A Novel Method for Mutation Analysis Using Genomic DNA Obtained from Immunohistochemistry-Stained Sections

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Background: Tumor biopsies obtained from patients are often limited in size and availability, and the ability to perform multiple diagnostic assays depends on the quantity and quality of the tissue. Here we describe and evaluate a method for performing DNA-based mutational analyses after immunohistochemistry analysis has been performed, using a single tissue section.

Method: Immunohistochemistry analysis was performed on 4-5 µm formalin-fixed paraffin-embedded tumor tissue sections and immunohistochemistry-stained sections were stored for subsequent genomic analysis. DNA was isolated from these immunohistochemistry-stained sections and DNA quality was assessed using a multiplex-polymerase chain reaction method as well as real time quantitative polymerase chain reaction of commonly used reference genes. Subsequently, genomic DNA was pre-amplified and mutations in KRAS, BRAF, NRAS and PIK3CA were detected by validated Taqman assays. Comparisons were made with results from unstained formalin-fixed paraffin-embedded sections obtained from the same paraffin block.

Results: Our results demonstrate that genomic DNA isolated from immunohistochemistry-stained and unstained formalin-fixed paraffin-embedded tissue sections are comparable in quality and are suitable for downstream analysis using polymerase chain reaction based assays. We also found that the sensitivity and specificity in detecting hotspot mutations are comparable in both sources of genomic DNA. This study reports 100% concordance in detecting hotspot mutations in KRAS, BRAF, NRAS and PIK3CA using quantitative real-time polymerase chain reaction between stained and unstained formalin-fixed paraffin-embedded sections.

Conclusion: We conclude that by using our novel approach, it is possible to perform immunohistochemistry staining followed by genomic analysis using a single 4-5 µm section of formalin-fixed paraffin-embedded tissue.

Keywords: Biopsy; Formalin-fixed paraffin-embedded tissue; Immunohistochemistry; Genotyping; DNA analysis

Abbreviations

Ct: Cycle Threshold; FFPE: Formalin-Fixed Paraffin-Embedded; FISH: Fluorescent in situ Hybridization; gDNA: genomic DNA; H&E: Haematoxylin and Eosin; IRB: Institutional Review Board; MND: Mutation Not Detected; PCR: Polymerase Chain Reaction; qRT-PCR: quantitative Real Time Polymerase Chain Reaction

Introduction

Histopathological and immunohistochemical (IHC) analysis have been the predominant methods used to diagnose cancer. A large number of genomic alterations such as gene amplifications, point mutations, translocations, deletions, or insertions have been extensively documented in various types of cancers [1,2].

However, only a small number of such alterations have been causally linked to cancer and they vary from tumor to tumor [3-5]. Identifying relationships between genomic alterations and cancer has provided a number of valuable targets for targeted therapies, such as BRAF mutations in melanoma [6,7] and ALK translocations in lung cancer [8,9]. Identifying genomic alterations along with histopathological and IHC analysis would enable clinicians to stratify patients based on the molecular characteristics of the tumor to deliver targeted therapies. Some well-known examples of such alterations and related therapies are vemurafenib for BRAF-mutant melanoma and crizotinib for lung cancers with EML4-ALK translocation.

The ability to perform multiple assays is often limited by the amount of patient sample available for biomarker assessments. Hence, being able to perform multiple assessments on a single section of tumor tissue would enable diagnostic testing in instances where the amount of tissue available is limited. Generally, clinical biopsies are preserved by fixing in formalin followed by embedding in paraffin for long-term storage. Formalin fixation greatly preserves the cellular architecture, which enables detailed histopathological and IHC analysis. Even though formalin-fixing is generally found to be deleterious for preserving the integrity of nucleic acids, DNA is relatively well-preserved in formalin-fixed paraffin-embedded (FFPE)
tissue compared to RNA. DNA from FFPE tissues have been reliably used for genomic analysis such as sequencing and polymerase chain reaction (PCR) in various tissue types [10-15]. Since FFPE specimens are easily obtainable from the tissue archives, they can serve as an excellent source of tumor DNA for genomic analysis in lieu of fresh or frozen samples.

