Measurement of Cyclin E Genomic Copy Number and Strand Length in Cell-Free DNA Distinguish Malignant versus Benign Effusions

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Abstract

Purpose: Previous studies have shown that the concentration of cell-free DNA was higher and its strand length longer in body fluids obtained from patients with cancer as compared to patients with benign diseases. We hypothesized that analysis of both DNA copy number and strand length of cell-free DNA from an amplified chromosomal region could improve the diagnosis of malignant diseases in body fluids.

Experimental Design: To test this hypothesis, we used ovarian cancer effusion as an example and applied a quantitative real-time PCR to measure the relative copy number and strand length of DNA fragments from one of the most frequently amplified genes, cyclin E, in ovarian serous carcinomas.

Results: As compared with nonamplified chromosomal loci, including β-actin, p53, 2p24.1, and 4p15.31, measurement of cyclin E DNA copy number (100 bp) had the best performance in distinguishing malignant (n = 88) from benign (n = 70) effusions after normalization to effusion volume or Line-1 DNA with areas under the receiver operating characteristics curve (AUC) of 0.832 and 0.847, respectively. Different DNA lengths of the cyclin E locus were further analyzed and we found that the AUC was highest by measuring the 400-bp cyclin E locus (AUC = 0.896). The AUC was improved to 0.936 when it was combined with the length integrity index as defined by the relative abundance of 400 bp cyclin E to 100 bp p53 loci. Cyclin E real-time PCR assay had a higher sensitivity (95.6%) than routine cytology examination (73.9%) and was able to diagnose false-negative cytology cases in this study.

Conclusions: The above findings indicate that measurement of the DNA copy number and strand length of the cyclin E locus is a useful cancer diagnostic tool.

The development of tumor biomarkers that can be clinically applicable in body fluids for cancer diagnosis is useful for clinical management in patients with cancer (1). It is well recognized that several solid malignant tumors release a significant amount of genomic DNA into body fluids including blood, urine, saliva, and effusion from cellular necrosis and apoptosis (2–5). Tumor-released DNA can be detected as a result of specific genetic and epigenetic alterations including point mutations, microsatellite alterations, allelic imbalance, translocation, promoter methylation, and the presence of viral sequences (6–9). In addition to those specific molecular genetic alterations, elevated DNA concentration and increased DNA strand integrity in cell-free DNA have been reported in plasma samples from patients with cancer. As compared with nonneoplastic controls, cell-free DNA concentration was higher in ovarian (7, 8), breast (10), prostate (11), esophageal, and lung (12) carcinomas, as well as in melanoma (13, 14). Furthermore, increased DNA strand length (integrity) of cell-free DNA was found in plasma samples from patients with ovarian cancer (15) and in the stool of patients with colorectal tumors (16).

Increased DNA copy number or genomic amplification at certain loci are frequently present in a variety of human cancers (17). As genomic amplification occurs only in cancer but not in normal tissues, measurement of a certain amplified chromosomal region may likely increase both the sensitivity and specificity of the DNA copy number and DNA strand integrity assays. In this study, we hypothesize that measurements of both DNA copy number and strand integrity of an amplified chromosomal locus in cancer could provide a highly sensitive and specific approach to diagnose malignant versus benign clinical samples in body fluids. To test this hypothesis, we used ovarian carcinoma effusion as an example because ovarian carcinoma is one of the most common causes of malignant effusion in women. Furthermore, the differential diagnosis of malignant from benign effusions would have a significant effect

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Table 1. Clinical diagnosis of benign effusions

Diagnosis	No. of patients
Cardiomyopathy	26
Liver cirrhosis	21
Pneumonia/tuberculosis	15
Others	
Renal failure	3
Pulmonary embolus	2
Meigs syndrome	1
Congenital chylothorax	1
Arthritis	1
Total	70

on the clinical management of patients (18). Based on our single nucleotide polymorphism array analysis (19), we identified cyclin E as the most frequently amplified chromosomal region in ovarian carcinomas which occur in nearly 40% of high-grade ovarian serous carcinomas. Thus, we measured the DNA copy number and strand length of the cyclin E locus and several other control regions that are rarely amplified in ovarian cancer. Our results showed that such an approach showed promise toward diagnosing ovarian cancer effusions.

