

# Detection of Allelic Imbalance in Ascitic Supernatant by Digital Single Nucleotide Polymorphism Analysis<sup>1</sup>

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## ABSTRACT

**Purpose:** Cytological examination of ascitic fluid is critical for clinical management of patients with peritoneal or pelvic diseases. Such morphological examination can only achieve a sensitivity of <62%, and thus a molecular test that is able to distinguish benign *versus* malignant ascites could be clinically useful. In this study we determined the presence of allelic imbalance (AI) in tumor-released DNA in ascitic supernatant by directly counting the alleles using a newly developed technology, digital single nucleotide polymorphism (SNP) analysis.

**Experimental Design:** Allelic status was assessed using a total of seven SNP markers that commonly demonstrated AI in ovarian, colorectal, and pancreatic cancers.

**Results:** With digital SNP analysis, AI in at least one SNP marker was found in 19 of 20 (95%) ascitic fluid DNA samples obtained from patients with cytologically proven carcinomas in ascitic fluid. In contrast, AI was detected in only 1 of 20 patients with negative cytology. This latter patient with AI in her ascites had known stage III ovarian carcinoma at the time of cytology sampling. The ascitic specimen of this patient demonstrated the presence of carcinoma cells in culture with an identical AI pattern found in the ascitic supernatant and surgical specimen.

**Conclusions:** These findings suggest that detection of AI using digital SNP analysis can be a useful adjunct for the detection of ovarian and other types of cancer in ascitic fluid.

## INTRODUCTION

Cytological examination of ascitic fluid is a frequently performed and important technique in the diagnosis and management of malignant diseases involving the peritoneal and

pelvic cavities. However, it often fails to detect malignant cells. The sensitivity of cytological examination has been estimated to be 60%, at best (1). This low sensitivity is related to several factors including the presence of a large amount of lymphocytes, mesothelial cells, and blood that can obscure the recognition of malignant cells in these specimens. For example, inflammation that is often associated with malignant ascites can lead to reactive changes in mesothelial cells that make their morphological distinction from metastatic carcinoma cells very difficult (Fig. 1; Ref. 1). Thus, a molecular test that is able to reliably distinguish malignant *versus* benign ascites could be very useful.

Previous reports have shown that tumors release substantial amounts of DNA into body cavity fluids, probably as a result of cellular necrosis and apoptosis (2–4). Tumor-released DNA can be detected as a result of specific genetic and epigenetic abnormalities such as AI<sup>3</sup> (or loss of heterozygosity), microsatellite instability, mutations, translocation, and aberrant methylation (5–7). Among these molecular genetic alterations, AI, characterized by losses or gains of defined chromosomal regions, is universally associated with malignant tumors from almost all types of cancer (8, 9). Traditionally, AI has been assessed by a PCR assay to analyze the length of polymorphism using microsatellite markers. Although several studies have demonstrated that AI can be detected in plasma from a variety of malignant tumors (6, 10–18), there are, however, two main problems associated with the current method. First, ascitic fluid DNA is a mixture of tumor-released DNA and normal background DNA from nonneoplastic cells. The latter can obscure AI because it is difficult to quantify the allelic ratio using microsatellite markers. Second, DNA in these specimens is often degraded to a variable extent, producing artifactual enrichment of smaller alleles when microsatellite markers are used for analysis.

In this study, to overcome the technical problems associated with the traditional analysis (19–23), we used a new technology, digital SNP analysis, to measure allelic ratios from 20 cytology-proven cancer ascites fluid samples compared with 20 benign ascites fluid samples. This technique has been developed based on the principle of digital PCR (23) and has been proven to be able to directly count two parental alleles and therefore precisely determine the allelic status in the presence of normal DNA.

## MATERIALS AND METHODS

**Samples and DNA Purification.** A total of 40 ascitic fluid samples were retrieved from the Cytology Laboratory in the Department of Pathology at the Johns Hopkins Hospital. These specimens included 15 sporadic ovarian serous carcino-

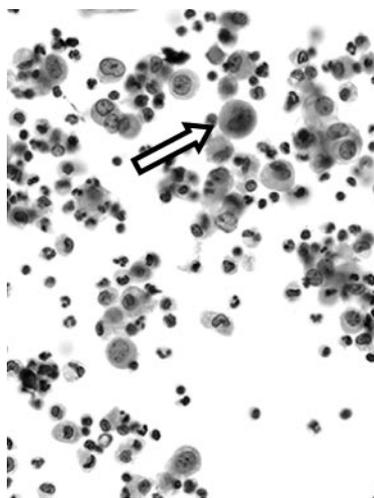
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<sup>3</sup> The abbreviations used are: AI, allelic imbalance; SNP, single nucleotide polymorphism; SPRT, sequential probability ratio test.



