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Original article

DNA methylation analysis of cancer-related genes from cell-free DNA of patients with cancer

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ABSTRACT

DNA methylation is a very important mechanism for regulating gene expression in normal development and cell differentiation. However, DNA methylation, when acting on the promoter region of a tumor suppressor gene, suppresses transcription and is one of the major epigenetic modifications involved in the development and progression of many cancers (Wajed et al., 2001; Morris and Chan 2015) [1,2]. In our laboratory, we have carried out methylation analysis of 14 different tumor suppressor genes in cell-free DNA from the peripheral blood of over 1400 cancer patients, and have detected more than a total of 1900 methylations. We performed an analysis of DNA methylations of 14 different tumor suppressor genes, by type of cancer, stage of cancer, function of tumor suppressor genes, and their correlation to genetic mutations.

1. Introduction

Genetic mutations have long been recognized as the cornerstone in the development of cancer, however, epigenetic processes having a key regulatory role in cancer is becoming increasingly apparent [1,2]. This epigenetic process acts as an alternative to mutations to disrupt tumor-suppressor gene function and can predispose to genetic alterations [3].

Of all epigenetic modifications, including histone modifications and nucleosome positioning, the hypermethylation of DNA has been the most extensively studied [4]. Hypermethylation in the CpG islands of the promoter regions of many tumor suppressor genes leads to the recruitment of co-repressors, altered chromatin structure, and ultimately transcriptional silencing [5]. To date, many genes with aberrant promoter hypermethylation have been identified at varying rates in essentially all forms of cancer. Some of these susceptible genes include those involved in cytostatic suppression (p14, p15, p16, RB1, APC), cancer invasion and metastasis (DCC, E-Cadherin, TIMP3), DNA repair (BRCA1, hMLH1), DNA damage response (ATM, RASSF1A), protein decomposition (VHL) and hormonal regulation (RAR-β2), among many others [6].

As methylation occurs early in oncogenesis and can be detected in bodily fluids, it has great potential for the use of early detection of tumors and for determining prognosis [4]. In our laboratory, cell-free DNA is extracted and measured from plasma in peripheral blood, and methylation analysis is carried out together with mutation analysis. It should be noted that there are limitations in using cfDNA, particularly with using quantitative measurements, as there is large variability in the total plasma cfDNA levels with cancer patients having higher overall concentrations of cfDNA [7–9]. As circulating tumor DNA is thought to originate from apoptotic tumor cells, due to tumor heterogeneity and depending on the patient's immune state, the detection of DNA hypermethylation could be affected [10].

In order to limit variability in the pre-analytic phase, we attempted to standardize the collection and processing of samples as much as possible, and have focused on the methylation patterns of the genes rather than the quantitative amounts. Despite these obstacles, as obtaining cell-free DNA is minimally invasive, giving us the opportunity to carry out examinations at desired intervals, such as pre- and post-treatment, in early detection, and in checking for recurrence, it has become an increasingly attractive model for use as a biomarker for cancer [11].

DNA methylation is linked to and is considered to be a precursor to genetic mutations. However methylation itself being a cause of cancer is particularly intriguing because unlike genetic mutations, DNA methylation is reversible [4]. This means there is great potential for cancer treatment by targeting DNA methylation with

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demethylating agents and indeed an increasing number of drugs targeting DNA methylation are being developed. Current efforts are underway to increase the efficacy and stability and to decrease the toxicity of such demethylating agents. Encouraging results from clinical trials have prompted further efforts to elucidate epigenetic alterations in cancer, and to subsequently develop new epigenetic therapies [12].

2. Methods and study selection

This observational study was performed between January 2011 and August 2016, in accordance with the amended Declaration of Helsinki. The protocol for the present study was approved by the Ethics Committee of the Hakushin Koseikai Medical Foundation. Written informed consent was obtained from all participants included in the present study.

2.1. Cell-free DNA extraction and bisulfite conversion

EDTA-2 Na-added blood samples were centrifuged at 2400 G at 4° C for 10 min, and the plasma was collected. The cell-free DNA was then extracted from the plasma using QIAamp Circulating Nucleic Acid Kit (QIAGEN). Bisulfite conversion was then performed using EpiTect Plus DNA Bisulfite Kit. In this reaction, the bisulfite in the reagent converts the unmethylated cytosine in the free DNA to uracil (C → U), while the methylated cytosine remains unchanged (mC → C). Following this reaction, the methylated DNA and unmethylated DNA will have different base sequences, and can thus be differentiated.

