

Quantitative and Sensitive Detection of Cancer Genome Amplifications from Formalin Fixed Paraffin Embedded Tumors with Droplet Digital PCR

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Abstract

For the analysis of cancer, there is great interest in rapid and accurate detection of cancer genome amplifications containing oncogenes that are potential therapeutic targets. The vast majority of cancer tissue samples are formalin fixed and paraffin embedded (FFPE) which enables histopathological examination and long term archiving. However, FFPE cancer genomic DNA is often degraded and generally a poor substrate for many molecular biology assays. To overcome the issues of poor DNA quality from FFPE samples and detect oncogenic copy number amplifications with high accuracy and sensitivity, we developed a novel approach. Our assay requires nanogram amounts of genomic DNA, thus facilitating study of small amounts of clinical samples. Using droplet digital PCR (ddPCR), we can determine the relative copy number of specific genomic loci even in the presence of intermingled normal tissue. We used a control dilution series to determine the limits of detection for the ddPCR assay and report its improved sensitivity on minimal amounts of DNA compared to standard. Real-Time PCR. To develop this approach, we designed an assay for the fibroblast growth factor receptor 2 genes (*FGFR2*) that is amplified in gastric and breast cancers as well as others. We successfully utilized ddPCR to ascertain *FGFR2* amplifications from FFPE-preserved gastrointestinal adenocarcinomas.

Keywords: Cancer; gene; Amplifications; Copy number variations; Archival cancer sample

Introduction

Genomic amplifications, a type of DNA Copy Number Variation (CNV), are common features of cancer genomes. These amplifications often lead to overexpression of specific cancer oncogenes that act as “drivers” in cancer development. As a result, these amplified oncogenes provide pro-growth signals that lead to tumor proliferation [1]. Inhibition of these amplified oncogenes and their effector signaling cascades often leads to arrest of tumor growth and cell death. Thus, oncogenes residing within cancer genome amplifications provide potential therapeutic targets that are specific to a given tumor [2]. For example, a significant proportion of breast and gastric cancers have amplifications of the gene *ERBB2*, otherwise referred to as *HER2*, which is an oncogenic growth factor receptor. The *ERBB2* gene product is a target for the therapeutic antibody, trastuzumab and other small molecule inhibitors in cancer [3]. Given the existence of therapeutics to treat specific gene amplifications in a “personalized medicine” approach, there is great interest in the accurate and timely identification of genomic amplifications of specific oncogenes. Current detection methods include real-time PCR, high-density array Comparative Genomic Hybridization (CGH) methods, Single Nucleotide Polymorphism (SNP) microarrays and Fluorescence In Situ Hybridization (FISH). While these approaches have generally performed well, they are handicapped by the issues of sensitivity of detection. For example, tumors are often associated with normal stroma that effectively dilutes the presence of genomic amplifications and thus makes detection of amplifications substantially more difficult.

Another challenge for cancer genome amplification analysis is that the vast majority of clinical cancer samples are processed as Formalin Fixed Paraffin Embedded (FFPE) tissues. For clinical pathology laboratories, this is a universal preservation method, because [1] it maintains morphological features of the tumor; [2] it enables histopathologic examination with a number of immunohistological

staining processes and [3] it can be stored indefinitely at room temperature. However, the FFPE fixation process causes irreversible damage to the sample genomic DNA via cross linkages and increased fragmentation. As a result, genomic DNA extracted from FFPE material is often of poor quality. Therefore, analysis of FFPE-derived genomic DNA using PCR-based or microarray-based assays for genomic amplification can be technically challenging.