Materials and Methods

Samples and genomic DNA isolation. The effusion samples were collected at the Innsbruck University Hospital in Austria and the Rikshospitalet-Radiumhospitalet Medical Center in Oslo, Norway. All specimens used were approved by the local institutional review boards or ethics committee and included a total of 268 anonymous effusions (140 ascites samples and 128 pleural effusions). The samples included 88 ovarian carcinoma effusions, 70 benign effusions (Table 1), and 110 effusions from other cancer types (34 lung carcinomas, 21 breast carcinomas, 10 endometrial carcinomas, 8 gastrointestinal carcinomas, 7 pancreatic carcinomas, 6 hepatocellular carcinomas, and 24 other miscellaneous cases). The diagnoses of effusions were based on clinical diagnosis and final pathology reports. There was no statistical significance in ages among the cancer and benign groups ($P > 0.5$, two-way ANOVA analysis). Effusion samples (3 mL-2 L) were collected from patients and samples were centrifuged for 5 min and the supernatants were aliquoted and frozen immediately. Before genomic DNA extraction, the effusion supernatant was centrifuged again and 200 μL of the supernatant, containing the cell-free DNA, was collected from tubes. The genomic DNA was extracted using a Qiagen DNA Blood Kit (Qiagen) according to the manufacturer's instructions. All samples were analyzed in a blinded fashion without prior knowledge of the specimen's identity.

Primer selection and real-time PCR. The primers that amplified each genomic locus were designed based on the Santa Cruz web site.[7] The sequences of all PCR primers used in this study are listed in Table 2. The genomic loci were selected based on the frequency of amplification in ovarian serous carcinomas (19). Among them, cyclin E locus was the most frequently amplified whereas the TP53 locus was commonly deleted (20, 21). Semiquantitative real-time PCR was done using locus-specific primers to obtain the cycle threshold and to calculate the DNA length integrity, which was defined as the ratio of the longer (400 bp) PCR products and the 100 bp PCR products. All samples were done in duplicate. For semiquantitative real-time PCR, an aliquot of 1 μL of the purified cell-free DNA was used in an 11.5 μL PCR mixture containing PCR buffer, 10 μmol/L of deoxynucleotide triphosphate, and 0.125 units/μL of Platinum Taq (Invitrogen). The PCR protocol for iCycler was denaturation for 1 min at 94°C followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 30 s, and extension at 70°C for 5 min. The Bio-Rad iCycler software monitored the changes in fluorescence of SYBR Green I dye (Molecular Probe) in each cycle. The cycle threshold (Ct) value for each reaction was calculated by the iCycler software package. Alternatively, the real-time PCR was done in ABI PRISM 7000 with Power SYBR Green PCR Master Mix (2×, Applied Biosystems) and the default program: 50°C for 2 min, 95°C for 10 min, and 40 cycles of the two-step reaction; 95°C for 15 s and 60°C for 1 min. Cyclin E DNA integrity index was modified from a previous report (15) and was defined as Ct of cyclin E (400 bp) - Ct of p53 (100 bp).

Statistical analysis. The results of the real-time PCR were analyzed using receiver operating characteristics (ROC) curves. ROC curves were constructed for cycle threshold of a given genomic locus and DNA integrity index as a diagnostic marker by plotting sensitivity versus 1- specificity, followed by the calculation of the area under the curve (AUC), which was done by a statistics program, MedCalc version 8.1.1.0.[8] Reproducibility of the real-time PCR assay was done by evaluating the coefficient of variation (CV) for each primer and disease subgroup. Linear regression was used to correlate the cyclin E copy number between ascites cell–free DNA and matched tumor cell pellets in 10 representative ovarian cancer effusions. The DNA copy number of a specific locus was normalized to the effusion volume or the level of the Line-1 DNA element because Line-1 represented the abundant repetitive sequences dispersed in the human genome and was thought to be a reliable reference marker to quantify specific genomic DNA fragments. Fisher exact test was used to determine the significance of the differences between cases and controls. CV was used to determine assay reproducibility between two institutions.