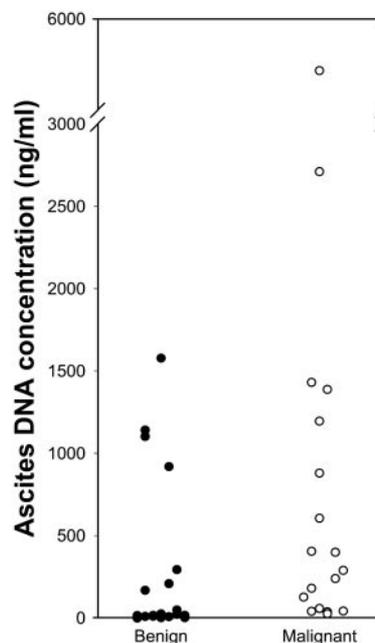
*Fig. 1* An ascitic fluid specimen containing a single ovarian serous carcinoma cell (arrow) in a background of reactive mesothelial cells and inflammatory cells.

mas, 3 pancreatic adenocarcinomas, 2 colorectal adenocarcinomas, and 20 benign ascitic fluids without any cytological evidence of malignancy. The cytological specimens were rereviewed by a cytopathologist (S. Z. A.) before molecular analysis.

Supernatant was prepared from the ascitic fluid samples after centrifugation at 750 rpm for 3 min. DNA was purified from 200–600  $\mu$ l of supernatant using a QiaAmp DNA blood kit (Qiagen, Valencia, CA). Formalin-fixed, paraffin-embedded tumor tissues were retrieved from the surgical pathology files that corresponded to the ascitic fluid specimens (Table 1), and sections were cut and stained with H&E. Tumor and normal tissues were microdissected from the sections under a phase-contrast microscope by a surgical pathologist (I-M. S.). The DNA was purified using the QiaQuick PCR purification kit (Qiagen). All of the procedures were performed following the manufacturer's instructions.

**DNA Quantitation.** The PicoGreen dsDNA quantitation kit (Molecular Probes, Inc.) was used to measure DNA concentration following the manufacturer's instructions. Purified DNA was dissolved in Tris-EDTA buffer and incubated with 2  $\mu$ g/ml PicoGreen dye at room temperature for 4 min. The fluorescence intensity was measured by a FLUOstar Galaxy fluorescence microplate reader (BMG Lab Technologies Inc., Durham, NC). The DNA concentrations were extrapolated from the standard curves and expressed as the average of six replicates for each sample.

**Digital SNP Analysis.** Seven SNP markers were retrieved from the National Cancer Institute SNP map,<sup>4</sup> based on their chromosomal locations with high frequent allelic losses in ovarian, colorectal, and pancreatic carcinomas (24–28). The sequences of the primers and molecular beacons were reported previously (20, 21, 29). Digital SNP analysis was performed as



*Fig. 2* Ascites DNA concentrations in 40 individuals (20 patients with benign ascitic fluid and 20 patients with a malignant neoplasms). Each symbol represents an individual ascitic DNA sample. The average DNA concentration was 820 ng/ml in cytology-positive specimens and 101 ng/ml in cytology-negative samples.

described previously with some modifications (19–22, 29). In brief, DNA samples were diluted and distributed to the wells of a 384-well plate at approximately 1 genomic equivalent ( $\sim$ 3 pg)/2 wells. In addition to all essential PCR reagents, the PCR mixture contained a pair of molecular beacons (Gene Link, Thornwood, NY) along with an excess of reverse primer that allowed the generation of single-stranded DNA complementary to the molecular beacons. PCR was performed in a single step with the following protocol: 94°C (1 min); 4 cycles of 94°C (15 s), 64°C (15 s), and 70°C (15 s); 4 cycles of 94°C (15 s), 61°C (15 s), and 70°C (15 s); 4 cycles of 94°C (15 s), 58°C (15 s), and 70°C (15 s); 60 cycles of 94°C (15 s), 55°C (15 s), and 70°C (15 s); 94°C (1 min); and 60°C (5 min). The fluorescence intensity in each well was then measured by a Galaxy FLUOstar fluorometer (BMG Lab Technologies Inc.), and the number of specific alleles in each sample was directly determined from the fluorescence measurements.