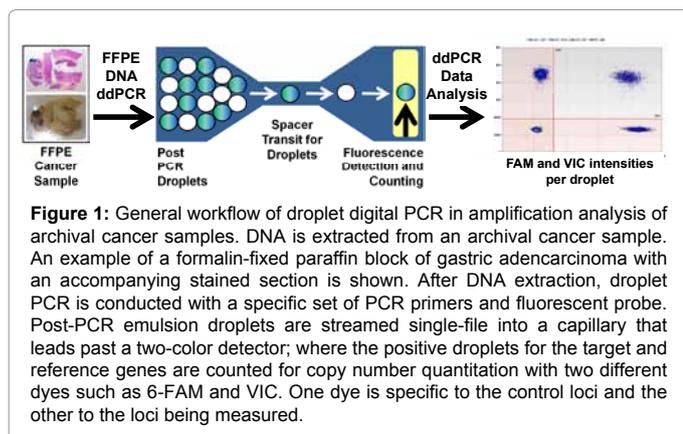
We developed a robust solution for measuring genomic amplifications in FFPE tumor samples using droplet digital PCR (ddPCR) [4] that is sensitive enough to detect abnormal genomic amplifications even if the sample contains only a small fraction of tumor cells. The ddPCR method requires nanogram amounts of genomic DNA, thus facilitating the study of rare samples. As described by Hindson et al. the ddPCR method involves emulsifying the sample which provides specific advantages for highly sensitive and specific detection of certain genomic events including CNVs such as amplifications [5]. In the case of our copy number assay in the post-amplification reaction, emulsion droplets are streamed single-file into a capillary that leads past a two-color detector; where the positive droplets for the target and reference genes are “counted” for quantitation as generally shown in figure 1. We demonstrate here the robustness of ddPCR for highly sensitive and

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specific detection of a cancer gene amplification specific for the *FGFR2* gene from minute amounts of genomic DNA derived from clinical cancer samples [6]. This gene encodes the fibroblast growth factor receptor 2 and is a potential therapeutic target for cancer in clinical trials [7].

Materials and Methods

Sample DNA preparation

This study was approved by the Institutional Review Board (IRB) at Stanford University School of Medicine. Samples were obtained from the Stanford Cancer Institute Tissue Bank. Frozen tissue sections were prepared and Hematoxylin-Eosin (HE) staining was performed on a single section from each tumor. On a subset of samples, we conducted our analysis on matched gastric cancer and normal tissue from the same individual. Tumor samples were macro-dissected from areas where tumor cellular composition was estimated to be 60% or higher. For a number of cases, we also used matched tissue that was confirmed to be normal using pathological examination. For FFPE cancer samples, genomic DNA was extracted using the E.Z.N.A.[®] SQ DNA/RNA Protein Kit (Omega Bio-Tek). For the gastric cancer cell line Kato III, we extracted genomic DNA with the DNAeasy Tissue Kit (Qiagen) following the manufacturer's protocol. Concentrations of genomic DNA were determined with a Nanodrop instrument (Thermo Scientific).

Standard real-time PCR for copy number analysis

We conducted traditional real-time PCR to detect copy number changes in *FGFR2*. Briefly, a 20 μ l reaction mixture was made up of 2X Quantitech SYBR Green PCR master mix (Qiagen), 1 μ M each forward and reverse primers, and 50 ng of template DNA. All real-time PCR assays were performed in triplicate with an iCycler thermocycler (BioRad). Thermal cycling conditions were as follows: 95°C at 15 min (1 cycle); 94°C for 15 s, 60°C for 30 s and 72°C for 15s (40 cycles). Melting curves and product validation proceeded per manufacturer's guidelines. Primers used in this study were ordered from Integrated DNA Technologies and had the sequences 5'-ACTTGGGCTGGAGTGATTTG-3' (forward primer), and 5'-AATCCCATCTGCACACTTCC-3', (reverse primer). Results were normalized using normal genomic DNA.

SNP array analysis

Standard protocols for DNA preparation, array hybridization and scanning were used to analyze the normal, primary tumor and metastatic tumor samples using SNP 6.0 arrays (Affymetrix, Santa Clara, CA). We

used 1 μ g of genomic DNA from our samples for array hybridization. We used the Matlab programming language (Mathworks) for quantile probe normalization, probe level summarization and estimation of the copy number between a tumor and normal samples using log₂ ratio of the normalized signals.