Results

We first tested whether the DNA copy number was different between cancer cases and nonneoplastic controls using primers that amplified 100 bp of cyclin E, β-actin, p53, 2p24.1, 4p15.31, and Line-1 (as the normalization control). We found that the copy numbers of all the genomic loci were higher in ovarian cancer effusions ($n = 88$) than in benign samples ($n = 70$) after normalization to effusion volume or Line-1 DNA ($P < 0.0001$; Table 3). ROC curves were used to compare the performance of the DNA copy number between cyclin E and nonamplified chromosomal loci in distinguishing ovarian cancer effusions from benign samples. As shown in Table 3, cyclin E primers demonstrated the highest performance in distinguishing ovarian cancer versus benign effusion samples with an AUC of 0.832 [95% confidence interval (95% CI), 0.762-0.889] when normalized to plasma volume and 0.847 (95% CI, 0.738-0.923) when normalized to Line-1 DNA.

We then assessed if a DNA length (integrity) index could improve the performance of cyclin E DNA copy number in the differential diagnosis of malignant effusions. We tested the performance of different lengths of cyclin E DNA fragments using different reverse primers that were located 100, 400, 600, and 800 bp downstream from the anchored forward primer. The AUC of the abundance PCR product in distinguishing ovarian cancer versus benign effusions was the highest using the 400 bp (AUC = 0.896) as compared with the 100 bp (AUC = 0.832), 600 bp (AUC = 0.813), and 800 bp (AUC = 0.825; Table 4). Thus,

[7] http://genome.ucsc.edu/

[8] http://www.medcalc.be/

Table 2. Primer nucleotide sequences used in the study

Gene	Product size (bp)	Forward primer	Reverse primer
β-Actin	100	5′-GCACCACACCTTCTACAATGA-3′	5′-GTCATCTTCTCGCGGTTGGC-3′
β-Actin	400	5′-GCACCACACCTTCTACAATGA-3′	5′-TGTCACGCACGATTTCCC-3′
Cyclin E	100	5′-TCATTTACAGCCTTGGGACAA-3′	5′-CTTGCACGTTGAGTTTGGGTA-3′
Cyclin E	400	5′-TCATTTACAGCCTTGGGACAA-3′	5′-AGCGAACAGGAAGACTCAAGC-3′
Cyclin E-DCB	400	5′-GTCCAAAGGCCTGCTCTCAG-3′	5′-AAAGTGGAAACTCGGGCTGTAAG′-3
Cyclin E	600	5′-TCATTTACAGCCTTGGGACAA-3′	5′-GCCAATTTTAAAACATCTGAAGGA-3′
Cyclin E	800	5′-TCATTTACAGCCTTGGGACAA-3′	5′-GGCCAGGAAAGTCACTTGAAC-3′
Line-1	100	5′-AAAGCCGCTCAACTACATGG-3′	5′-TGCTTTGAATGCGTCCCAGAG-3′
p53	100	5′-TCAGATAGCGATGGTGAGCAG-3′	5′-GGCCAGACCTAAGAGCAATCA-3′
p53-DCB	100	5′-TGCAATAGGTGTGCGTCAGAA-3′	5′-AAACCTTAAAATCTAAGCTGGTATGTCCTACT-3′
2p24.1	100	5′-TTCACTCAGGAAACCACATGC-3′	5′-ATTGAACACGGCTCTCCTCTC-3′
4p15.31	100	5′-GGGATCTGACAAACAATCGAA-3′	5′-TGGCTTCTATAACCCCTACAGTTG-3′

we defined the cyclin E DNA strand length (integrity) index as the difference of Ct of cyclin E (400 bp) and Ct of p53 (100 bp). The cyclin E integrity index was significantly higher in malignant effusions than in benign effusions ($P = 0.0014$). As compared with the DNA length integrity index using β-actin (400 bp) - β-actin (100 bp) as we previously described (15), we found that the AUC of cyclin E DNA integrity index defined in this study (cyclin E 400 bp - p53 100 bp) was higher (0.921) than the β-actin DNA integrity index (0.851). Furthermore, combining the cyclin E copy number and length index, we found that the AUC for the cyclin E integrity index increased to 0.936 (95% CI, 0.868-0.975; $P < 0.0001$), with a sensitivity of 73% and a specificity of 97% (Fig. 1).