**Culture of Tumor Cells from Ascitic Fluid.** After centrifugation of the ascitic fluid, cell pellets were dispersed and cultured in DMEM supplemented with 10% fetal bovine serum and antibiotics (Invitrogen, Carlsbad, CA). Cells were then enriched by immunosorting using the immunosorting system (Dyna, Oslo, Norway). Tumor cells were grown in the culture medium for 5 days before harvesting for molecular genetic analysis.

**Statistical Analysis.** To determine whether there was statistical significance for AI, we used the SPRT (30). This method allows two probabilistic hypotheses to be compared as data accumulate and facilitates decisions about the presence or absence of AI after study of a minimum number of samples. The

<sup>4</sup> <http://lpg.nci.nih.gov/html-snp/imagemaps.html>.

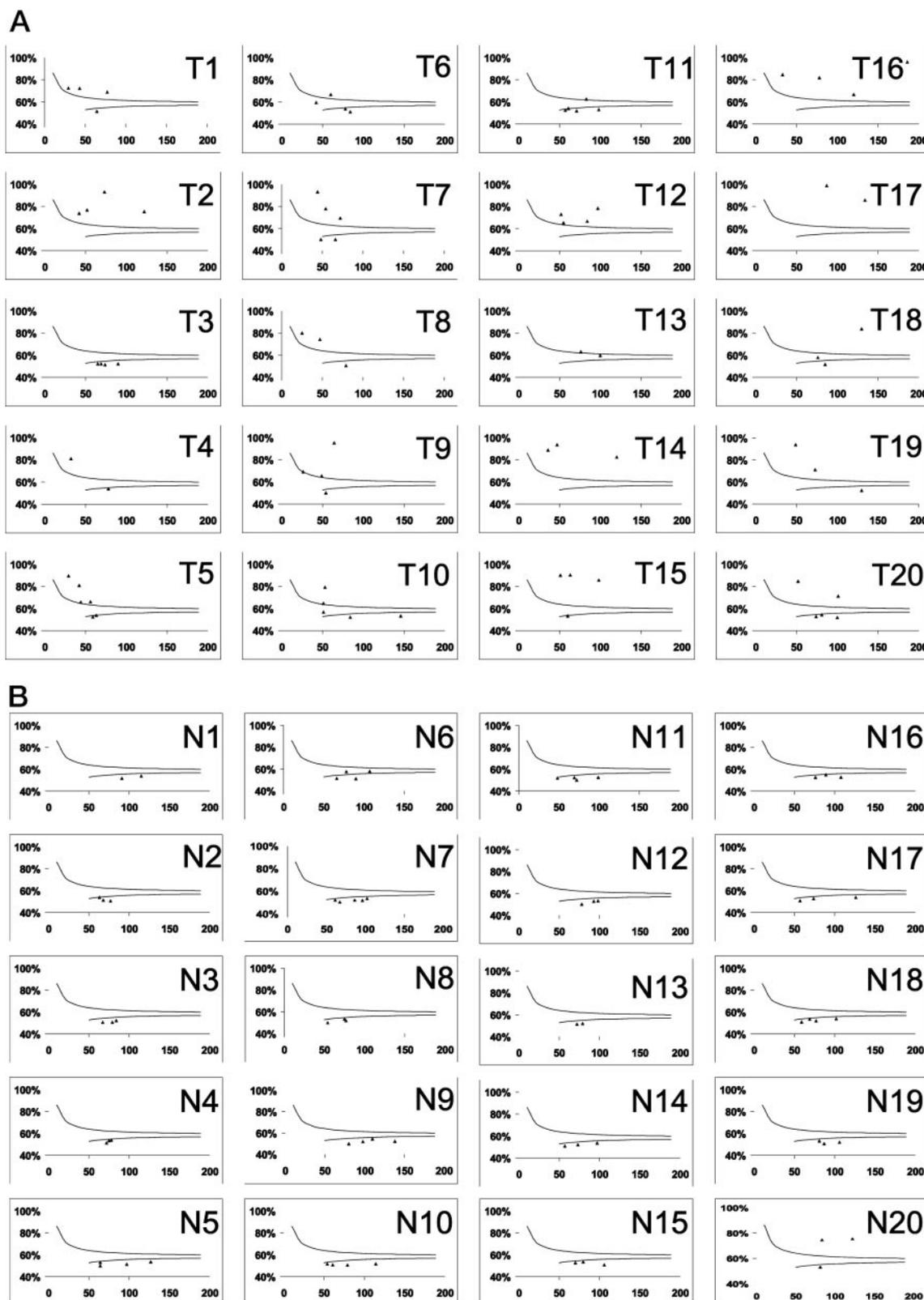


Fig. 3 SPRT of AI. The X axis represents the total number of alleles counted, whereas the Y axis represents the observed proportion of the two alleles. Each symbol represents one informative SNP that is either allele imbalanced (above the top curve) or allele balanced (below the bottom curve) from one specimen. The specimen number is shown in the top right corner of each panel. A, ascitic supernatants with malignant cytology. Specimen T3 is the one without AI. B, ascitic supernatants with negative cytology.