Droplet digital PCR workflow

Digital PCR was performed as previously described on the QX100 droplet digital PCR system (Bio-Rad). Briefly, Taq polymerase PCR reaction mixtures were assembled using 2X ddPCR Supermix for Probes, 20X assays (18 μ M primers and 5 μ M probe) and restriction digested FFPE sample. DG8 cartridges were loaded with 20 μ l PCR reaction mixtures and 70 μ l of droplet generation oil for each sample. The cartridges were placed into a droplet generator for emulsification and the emulsified samples were transferred onto 96-well plate as per manufacturer's instructions. Plates were heat-sealed and underwent 40 cycles in a C-1000 thermal cycler (Bio-Rad). After PCR, the 96-well droplet PCR plates were loaded into a droplet reader, which sequentially reads the droplets from each well of the plate. Analysis of the ddPCR data was performed using the CNV mode of the QX100 analysis software (version 1.2.9.0), relying on two probes, one specific to the target with a FAM fluorescent signal and the second representing a control with a VIC fluorescent signal. We had replicates for all of the ddPCR wells for any given sample. A dilution series with the genomic DNA from Kato III and NA18507 involved seven replicates.

Determination of cancer genomic amplifications in FFPE tumor samples

For the assessment of copy number on high quality genomic DNA samples, we digested the samples with a restriction enzyme in order to unlink tandem gene copies for random distribution into droplets for precise DNA quantitation using Poisson statistics. The digestion step was not required for FFPE samples as the DNA tends to be highly fragmented, but was performed here to maintain experimental consistency. To assess *FGFR2* copy number, 125 ng of each FFPE sample was digested with 1.25 units of BsaJI (New England BioLabs) in 15 μ l for 1 h at 60°C. The restriction enzyme reactions were diluted 1.67-fold to 25 μ l with nuclease free water then 25 ng (5 μ l) was assayed per 20 μ l ddPCR reaction. *FGFR2* assay sequences were (forward primer) 5'-GGCTGGCTGCTGAAGTCT-3', (reverse primer) 5'-CTTAATCGCCTGTATGGTGGTAACA-3', and (probe) 5'-FAM-TCTTGTCGTGTTCTTCATTCGGCACAG-BHQ1-3' (Biosearch Technologies). This sequence localizes around genome location chr10:123274680-123274760 based on the NCBI build 37. The *FGFR2* assay was duplexed with a reference assay targeting highly conserved sequence on Chromosome 1 that was used for comparison during the counting assay. This reference assay used the following primers: (forward primer) 5'-TGAGGGATTTCGGCAGATGTTG-3', (reverse primer) 5'-CTGAAAGGCTGGACTTGACAGA-3', and (probe) 5'-VIC- ACTGTGTGCTGGACCT-MGB-3' (Life Technologies). All assay primers were ordered from Integrated DNA Technologies. Thermal cycling conditions were 95°C, 10 min (1 cycle); 94°C, 30 s and 60°C, 60 s (40 cycles); 98°C, 10 min (1 cycle) and a 12°C hold. *FGFR2* copy number per cell was estimated as the ratio of the *FGFR2* and chromosome 1 marker concentrations multiplied by two to account for the two copies that are expected per a normal diploid genome.

Results

As previously described, a major issue in the detection of gene amplifications is the heterogeneous nature of tumor samples. Tumor and normal cells are admixed and this can reduce the sensitivity

of detection when determining the presence or extent of genomic amplification. To address this challenge of mixed sample copy number measurement, we determined the limits of detection of the ddPCR assay for *FGFR2* amplification. We identified a gastric cancer cell line, Kato III, containing a well-described amplification of the *FGFR2* gene [6]. Kato III genomic DNA was diluted in decreasing ratios with DNA from a well-described normal diploid genome sample (NA18507, Coriell Institute). After we verified the *FGFR2* amplification in Kato III with ddPCR, we calculated the predicted *FGFR2* amplification copy number when diluted with a normal diploid genome in various ratios. The equation for the predicted copy number for the dilutions series is expressed as follows: $CN = X * mutF + 2 * (1 - mutF)$ where CN is the predicted copy number of the mixed sample, X is the copy number of the genomic loci from a pure “mutant” cell line and mutF is the mutant fraction of the mixed sample. The assumption is that the normal cells in the sample have 2 copies per cell for the gene or loci of interest and the copy number estimate is a weighted average of mutant and normal cells.

