\) |                 |
| Female                                          | 39 \((28.7\%)\) |                 |
| Histological classification                     |                 |                 |
| Papillary adenocarcinoma                        | 2 \((1.5\%)\)  |                 |
| Tubular adenocarcinoma                          | 72 \((52.9\%)\) |                 |
| Poorly differentiated adenocarcinoma             | 49 \((36.0\%)\) |                 |
| Signet-ring cell carcinoma                      | 12 \((8.8\%)\)  |                 |
| Mucinous adenocarcinoma                         | 1 \((0.7\%)\)   |                 |
| Stage                                           |                 |                 |
| I                                               | 57 \((41.9\%)\) |                 |
| II                                              | 34 \((25.0\%)\) |                 |
| III                                             | 45 \((33.1\%)\) |                 |

\(^a\) Median \((\text{Min.-Max.)}\)

Figure-6 Cancer-specific survival curves according to CHFR-RMV

CHFR-RMV: Checkpoint with Forkhead and Ring Finger Domains–relative methylation value.
based therapy (n=129) are shown in Table-1. In univariate analysis, significant differences in cancer-specific survival were observed with respect to age (p=0.01), location (p=0.03), maximal tumor diameter (p<0.001), invasion depth (p<0.001), lymphatic invasion (p=0.03), lymph node metastasis (p<0.001) and CHFR-RMV (p=0.002) (Table-1). There were no significant differences with respect to the other clinicopathological factors. A comparison of the cancer-specific survival curves according to CHFR-RMV is shown in Figure-6. The cancer-specific survival among the patients in the high group was significantly worse than that of the patients in the low group (p=0.002).

5. The Prognosis in Patients Who Were Treated with Taxane-based Regimen

In this cohort, only seven patients underwent taxane-based chemotherapy for their recurrent diseases. When the same cut-off value for CHFR-RMV was used, there were four patients in the low group and three patients in the high group. There was no significant difference in cancer-specific survivals between the two groups (p=0.98).

Discussion

CHFR is an early mitotic gene that plays a crucial role in controlling chromosomal integrity. CHFR is expressed in the cytoplasm of all normal tissues and accumulates in the nucleus in response to microtubule poisoning or radiation damaging stress. That is, microtubule stress will lead to an elevation of CHFR expression levels and a mitotic arrest. For this reason, inactivation of CHFR can result in an increased vulnerability to microtubule stress. In addition, CHFR is able to inhibit the NFκB signaling pathway which subsequently results in decreased neovascularization and cell migration. Therefore, inactivation of CHFR is thought to play a crucial role in cancer aggressiveness. So far, decreased CHFR expression has been identified in multiple cancer tissues mainly due to promoter CpG island methylation. Furthermore, several studies showed that CHFR promoter methylation is correlated with a poor prognosis and increased sensitivity to taxane in multiple types of cancers. We and colleagues showed the clinical significances of CHFR promoter methylation in non-small cell lung cancer, esophageal squamous cell cancer and colorectal cancer. Similarly, in gastric cancer some studies revealed the correlation between CHFR promoter methylation and a poor prognosis or increased sensitivity to taxane. Li Y and colleagues showed that in docetaxel-treated gastric cancer patients, overall survival was significantly longer in the CHFR methylated group compared with the CHFR unmethylated group. As far as we know, our study is the first report which demonstrates the capability of the prognostic prediction of CHFR promoter methylation by means of qMSP. Although MSP is the most diagnostically sensitive assay type, qMSP is usually desirable because optimal thresholds can be determined to maximize the performance of the assay for specific clinical applications. In this study, we determined the optimal cut-off value of CHFR-RMV in evaluating cancer-specific survival based on AIC. Since AIC provides an objective means for choosing between competing models and the lowest value of AIC indicates the preferred model, our results, which show the capability of the prognostic prediction of CHFR-RMV with its cut-off value determined by AIC, are significant. With regard to the sensitivity to taxane, Satoh A and colleagues stated that the inactivation of CHFR due to promoter methylation in gastric cancer cell lines is associated with increased sensitivity to mitotic stress, which could be restored by treatment with 5-aza-2'-deoxycytidine. Koga Y and colleagues also reported that gastric cancer patients with CHFR promoter methylation showed a better clinical response to taxane than those without CHFR promoter methylation. On the other hand, Yoshida K and colleagues mentioned that CHFR methylation alone may not be a good predictive marker in advanced and recurrent gastric cancer to taxane-based therapy. They stated that the reason for the inconsistency with other reports was because they evaluated the response to taxane-based therapy in metastatic lesions while they investigated the CHFR promoter methylation in primary gastric cancers. In this study, there was no significant difference in cancer-specific survival rates among patients who received taxane-based therapy between the CHFR-RMV-low and -high groups. The main reason for this was because of our small sample size.
Also, methylation status of lesions might differ according to the organ location of the distant metastases. Moreover, the optimal cut-off value of CHFR–RMV in evaluating the response to taxane-based therapy should be determined.

Finally, there are several limitations in this study that should be considered, which are inherent to retrospective studies. Firstly, the data were collected and examined at a single institute, and only a small number of patients were enrolled. Therefore, as mentioned before, we couldn’t investigate the sensitivity to taxane-based chemotherapy with enough patients who had undergone taxane-based chemotherapy in this study. Secondly, the optimal cut-off values in CHFR–RMV may vary among the study cohorts and study aims. So far, there has been only one paper by Hiraki M and colleagues which employed qMSP in CHFR promoter methylation \(^{30}\). They determined the cut-off value of CHFR–RMV in patients’ peritoneal lavage fluid samples based on their previous experiments which showed that no increased methylation value was observed in the negative controls. As a result, they set up more than 1% of CHFR–RMV as positive in the detection of CHFR promoter methylation in peritoneal lavage fluid samples. Thus, the aim in their study was different from ours which focused on the prognostic prediction in cancer-specific survival. Therefore, a prospective validation study with a large enough cohort to investigate the prognostic capability of CHFR promoter methylation is anticipated in the future.

**Conclusions**

This study demonstrated the capability of the prognostic prediction in CHFR promoter methylation in gastric cancer. A prospective validation study in a large cohort of gastric cancer patients is needed to validate these findings.

**Conflict of interest**

The authors declare that they have no conflict of interest.

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**References**

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