In the ovarian carcinoma group, 74 samples were obtained from ascites and 14 samples were from pleural effusions. There was no difference in cyclin E copy number and strand length index according to sample origins ($P > 0.5$). Similarly, in the benign disease groups, our results also showed no difference between ascites ($n = 23$) and pleural effusions ($n = 47$; $P > 0.3$). Twenty-three effusion cases were selected in consecutive sequences of collections regardless of their cytology diagnoses and the remaining ovarian cancer cases were selected based on positive cytology. Therefore, only the former group of specimens were used to compare the results of the cyclin E assay with the traditional cytology. The cyclin E real-time PCR was able to identify 22 (95.6%) of 23 cases of ovarian carcinomas that were confirmed by clinical and surgical pathology findings using the cutoff value of 30.705, which was selected as the best value in distinguishing malignant cases versus benign controls based on the ROC curve analysis. In the same group, cytology results were positive in 17 (73.9%) of 23 cases. Therefore, the cyclin E assay was able to diagnose five additional cases of ovarian carcinoma which were not diagnosed by routine cytology. Combining the cyclin E assay with traditional cytology, all 23 cases of ovarian cancer were correctly identified, achieving 100% sensitivity in the diagnosis of malignant effusions.

Ten representative pairs of ascites samples and matched ascites tumor cell pellets from the same patients were compared to determine if there was a correlation in the cyclin E DNA copy number (normalized to Line-1 DNA copy number) between ascites cell–free DNA and the corresponding tumor cell DNA. Linear regression showed a significant correlation ($r = 0.946$) of the cyclin E copy number between ascites cell–free DNA and the genomic DNA from tumor cell pellets of the matched cases.

To determine whether the cyclin E assay was specific to ovarian carcinoma, the effusion specimens of ovarian carcinoma were compared with 110 malignant effusion samples of other cancer types. Using a cyclin E Ct of 30.705, which was used as a cutoff to distinguish ovarian carcinoma from benign samples, the percentage of cases with Ct below the cutoff (i.e., higher copy number) was significantly higher in ovarian cancer cases (88.6%; 95% CI, 95.6-81.6%) than in non–ovarian cancer cases (47.2%; 95% CI, 58.7-35.7%; $P < 0.001$). The reproducibility of the real-time PCR assay was assessed and the overall mean CV for the cyclin E and p53 PCRs were 1.31% and 1.02%, respectively. The reproducibility assay remained consistent and showed minimal variation between samples evaluated from day to day or batch to batch; CV for cyclin E ranged from 0.84% to 1.75% and CV for p53 ranged from 0.59% to 1.52%. Furthermore, the real-time PCR in representative specimens were analyzed in a separate institution (Development Center for Biotechnology, DCB) to assess the reproducibility of the assay for DNA integrity. A different experimental protocol was used for real-time PCR, including different primers, reagent composition, reaction programs, and instruments. We found that the CV for cyclin E was within 3.19 ± 2.3%, whereas for Line-1 it was 8.4 ± 4.8%.

Discussion

In this study, we show that the measurement of quantity and quality of a specific amplified genomic region in cell-free DNA of body fluids can be a useful approach to distinguish a cancerous specimen from a benign one. We showed that the copy number of the cyclin E genomic fragments and its DNA

Table 3. DNA copy number (100 bp) of different loci in the diagnosis of ovarian cancer effusions

Locus	AUC (effusion volume)*	AUC (Line-1) †	*P*
Cyclin E	0.832	0.847	<0.0001
β-Actin	0.816	0.811	<0.0001
p53	0.815	0.807	<0.0001
2p	0.693	0.685	<0.0001
4p	0.657	0.703	<0.0005

*Normalized to plasma volume.
† Normalized to Line-1 DNA copy number.