Using the equation described above, we determined the predicted *FGFR2* copy number for various dilutions of the Kato III: NA18507 dilution as indicated in table 1. For example, in the case of 90% wild type genome (NA18507) admixed with 10% mutant genome (Kato III), X is equal to 310 (the *FGFR2* copy number), mutF is equal to 0.1, and CN is the predicted *FGFR2* copy number. Solving for CN confirms that the *FGFR2* CN is predicted to be 32.8. This calculation was repeated for each dilution to determine the predicted copy number.

Seven replicates were run for each dilution point to empirically determine the actual *FGFR2* copy number values. The reproducibility between replicates was tight, as evidenced by the low coefficient-of-variance. This analysis confirmed the accuracy and sensitivity of ddPCR in quantifying the *FGFR2*-amplified locus even when there was 1,000-fold dilution with normal diploid genomic DNA (Figure 2). Using this cell line with an amplified locus, the 0.1% dilution was found to be statistically distinguishable from a pure normal DNA source. The empirically measured copy number versus the extent of dilution with normal genomic DNA demonstrated a linear relationship with a linear regression R coefficient of 0.99. Based on our analysis, the level of detection based on dilution is thus related to the level of amplification and the ratio of tumor to normal DNA.

Amplification analysis comparison between a matched FFPE and flash frozen tumor sample

In contrast to the high quality genomic DNA obtained from cell lines, genomic DNA obtained from FFPE samples is highly degraded, making standard molecular biology techniques, such as PCR, more difficult. We sought to determine the ability of ddPCR to detect amplification in FFPE-derived cancer and normal tissue samples. As a test case, we analyzed matched FFPE and flash frozen gastric cancer samples from one individual (ID 525). Using high quality genomic DNA from the flash frozen gastric tumor, we identified a 6-fold amplification

of the *FGFR2* locus using an Affymetrix 6.0 microarray. From the same tumor which had a portion that was FFPE processed, we used ddPCR to measure the *FGFR2* amplification and confirmed the presence of an approximate 7-fold amplification of the *FGFR2* locus (Figure 3A). As a performance comparison, we used a standard real-time PCR assay on the same FFPE tumor sample and measured a copy of number estimate of 35 (Figure 3B). These results suggest ddPCR is more accurate than real-time PCR for determining copy number variants in FFPE-derived samples. As a control, we also analyzed a normal gastric tissue sample (ID 2502) from an FFPE block with ddPCR (Figure 3A) and real-time PCR (Figure 3B). In both cases, there was no evidence of the *FGFR2* amplification, supporting the specificity of our method.

Genomic amplification analysis in FFPE gastrointestinal cancer samples

Given that ddPCR accurately detects genomic amplifications from archival FFPE tumors, we analyzed FFPE colorectal and gastric adenocarcinomas for the presence of *FGFR2* amplification. This included two different subtypes of gastric cancer referred to as intestinal versus diffuse. We also analyzed diffuse gastric cancer samples that were either sporadic or originated from individuals with Hereditary Diffuse Gastric Cancer, an inherited cancer syndrome. Germline carriers of a mutation in the *CDH1* gene are at increased risk for the diffuse subtype.

