Tumors release genomic DNA into the circulation of cancer patients after cellular necrosis and apoptosis. Isolation of the apoptotic fraction of plasma-circulating DNA can enhance detection of low-level mutations that can serve as tumor biomarkers (1). Because the amount of DNA circulating in the plasma of cancer patients is low, on the order of a few nanograms per milliliter of blood, the number of genes that can be examined for tumor-specific alterations is limited, a situation that reduces biomarker sensitivity. We recently applied whole-genome amplification of plasma-circulating DNA to increase the number of targets that can be analyzed from each sample, thus potentially increasing biomarker sensitivity (2). This approach yields highly-expanded DNA amounts for performing genetic screening; however, there is no preferential enrichment of smaller sized DNA fragments. We report a new method for whole-genome amplification of plasma-circulating DNA, based on ligation-mediated PCR of blunted DNA fragments (BLM-PCR)\(^1\), which results in preferential amplification of smaller size, apoptotic DNA fragments.

Plasma-circulating DNA was extracted from blood obtained from radiation therapy patients after the patients gave informed consent and the study received institutional review board approval. Within 2–3 h of collection, whole blood was centrifuged at 2000g for 15–30 min, plasma was separated, and plasma-circulating DNA was purified by use of a QIAamp\(^{TM}\) MinElute Virus Spin Kit (Qiagen) and quantified via Taqman real-time-PCR. To test for v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) codon 12 mutations, we used a simplified version of the recently reported fluorescent amplicon-generation method (3), which employs the highly thermostable PspGI enzyme to destroy wild-type alleles during PCR. Of the 15 plasma samples studied, plasma samples from 3 patients (#5, #6, and #15) were positive for KRAS mutations when unamplified DNA was used. Tumor-derived DNA from these patients also contained the plasma-identified KRAS nucleotide changes. These KRAS-positive samples (#5, #6, and #15) plus 2 KRAS-negative samples (#12 and #13) were chosen for further study.

To apply BLM-PCR to these plasma-circulating-DNA samples, we generated blunt ends on 2–5 ng plasma-circulating DNA using 0.6 U T\(_4\) DNA polymerase at 12 °C for 15 min in 5 μL ligase buffer supplemented with dNTP at a final concentration of 100 μmol/L. T\(_4\) DNA polymerase was then heat inactivated. Double-stranded adaptors were prepared by annealing the following primers at 55 °C: 5’-TTCCCTCGGATA-3’ and 5’-AGGCACTGTGCTATCAGGG GAA-3’. 5 μL of blunted DNA and 0.8 μL adaptors were then ligated via T\(_4\) DNA ligase. 60-μL final reagent concentrations of the PCR reactions were as follows: 1X GoTaq Flexi™ buffer, 1.5 mmol/L MgCl\(_2\), 2 mmol/L each dNTP, 0.2 μmol/L 24-mer primer, and 6 μL adaptor-ligated product. The reaction was incubated at 72 °C for 3 min followed by rapid cooling on ice. 1.3 U GoTaq Flexi DNA polymerase (Promega) was used. PCR-cycling was: 72 °C, 5 min; 95 °C, 2 min; (95 °C, 15 s; 72 °C, 15 s) × 25 cycles. When we examined the BLM-PCR product via gel electrophoresis, a pattern of discrete DNA sizes of approximately 200 and approximately 400 bp was observed uniformly for all samples (Fig. 1A). Plasma-circulating DNA consists of a mix of small fragments consistent with apoptotic DNA the size of mono- or di-nucleosomes and of large DNA fragments (1, 4, 5). As we demonstrated using larger size DNA, the conditions applied for BLM-PCR preclude amplification of DNA in excess of a few hundred base pairs. We observed a similar ladder-like pattern when we amplified plasma-circulating DNA from healthy volunteers via BLM-PCR (data not shown). When BLM-PCR samples were tested for KRAS mutations, we found that KRAS mutations were absent for samples #12 and #13 (no PCR product in the presence of PspGI), but these mutations were clearly present in samples #5, #6 and #15 (Fig. 1B). Two additional methods of whole-genome-amplification were also applied to the 5 plasma-circulating DNA samples, BL-whole-genome amplification (which amplifies all DNA fragment-sizes), and multi-displacement amplification (which amplifies only the large DNA fragment-sizes) (2); KRAS-mutation detection was also repeated. The KRAS mutation load (fraction of DNA product containing KRAS mutations) was semiquantitatively estimated by measuring the fraction of endpoint PCR product surviving PspGI digestion. For this purpose, samples were amplified either in the presence or in the absence of PspGI, and relative PCR product was quantified via denaturing HPLC. After BLM-PCR, results for the 3 samples that were KRAS-mutation positive demonstrated an increased KRAS mutation load relative to results obtained with other methods of amplification or relative to unamplified plasma-circulating DNA,