Table 4. Results of the DNA copy number and DNA length index in the diagnosis of malignant effusions

PCR product	Cycle threshold	DNA length index	AUC (95% CI)	*P*
Cyclin E 100	31		0.832 (0.762-0.889)	<0.0001
Cyclin E 100*	27.42	<0.35	0.879 (0.781-0.944)	<0.0001
Cyclin E 400	30.93		0.896 (0.838-0.939)	<0.0001
Cyclin E 400*	30.705	<0.90	0.936 (0.868-0.975)	<0.0001
Cyclin E 600	31.865		0.813 (0.741-0.872)	<0.0001
Cyclin E 600*	30.82	<0.32	0.914 (0.813-0.971)	<0.0001
Cyclin E 800	31.11		0.825 (0.752-0.883)	<0.0001
Cyclin E 800*	31.11	<0.75	0.905 (0.812-0.962)	<0.0001

*Combination of cyclin E copy number and DNA length index.

length integrity were significantly higher in effusion samples of ovarian cancer than in benign effusions. This finding suggests that increased DNA levels and strand length (integrity) of a specific amplified genomic locus in body fluid are unique characteristics of tumor-released DNA besides molecular genetic changes such as sequence mutations, microsatellite alterations, and allelic imbalance. Our results also imply that measurement of cyclin E DNA fragments using real-time PCR provides a simple diagnostic test for malignant effusions.

Effusions in the abdominal cavity (ascites) and the pleural compartment are associated with a variety of clinical conditions, including inflammatory disorders, infectious diseases, cardiac, liver, and renal diseases, as well as malignant neoplasms (22–24). Cytologic examination is routinely done to distinguish malignant from benign diseases. Although the sensitivity and specificity of cytology, when combined with immunocytochemistry, are generally high in the diagnosis of malignant effusions, they can be variable in some cases (25). This can be a result of a small number of tumor cells in some of the effusion samples or the presence of a large amount of leukocytes, mesothelial cells, and blood which obscure the detection of malignant cells. For example, inflammation, which is commonly associated with a malignant effusion, could result in reactive changes in mesothelial cells that make their morphologic distinction from carcinoma cells difficult. Thus, the cyclin E real-time PCR assay described here may provide an adjunct molecular test to distinguish malignant from benign effusions and could have potential clinical utility.

We first compared DNA copy number of cyclin E and other nonamplified chromosomal loci in effusion DNA samples using a quantitative real-time PCR assay of the 100 bp products. We showed that measurement of the cyclin E DNA copy number has the best performance in the diagnosis of ovarian cancer effusions. Next, we focused on the cyclin E locus to improve its diagnostic efficacy by testing different lengths of PCR products and by combining the DNA copy number and the DNA integrity index of cyclin E. The significant correlation of cyclin E copy number between cell-free DNA from effusion and DNA from the corresponding tumor cell pellets supports the view that a significant amount of cell-free DNA in body fluids was likely derived from tumor cells (7, 8). Results showing that measurement of the cyclin E DNA copy number was better than other genomic loci that are rarely amplified in ovarian cancer suggests that the amplified genomic region was a better marker for cancer diagnosis, as it was released at higher levels from tumor cells into body fluids than the nonamplified chromosomal regions. Based on our previous report, we found that increased DNA copy number in the cyclin E locus was observed in 43.3% of high-grade ovarian serous carcinomas (36.1% of cases showed amplification and 7.2% showed low copy number gain; ref. 19). Thus, many ovarian serous carcinomas we analyzed presumably did not have increased cyclin E DNA copy number and this could explain a mild increase of AUC by analyzing cyclin E as compared with the *β-actin* gene. The current finding was also consistent with our previous report showing that the level of total cell-free DNA was higher in malignant effusions than in benign effusions; however, the performance of total cell-free DNA concentration as a marker was not satisfactory as a sensitive or specific stand-alone assay to differentiate malignant from benign effusions (7). The selection of the cyclin E locus also facilitated a differential diagnosis in malignant effusions which resulted from different types of cancer because cyclin E was more frequently amplified in ovarian cancer than in other types of