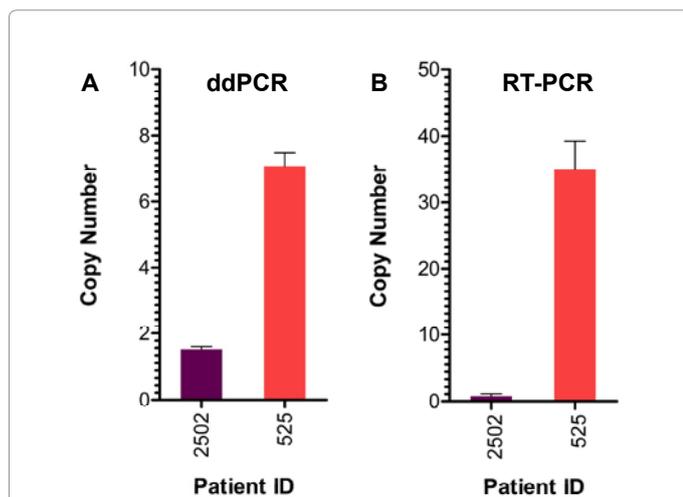
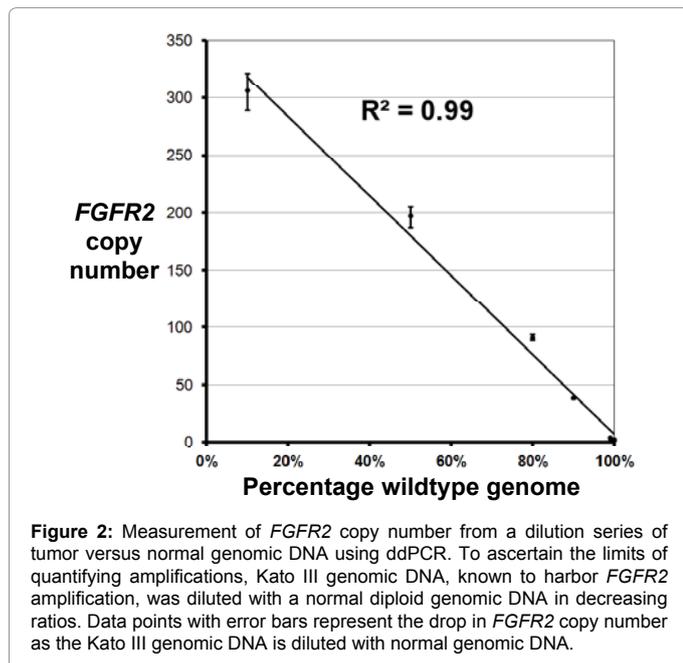
Analysis of four separate colorectal carcinoma samples (IDs 602, 1428, 1427, 1585) did not identify any instances of *FGFR2* amplifications, an expected result given that this amplification has not been described in colon cancer. We assessed the presence of *FGFR2* amplifications in six sporadic gastric carcinomas (IDs 2521, 2512, 709, 2505, 2502, 2504) (Figure 4). *FGFR2* amplifications were detected in two (IDs 2521, 2512) of the six. For five of the diffuse gastric cancer samples, we had matched normal FFPE tissue from the same individual (IDs 2521, 2512, 2505, 2502, 2504) with the exception of one sample (ID 709). Every tested, matched normal gastric tissue contained no *FGFR2* amplification, thus confirming that the amplifications in 2521 and 2512 were somatic in nature and related to the tumor (Figure 4). We also evaluated seven hereditary diffuse gastric carcinomas (IDs 525, 2618, 379, 2619, 561, 778 and 619) of the diffuse subtype and identified a single *FGFR2* amplification (Figure 4). All six of these individuals inherited a germline *CDH1* mutation and thus are at risk for developing gastric cancer. With the exception of ID 525 as previously described, none of these samples had *FGFR2* amplifications. From the intestinal subtype (IDs 15314, 15290, 15313, 15289) of the gastric cancer samples, none had *FGFR2* amplifications (Figure 4).

Discussion

We developed a droplet digital PCR assay method of determining genomic amplifications from archival FFPE cancer samples. We demonstrated ddPCR offers improved accuracy and precision in measuring genome amplification from FFPE samples over traditional real-time PCR and other techniques that are adversely affected by PCR

| % Kato III (tumor DNA) | % Wildtype (normal DNA) | Predicted <i>FGFR2</i> Copy Number | Measured average <i>FGFR2</i> Copy Number | Coefficient of variation for measured <i>FGFR2</i> CN |
|------------------------|-------------------------|------------------------------------|---|---|
| 0.00 | 100.00 | 2.00 | 1.99 | 0.02 |
| 0.10 | 99.90 | 2.30 | 2.16 | 0.03 |
| 1.00 | 99.00 | 4.80 | 3.88 | 0.02 |
| 10.00 | 90.00 | 29.80 | 38.66 | 0.02 |
| 20.00 | 80.00 | 57.60 | 91.30 | 0.03 |
| 50.00 | 50.00 | 141.00 | 196.57 | 0.05 |

Table 1: Predicted versus measured *FGFR2* copy number across a dilution series with Kato III and normal diploid genomic DNA.