Table 1 Summary of digital SNP analysis in ascitic supernatants

Case No.	Diagnosis	Age	DNA concentration	SNP markers							
				8118	486	273	2952	331	852	1085	
T1	Ovarian CA	45	1388		NI					NI	NI
T2	Ovarian CA	55	127			NI				NI	NI
T3	Ovarian CA	71	240	NI	NI		NI			NI	
T4	Ovarian CA	73	606			NI	NI			NI	NI
T5	Ovarian CA	44	31				NI				?
T6	Ovarian CA	60	405		NI	?				NI	NI
T7	Ovarian CA	53	42				NI			NI	
T8	Ovarian CA	36	1430				NI	NI	NI	NI	
T9	Ovarian CA	45	550	NI				NI		NI	
T10	Colon CA	69	2711			?				NI	
T11	Ovarian CA	48	180					NI	NI		
T12	Ovarian CA	51	58	NI		NI				NI	
T13	Pancreatic CA	53	37	NI		NI	NI	NI	?		NI
T14	Ovarian CA	53	399	NI	NI		NI				NI
T15	Colon CA	46	289	NI			NI	NI			
T16	Ovarian CA	53	880	NI			NI				NI
T17	Ovarian CA	74	5774	NI		NI	NI	NI	NI		
T18	Ovarian CA	59	40	NI	NI	NI	NI				?
T19	Pancreatic CA	63	1195		NI		NI			NI	NI
T20	Pancreatic CA	56	26		NI	NI					
N1	NFT	49	10	NI	NI		NI	NI	NI		
N2	NFT	48	168		NI			NI	NI		NI
N3	NFT	58	2	NI			NI			NI	NI
N4	NFT	73	8		NI		NI			NI	NI
N5	NFT	55	22	NI			NI			NI	
N6	NFT	41	2		NI	NI	?			?	NI
N7	NFT	62	15					NI	NI		
N8	NFT	44	9		NI		NI	NI			NI
N9	NFT	62	16	NI		NI	NI				
N10	NFT	41	25	NI			NI	NI			
N11	NFT	75	19		NI	NI		NI			
N12	NFT	38	293	NI				NI	NI	NI	
N13	NFT	53	7	NI		NI	NI	NI			NI
N14	NFT	44	4	NI	NI	NI				NI	
N15	NFT	34	1141				NI	NI	NI	NI	
N16	Ovarian cyst	45	10	NI	NI	NI	NI				
N17	Ovarian cyst	65	6				NI	NI	NI	NI	
N18	NFT	63	208		NI	NI				NI	
N19	NFT	32	49	NI	NI	NI				NI	
N20	NFT	37	16		NI		NI	NI			NI

CA, carcinoma; NFT, negative for tumor; NI, not informative; black square, allelic imbalance; gray square, allelic balance; ?, allelic status indetermined.

details and application of the SPRT in allelic counting have been reported previously (20, 21, 30). Student's *t* test was used to analyze the difference in DNA concentration between malignant and benign ascites fluid samples.

## RESULTS

The DNA concentrations in the ascitic fluid specimens from 40 patients ranged from 2 to 5774 ng/ml (Fig. 2; Table 1). The average  $\pm$  1 SD of DNA concentrations was  $820 \pm 1349$  ng/ml in cytology-positive specimens, which was significantly higher than the  $101 \pm 257$  ng/ml in cytology-negative samples ( $P < 0.05$ ). The DNA concentrations in ascitic fluid samples did not appear to be correlated with the grade or amount of necrosis and inflammatory cells in the corresponding tumor tissues.

Analysis of AI in ascitic supernatant DNA was performed in all 40 samples, and the results are summarized in Table 1. All samples, except one (case No. T3), had AI in at least one of the informative SNP markers in ascitic fluids containing malignant tumor cells (Fig. 3A). In contrast, only 1 of 20 ascitic fluid DNA samples from cytology-negative patients demonstrated AI in two SNP markers (Fig. 3B). None of the patients with negative cytology were known to have malignant disease except one patient (case N20), who was known to have stage III ovarian carcinoma at the time of sample collection (Table 1). Further analysis of this specimen revealed the presence of tumor cells in primary culture that were immunoreactive to cytokeratin 7 and CA125, markers associated with ovarian carcinoma (data not shown). The cultured tumor cells demonstrated AI in SNPs 8118

and 825 and allelic balance in SNP 273, a pattern identical to the ascitic supernatant and tumor tissue from the resected ovarian carcinoma. This case was interpreted as a false negative cytology.