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\(^{1}\) Nonstandard abbreviations: BLM-PCR, ligation-mediated PCR of blunted DNA fragments.
indicating an enhanced ability to detect KRAS mutations (Fig. 1C).

In summary, BLM-PCR provides selective amplification of apoptotic DNA and potentially a method to improve identification of rare alleles in plasma-circulating DNA while also providing ample DNA for testing an almost unlimited number of biomarkers for monitoring cancer patients. This technique is anticipated to be equally applicable to other applications such as prenatal diagnosis.

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**References**


2. Li J, Harris L, Mamon H, Kulke M, Liu W, Zhu P, Makrigiorgos GM. Whole genome amplification of plasma-circulating DNA enables expanded
Hepcidin-25, a liver-produced peptide hormone, was initially isolated from human urine and blood ultrafiltrate. Hepcidin-25 is thought to be the central regulator of iron metabolism (1). Iron deficiency is associated with low hepcidin-25 concentrations and anemia of chronic disease, but the true diagnostic value of hepcidin-25 is still under investigation. A recently published review (2) stated that only semiquantitative methods for comparative studies based on mass spectrometry (MS) have been used for the determination of hepcidin in serum and urine.

During sample preparation, hepcidin-25 undergoes strong nonspecific binding to surfaces. A stable isotope-labeled internal standard helps to compensate for any matrix effects. Micro-HPLC-MS/MS with monolithic capillary columns ensures highest resolution and limits of quantification (3).

Native hepcidin-25 (M, 2789) was purchased from Bachem AG. The internal standard, hepcidin-25 [DTHFPI(15C6N1)Cl(15C6N1)]FCCGCCHRSCGMCCKT-OH] was synthesized with isotopically labeled isoleucines by use of the FastMoc/tBu-strategy. Renaturation and purification were performed according to a previously reported method (4). Correct folding was verified by testing bioactivity with an in vitro ferroportin internalization assay (1).

Blood samples were collected and anonymized in-house according to the Roche Diagnostics policy, and informed consent was obtained from all sample donors; results were not used for regulatory purposes.

Calibrators with concentrations of 0.1–100.0 nmol/L were prepared from hepcidin-25. For sample preparation we added 5 μL concentrated formic acid and 50 μL internal standard solution (1450 nmol/L in water, 0.04% acetic acid) to 45 μL human serum or calibrator. Samples were ultrafiltered with a Microcon Ultracel YM-10 filter (Millipore). The flow-through was transferred into HPLC vials. A Thermo Electron Quantum-Ultra triple-quadrupole mass spectrometer, Dionex Ultimate 3000 micro-HPLC, and PS-DVB Monolithic 200-μm internal diameter × 5 cm column (P/N 161409) were used for online micro-HPLC-MS/MS. A 2-μL sample was injected. The mobile phases were (eluent A) 1% formic acid/0.025% trifluoroacetic acid in water and (eluent B) 1% formic acid/0.025% trifluoroacetic acid in acetonitrile, flow rate 3 μL/min, with a linear gradient from 0% to 80% eluent B during 7 min and then held at 80% eluent B until minute 11.

We used microelectrospray ionization in the positive mode; recorded selected reaction-monitoring transitions were m/z 930.8→1145.5 and m/z 935.5→1152.6, collision energy 33 V, and argon collision gas 2.0 mTorr. Carryover was excluded by blank injections. Samples were kept in long-term storage at −80 °C and were stable for at least 2 days at 6 °C. Processed samples were stable for 48 h at 6 °C. We observed no ion suppression attributable to changing elution conditions and monitoring-signal intensities; postcolumn infusion ex-