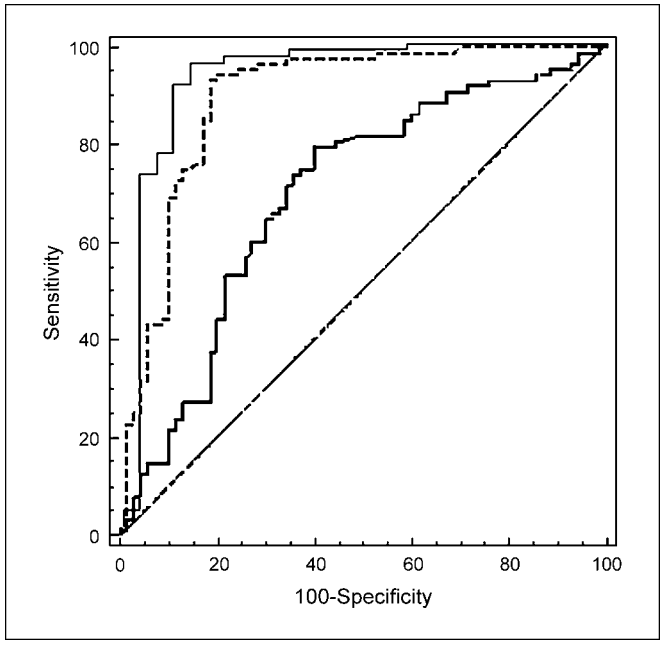

Fig. 1. ROC curve comparison in the diagnosis of ovarian cancer cases versus benign controls. Using cyclin E copy number and strand length integrity index (——), the AUC is 0.936; cyclin E copy number only (– – –), AUC is 0.896; and DNA strand length integrity index only (——), AUC is 0.698.

cancer analyzed in this study. As the current report is a proof-of-concept study, future efforts are required to extend the current findings by analyzing an array of amplified loci in a specific type of cancer to determine if such approaches have value in differential diagnosis among cancer types.

In this study, we combined the cyclin E DNA copy number and the integrity index to improve performance in the diagnosis of ovarian cancer effusions. This is based on the hypothesis that malignant effusions contain longer DNA fragments than benign effusion because tumor cell necrosis may release a spectrum of DNA strand lengths including the longer ones. Indeed, the AUC in differential diagnosis of ovarian versus benign effusions was improved to 0.936 by combining the cyclin E copy number (400 bp) and DNA length index (Ct of cyclin E-p53, 400 - 100 bp; Fig. 1). The use of the longer cyclin E PCR products such as 600 and 800 bp might be seemingly more specific to differentiate cancer cases versus benign controls (Table 2). However, the longer the DNA strands, the lower their abundance in effusion supernatants because of DNA fragmentation, which may decrease the overall assay sensitivity.

In summary, malignant effusions of ovarian cancer contained a much higher copy number of longer cyclin E DNA fragments than benign effusions. There are several molecular approaches that were previously reported to be useful in the diagnosis of malignant effusions and they included fluorescence *in situ* hybridization to detect aneuploidy cells, reverse transcription-PCR to detect mammaglobin expression (26), and digital PCR to detect allelic imbalance (7). Compared with other molecular techniques, the method described here would likely provide a simple assay system that would facilitate the differential diagnosis of malignant effusion besides conventional cytology. In order for this new assay to have clinical utility, several issues should be addressed. Although the sensitivity and specificity of the assay in diagnosing ovarian cancer effusion seemed satisfactory, higher sensitivity and specificity would be desirable. Sensitivity and specificity could be improved by combining the measurement of cyclin E DNA fragments with other amplified genomic loci, secretory tumor-associated markers (27–29), and other molecular assays (26). It seems necessary to compare the performance of the cyclin E real-time PCR assay and routine cytology by prospectively testing a large number of cytology-negative but clinically positive samples. It is also important to address how age, menopausal status, histologic grade, and other clinical variables affect cyclin E copy number and length integrity in effusions. Lastly, the potential use of cyclin E copy number and strand length in other body fluids such as plasma should be investigated.

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