To compare the allelic status in the ascitic supernatant DNA with cell pellet DNA, we performed digital SNP analysis on 10 representative DNA samples prepared from cell pellets of ascitic fluids. We found that AI could only be detected in the cell pellet samples containing at least 30% tumor cells as determined by direct counting of the tumor cells on the cytology slides (result not shown). In contrast, all of the corresponding ascitic supernatant samples in which the cell pellets contained <30% tumor cells showed AI.

To assess the AI pattern in the corresponding primary tumors, we analyzed allelic status using the same seven SNP markers in 12 representative tumors in which tumor tissue was available for study. Of these 12 tumors, 11 demonstrated the identical AI patterns seen in the ascitic fluid DNA samples. In one tumor, there was a discordant AI pattern in one of the three informative SNP markers.

## DISCUSSION

The results of this study provide preliminary but cogent evidence that measurement of AI using digital SNP analysis is a highly sensitive technique to detect malignant cells in ascitic fluid. A total of 95% of specimens with malignant cells identified by cytology showed evidence of AI, as did one specimen that was determined to be a false negative case by cytological examination.

There are several advantages of digital SNP analysis compared with other types of molecular analyses in assessing AI in ascitic fluid. First, as compared with microsatellite markers, the PCR products derived from the two SNP alleles at every locus are of the same size, and therefore their analysis is not biased by the preferential DNA degradation of larger alleles. Second, the digital PCR approach, which amplifies single allele templates in the PCR reaction, can precisely determine the number of alleles examined in each experiment. Accordingly, SNP genotyping is digital, involving the detection of the presence or absence of a specific allele, rather than analogue, as is microsatellite genotyping that measures the length of microsatellites (23). Third, a statistical method, SPRT, can be used to conclude whether AI is present in the background DNA. Indeed, it has been shown that AI can be demonstrated in a much higher percentage of colorectal carcinomas using digital SNP analysis as compared with the traditional methods using microsatellite markers (21, 27).

The sensitivity of digital SNP analysis to detect AI in ascitic fluid supernatant is much higher than that in the cell pellets, suggesting that tumor DNA is more abundant in the ascitic supernatant than in the cell pellet. This is likely due to the fact that a large number of tumor cells in the ascitic fluid or peritoneal wall undergo necrosis and apoptosis and release a higher level of tumor DNA into the ascitic supernatant, although tumor cells present in ascitic fluid may only represent a minor cell population. This may account for the capability of digital SNP analysis to detect the cytologically false negative case in this study. This feature of digital SNP analysis is especially important when a limited amount of ascitic fluid can be obtained

and only a few cells are available for cytological examination because no molecular tests are currently available to improve the sensitivity of routine cytological examination.

AI as determined by digital SNP analysis was demonstrated in all malignant ascitic fluid samples except one case with allelic balance in all of the informative SNP markers. This finding can be explained by the fact that the corresponding resected tumor tissue exhibited the identical allelic status and that the SNP markers chosen in this study were not located in the chromosomal regions showing AI in this particular tumor. Although the AI patterns in ascites and the corresponding tumor tissue of the surgical specimen were concordant in most cases, one case did not show an identical pattern. The discordant allelic patterns in the ascites and the corresponding tissue specimen have been reported recently. It may reflect intratumoral clonal heterogeneity and biased tissue sampling for analysis (31).

In summary, this report provides cogent preliminary evidence that AI in ascitic supernatant DNA can be detected in a significant percentage of patients with malignant cytology using digital SNP analysis. For this new technology to have clinical utility, several issues must be addressed. Although the sensitivity of digital SNP analysis in diagnosing malignant ascites in this study was 95%, a higher sensitivity would be desirable. Sensitivity could be improved by including additional SNPs in the assay, and digital SNP analysis could be combined with other molecular tests for mutant genes, for example, *p53* and *K-ras*. Before clinical implementation of this technique, it would be necessary to compare the performance of the new method and routine cytological examination by testing a large series of cytology-negative but biopsy-positive samples using digital SNP analysis. Besides, the value of this new technique should be assessed in the differential diagnosis of benign *versus* malignant specimens. For example, it is important to determine the specificity of digital SNP analysis in distinguishing benign ovarian tumors from carcinomas. Finally, it should be noted that digital SNP analysis could be fully automated on a high-throughput format to reduce labor and reagent cost.

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