Various ways of multimodal analysis of solid tumors have been reported. One example successfully combined immunostaining and fluorescent in situ hybridization (FISH) to co-visualize protein expression and chromosomal aberrations [16-19]. Zhang et al [19] reported a study combining estrogen receptor expression and the detection of partial deletion in a tumor suppressor chromosomal region in breast carcinoma cell lines. Ye et al. [17] reported the use of combined multi-color FISH and immunostaining and its importance in future and clinical cancer research. A combined morphological and cytogenetic approach to detect minimal residual disease in leukemia was reported by Grimwade and Freeman [20]. Similarly, a simultaneous visualization of HER2 protein by IHC and gene copy number variation by in situ hybridization has been reported by Nitta et al. [21].

Even though there have been several reports of combining IHC and FISH analysis, no systematic study has been reported evaluating the integrity of DNA obtained from IHC-stained sections. The suitability of using such DNA in PCR-based applications, compared to DNA obtained from unstained sections has not been established. In this study, we evaluated the ability to isolate genomic DNA (gDNA) from tissue sections that have previously been used for IHC. We compared the quality and quantity of gDNA recovered from IHC-stained sections to that obtained from unstained sections. Subsequently, we studied the sensitivity and specificity of detecting oncogenic hotspot mutations using quantitative real-time PCR (qRT-PCR) on gDNA obtained from each section.

Materials and Methods

Tumor specimens

Matched unstained and IHC-stained sections from FFPE tissues derived from 31 patients were obtained from the Genentech human tissue repository for performing this study. From an additional 68 patients, IHC-stained sections were obtained where additional unstained sections were not available. All patients had appropriate IRB (Institutional Review Board) approval and informed consent.

Sample preparation

FFPE tissue sections of 4−5 μm thickness were cut from an archival tissue block and mounted on microscope slides. One section was used for hematoxylin and eosin (H&E) staining and evaluated for histopathological features to confirm diagnosis, tumor content, and was marked to exclude non-tumor tissue in downstream analysis. IHC was performed on tumor tissue sections mounted on glass slides. All IHC steps were carried out on the Ventana Discovery XT automated staining platform (Ventana Medical Systems, Tucson, AZ). Sections were treated with cell conditioning solution, then incubated with specific primary antibody for 1 to 2 hours. Specifically bound primary antibody was detected using the Ultraview detection system (Ventana Medical Systems, Tucson, AZ) and counterstained with Hematoxylin II (Ventana Medical Systems, Tucson, AZ), dehydrated, and coverslipped. Tumors were scored 0 (no signal) to 3 (strong signal) based on staining intensity in ≥50% of tumor cells. Following IHC staining and scoring, stained slides were stored at ambient temperature for 1 to 3 years.

Genomic DNA isolation

IHC-stained sections were immersed in xylene for 1 to 5 days until the coverslips fell off the microscope slides. IHC-stained and unstained sections were treated with a xylene substitute (Envriene, Hardy Diagnostics, Santa Maria, CA) to remove paraffin, followed by two ethanol washes for 2 minutes and 3 minutes, respectively. Sections were air-dried and non-tumor areas removed using a sterile scalpel. The remaining tumor tissue was scraped into a tube containing Proteinase K lysis buffer and gDNA was extracted using the QiAamp DNA FFPE Tissue Kit following manufacturer’s instructions (Qiagen, Valencia, CA). The gDNA isolated was quantified using Nanodrop (Nanodrop Products, Wilmington, DE). The quality of gDNA was assessed using multiplex PCR assays as described below.