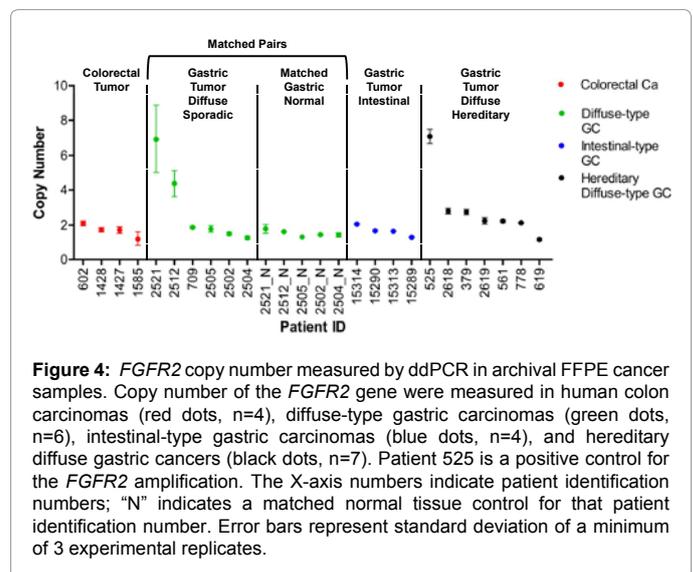


amplification inequalities, sample quality, sample quantity, and sample purity. Clinical cancer specimens from surgical resections and biopsy are frequently comprised of both malignant and adjacent normal tissue. This admixture of tissues with inherently different genetic compositions can subsequently confound copy number estimations. The digital PCR method employed here is highly sensitive and capable of accurately detecting amplified loci present at ratios less than 1:100. Thus, biopsy specimens of tumor admixed with significant amounts of surrounding normal tissue can be accurately evaluated for the presence of an amplified locus. However, the detection limit of ddPCR is dependent on the extent of target locus amplification. Highly amplified genomic loci are more readily detected in highly heterogeneous

populations than low-level amplification events which require a more pure sample for accurate detection.

CNVs, such as genomic amplifications, represent critical genetic events that contribute to the development and progression of human malignancy. In this regard, accurately identifying and characterizing copy number alterations are important for understanding the genetic, genomic and underlying biology of human tumors. Of significant clinical relevance is the detection of genomic amplifications containing oncogenes that can be inhibited with targeted therapies. This represents an increasingly important approach for identifying personalized targets for pharmaceutical intervention. This principle is exemplified by the detection of *ERBB2* (e.g., *HER2*) amplification in gastric and breast tumors with subsequent targeting through small molecules (e.g., lapatinib) and biologics (e.g., trastuzumab), which result in improved patient outcomes and survival. As a demonstration of the translational utility of the ddPCR analysis in cancer studies, we demonstrated reliable detection of amplification of the *FGFR2* gene. Several small molecule *FGFR2* inhibitors have been recently developed for clinical use in cancer patients whose tumors harbor amplification or activating mutations of the *FGFR2* gene. The reliable detection of an *FGFR2* amplification event will be of critical importance in appropriately selecting individuals with cancer that might benefit from pharmacological blockade of the FGF receptor signaling cascade. Overall, the ddPCR approach is another tool for evaluating and measuring the diverse genomic events that drive human malignancies such as *FGFR2* amplifications among others [8].

During the course of our study, we analyzed gastric adenocarcinoma samples. Based on specific histopathological and clinical features, gastric carcinoma is generally categorized into diffuse and intestinal subtypes [9]. Compared to the intestinal subtype, diffuse gastric cancer has a higher incidence of metastatic disease and a generally worse prognosis [10,11]. The amplification events reported in this study were restricted to diffuse-type gastric tumors, but not the intestinal type. Although the sample size in this study was too small to extrapolate a significant difference in amplification frequency or elevation in copy number between these types of gastric cancer, the trend of this small data set is intriguing and warrants additional study. The presence of the amplification events in gastrointestinal cancers reported here and elsewhere suggests that targeted treatments of these solid tumors are feasible and represents an alternative to cytotoxic chemotherapies. Several early-phase trials evaluating the role of oral tyrosine-kinase



inhibitors, including *FGFR2* inhibitors, in gastrointestinal malignancies are underway.

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