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# Identification of the bleomycin hydrolase gene as a methylated tumor suppressor gene in hepatocellular carcinoma using a novel triple-combination array method

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ABSTRACT

In the present study, we sought to identify novel suppressor genes of hepatocellular carcinoma (HCC) using our newly designed triple-combination array. Using this method, the bleomycin hydrolase gene (*BLMH*) was detected as a candidate suppressor gene. We found that 28 of 48 (58.3%) tumor tissues showed *BLMH* promoter hypermethylation, and its expression level was significantly reduced in tumor tissues ($P = 0.001$). The present study suggests that our new method can detect novel genes of interest and that *BLMH* is a suppressor gene in HCC.



## 1. Introduction

Hepatocellular carcinoma (HCC) is one of the top five causes of cancer-related deaths worldwide [1]. The long-term prognosis of HCC is generally poor, despite surgical or locoregional management, in part because of the late stage at which patients are first diagnosed [2]. As with other cancers, HCC is a genetic disease of somatic cells that arises because of an accumulation of genetic changes, particularly abnormal activity of suppressor and oncogenes [3–9]. Therefore, to further improve HCC detection and patient survival, identifying the genetic markers associated with the prognosis of HCC is vital. The development of high-throughput technologies such as single nucleotide polymorphism (SNP) arrays and gene expression microarrays that can simultaneously screen thousands of genes has opened a new era in translational research [10–12]. The results of these labors, molecular-targeted therapies, have markedly improved the prognosis for patients with cancer. Clinical studies with the multitargeted kinase inhibitor sorafenib have shown the potential of this approach in HCC [13,14]. Surgeons can readily obtain fresh surgical specimens and use these specimens to determine the activity of cancer-related genes. However, for this the development of efficient and low-cost methods for evaluation of the surgical specimens is also important. We recently reported a new method, which combines an SNP array with a gene expression microarray, called the 'double-combination array' [15–19]. Although this method does not always guarantee the presence of hypermethylation, we can hypothesize that decreased gene expression is due to hypermethylation of the CpG islands and evaluate this by examining the sequences of the promoter regions of the

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candidate genes. A novel genome-wide DNA methylation platform was recently developed to determine the DNA methylation status of selected promoter regions [20]. Aberrant DNA methylation of the promoter and other genomic regions can lead to changes in gene expression. Therefore, in this study, we used our double-combination in conjunction with the Ilumina Infinium HumanMethylation 27 BeadChip platform (Ilumina, San Diego, CA) to identify genes displaying altered gene expression in tumor tissue. Using this approach, called the "triple-combination array", we found that many genes showed differential expression between normal tissue and tumor tissue. We also identified many genes considered as candidates for tumor suppressor genes. Using these candidate genes, we sought to identify the novel genes associated with HCC responses to treatment in the present study. Analyzing the data derived from the triple-combination array, the bleomycin hydrolase (*BLMH*) gene was given the highest priority of all candidate genes, because bleomycin is widely used as anti-cancer drug. Therefore, the BLMH gene was chosen as a candidate tumor suppressor gene to be investigated in the present study.

## 2. Materials and methods

### 2.1. Sample collection and DNA preparation

Five HCC cell lines (HuH1, HuH2, HuH7, HLE, and SK-Hep1) were obtained from the American Type Culture Collection (Manassas, VA). The cell lines were cultured in RPMI-1640 supplemented with 10% fetal bovine serum, and incubated in 5% $CO_2$ at 37 °C.

A 68-year-old woman with chronic hepatitis C was diagnosed as with HCC in the right lobe and underwent liver resection. Specimens of her tumor and adjacent non-tumorous tissues were excised, and total RNA and DNA extracted. Total RNA was sent to the manufacturer to prepare it for expression array analysis. Genomic DNA was used for a SNP-Chip array, and bisulfite-converted DNA was used for the Ilumina Infinium HumanMethylation 27 BeadChip. The tumor was pathologically confirmed to be HCC. RNA and DNA were extracted from an area consisting of >80% cancerous cells.

HCC tissue (HTs) and normal tissue (NTs) samples were obtained from 48 patients (43 males, 5 females) who underwent liver resection at Nagoya University Hospital, Nagoya, Japan, between 1994 and 2001. The patients were aged from 39 to 77 years (mean ± SD, 62.4 ± 7.9 years). Thirty-eight patients had hepatitis C and seven had hepatitis B. The median duration of follow-up was 63.6 months (range 17.9–164.7 months). All tissues were reviewed pathologically to confirm the diagnosis of HCC. Written informed consent, as required by the institutional review board, was obtained from all patients. The tissue samples were immediately frozen in liquid nitrogen and stored at −80 °C until required. Genomic DNA was obtained from the tissue samples by proteinase K digestion, followed by phenol/chloroform extraction.

### 2.2. RNA isolation, microarray and gene chip Affymetrix procedures

The expression array and SNP Chip array were performed, as previously described [15–17], using total RNA and DNA extracted from the 68-year-old woman's tissue sample.

### 2.3. Methylation array platform

The Illumina Infinium HumanMethylation 27 BeadChip protocol requires 500 ng to 1 μg of bisulfite-converted DNA [20]. Of the ~28 million CpG sites found throughout the haploid human genome, Illumina initially designed Infinium methylation probes for 27,578 CpG sites located in promoter regions (up to 1 kb upstream or 500 bp downstream of the transcription start sites). Of these, 27,324 CpG sites relate to 14,475 consensus coding sequences including ~1000 cancer-associated genes, and 254 CpG sites relate to ~100 micro-RNA genes. The probes were preferentially selected to occur within CpG islands using the NCBI "relaxed" definition of a CpG island: CpG islands identified bioinformatically with a CpG content of >50% and an observed/expected ratio of >0.6 [21].

Bisulfite-converted DNA is then whole-genome amplified, enzymatically fragmented, and hybridized to the array. During hybridization, the bisulfite-converted DNA anneals to methylation-specific probes on the chip. Each CpG locus is represented by two bead types, one of which is specific to the methylated state and the other is specific to the unmethylated state, which is directly related to the underlying sequence change catalyzed during bisulfite conversion. Therefore, for each CpG site, a possible C/T variant can be assayed through the single-base extension step, which is possible because of the ability to hybridize to either the "protected" methylated cytosine or the converted (unmethylated) thymine.

After hybridization, a single-base extension step is carried out using by a multi-layer staining process, as described below. The BeadChip is then scanned on the Illumina iScan and the resulting "idat" files are analyzed using BeadStudio software. The output of the BeadStudio analysis is a β-value for each CpG site. This is a continuous value between 0 and 1 where 0 = 0% methylation and 1 = 100% methylation at a given CpG site. Therefore, this assay enables quantitative analysis of methylation at individual CpG sites.

### 2.4. Reverse transcription-polymerase chain reaction (RT-PCR) BLMH

mRNA expression was analyzed by RT-PCR and real-time RT-PCR. Total RNA (10 μg) isolated from five HCC cell lines, primary HTs and NTs were used to generate cDNAs. The resulting cDNAs were then amplified by PCR primers for *BLMH* (sense, 5′-TGG CCC CAT AAC ACC CTT GG-3′ in exon 7; antisense 5′-GTA CTT GTG CTG GGG CCT AG-3′ in exon 8), which amplified a 106-bp product. Initial denaturation at 94 °C for 5 min, was followed by amplification consisting