2.2. Methylation-specific polymerase chain reaction and electrophoresis

Following bisulfite conversion, methylation-specific polymerase chain reaction (MSP) was carried out. In this reaction, after the bisulfite treatment, PCR is carried out using unmethylated DNA and methylated DNA specific primers which have different base sequences, and each is amplified. Unmethylated DNA and methylated DNA amplified by MSP are applied to agarose gel and electrophoresis was carried out. As the nucleic acid in the buffer solution is negatively charged by phosphate residues, the nucleic acid migrates to the positive side upon electrophoresis according to its chain length (by molecular weight). Following electrophoresis, the gel is stained with ethidium bromide (EtBr). EtBr enters the interstices of the double-stranded DNA (by intercalation), and when irradiated by UV, fluorescence is emitted and the presence of the nucleic acid can be confirmed. An example of the analysis image is shown (Figs. 1-1 and 1-2).

2.3. Direct sequencing for mutation analysis

Mutation analysis was carried out using real-time PCR to analyze 4 genes of interest (p53, APC, EGFR, K-ras) using Applied Biosystems® 3500 Genetic Analyzer. Using this fluorescence-based sequencing method we searched for mutations in exons 4, 5, 6, 7, 8 of the tumor suppressor gene p53, exons 15a, 15b of the tumor suppressor gene APC, exons 18, 19, 20, 21 in the oncogene EGFR, and exons 1, 2 in the oncogene K − ras.

2.4. Study selection and data analysis

The DNA methylation analysis and mutational analysis of cell-free DNA was measured in 1477 specimens between January 2011 and August 2016. Of these, 1410 individuals had cancers in various stages (stage I: n = 11, stage II: n = 23, stage III: n = 53, stage IV: n = 219), and 67 individuals did not have cancer.

The number of methylated DNA genes, number of individuals with DNA methylated genes, and the detection rate (detection ratio) was calculated for each individual cancer type. We divided sample populations into cancer-free and cancer-bearing patients, and the number of detected DNA methylations per person was compared.

We compared DNA methylation detection counts of 14 types of cancer suppressor genes that were analyzed and correlated against various cancer types. The following genes (p14, p15, p16, RB1, APC, DCC, E-Cadherin, TIMP3, ATM, RASSF1A, BRCA1, hMLH1, VHL, RAR-β2) were chosen for their well-known activity to suppress cancer growth on the basis that methylation of these genes shuts off its promoter function, allowing for favorable conditions for cancer to flourish [13,14].

6 types of cancers (lung, colon, pancreas, breast, stomach, prostate) were singled out for their large sample sizes in our patient population and the number of DNA methylation positive genes, their function, and detection rates were compared for analysis. The cancer-bearing patient group was further divided by cancer stage and again the number of DNA methylation positive genes and detection rates were compared for analysis. The oncogenes p53, APC, EGFR, K-ras were chosen for their well-documented causal relationships with many types of cancers. For the 6 most common types of cancers, it was investigated whether there was a link between the number of DNA methylations and genetic mutation rate.

3. Results

DNA methylation was detected not only in cancer patients but also in the cancer-free population, in 26 out of 67 individuals, or at a rate of 38.8%. Cancers with the highest DNA methylation rates were liver cancer (82.8% or 48/58 cases), bile duct cancer (80.0% or 28/35 cases), and skin cancer (85.7% or 12/14 cases). Due to the difficulty of showing statistical significance with a small sample size, we did not include the results for cancer types with fewer cases than 10.

The cancer types with the lowest DNA methylation detection rates were salivary gland cancer (47.4% or 9/19 cases), pharyngeal cancer (43.8% or 7/16 cases), and thyroid cancer (46.7% or 7/15 cases). See (Table 1) for full results.

The DNA methylation detection rate of a single tumor suppressor gene was not significantly different in cancer-free individuals (31.3%) when compared with cancer-bearing patients (27.7%). However, the detection rate for having two methylated tumor suppressor genes was significantly increased from 4.5% in cancer-free individuals to 21.8% in cancer-bearing patients (Fig. 2).

High methylation rates are found in APC, DCC and RASSF1A genes in many cancer types [15–18]. Although mutations in the BRCA1 gene are well known to be involved in familial breast cancer and ovarian cancer [19], our findings show that in ovarian cancer, DNA methylation was also detected in APC, DCC, RASSF1A and p14. DNA methylation of the APC gene was detected most frequently, both in cancer-free and cancer-bearing individuals (Fig. 3).

In our six most common cancer types (lung, colon, pancreas, breast, stomach and prostate), DNA methylation was seen predominantly in genes responsible for cytostatic suppression (p14, p15, p16, RB1, APC) ranging from 42.3% to 54.1%, invasion and metastasis suppressor genes (DCC, E-Cadherin, TIMP3) from 12% to 24.1%, and DNA damage response genes (ATM, RASSF1A) from 17.2% to 26.8%. In addition, in prostate cancer, the methylation of a hormone-related gene RAR-β2 was detected with relatively high frequency (18%) (Fig. 4).

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