Determination of DNA quality

DNA quality was assessed using a multiplex-PCR method [22] as well as qRT-PCR of commonly used reference genes. The multiplex PCR assay consisted of five primer sets derived from the NCBI UniSTS database in which 5 amplicons of increasing size, from 135 bp to 295 bp were amplified by PCR. A pre-amplification step was added to reduce the amount of DNA required to perform the assays. Multiplex primer mix, 10 μM, was prepared by combining 10 primers at a final concentration of 1 μM per primer. Each PCR reaction contained 25 μL of JumpStart RedTaq ReadyMix (Sigma-Aldrich, St. Louis, MO), 3 μL of 25 mM MgCl₂ (1.5 mM final), 1 μL of primer mix (0.2 μM final) and 5 μL of template DNA (25 to 100 ng) in a final volume of 50 μL. Reactions were assembled at room temperature. PCR reactions were run as follows: 94°C (2 min), then 35 cycles at 94°C (1 min), 60°C (1 min), and 72°C (1 min), followed by a final extension at 72°C (7 min). 5 μL of each PCR product was loaded directly onto a 4% agarose gel for electrophoresis. qRT-PCR was performed using equal amounts of DNA (25 ng) from both IHC-stained and unstained FFPE sections on the ABI 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). TaqMan amplification reactions were set up in a reaction volume of 10 μL using the SYBR® Green PCR Master Mix (Applied Biosystem, Foster City, CA) and 200 nM of each primer for GAPDH, Beta Actin and LINE1 genes. qRT-PCR was performed in 384-well reaction optical plates in duplicate. Thermal cycling conditions were 95°C (10 min), then 40 cycles at 95°C (15 sec) and 60°C (1 min). Data was analyzed using SDS analysis software (Applied Biosystems, Foster City, CA) to determine cycle threshold (Cₖ) values.

gDNA pre-amplification for mutation analysis

Twenty ng of gDNA was pre-amplified in 10 μL reactions on a 96-well plate, using a pre-amplification primer cocktail [15] in the presence of 1x TaqMan® PreAmp Master Mix (Applied Biosystems; Foster City, CA). Primer concentrations were maintained at 100 nM during the amplification reaction. Samples were pre-amplified using a Tetrad Thermal Cycler (BioRad; Hercules, CA) using the following protocol: 95°C (10 min), followed by 16 cycles at 95°C (15 sec) and 60°C (2 min). Samples were diluted 10-fold, mixed, centrifuged at 3500 rpm and stored at −20°C. To prevent amplion contamination, separate workspaces and pipettes were used for pre-amplification reaction setup and for dilutions following pre-amplifications. Pre-amplified samples were diluted 1:10 inside PCR.
hoods that were UV-irradiated before each use to prevent amplicon contamination.

**Mutation analysis**

Mutations in *BRAF*, *NRAS*, and *PIK3CA* were detected using Taqman assays that were developed and validated in-house [23]. Details of primers and probes sequences are described by Patel et al. [15]. 1.25 µL of the pre-amplified, diluted DNA was run in each mutation assay reaction along with TaqMan Master Mix (Applied Biosystems, CA) and 900 nM each of forward and reverse PCR primers were added. 200 nM of two TaqMan MGB probes: one specific to the wild-type allele labeled with VIC, and the other specific to the mutant allele labeled with 6-FAM were added. Reactions were carried out in 384-well plates using an ABI 7900HT Sequence Detection System (Applied Biosystems, CA) in duplicate. The following thermal cycling conditions were used: 50°C (2 min) and 95°C (8 min), followed by 40 cycles at 95°C (10 s) and 61°C (30 s).

Mutations in *KRAS* were detected using the Therascreen® KRAS Mutation kit (Qiagen, Velencia, CA) following the manufacturer’s instructions using pre-amplified DNA.

$C_T$ values were determined for each qRT-PCR assay using SDS analysis software (Applied Biosystems, Foster City, CA), and mutation calls were made based on the $\Delta C_T$ values between wild-type and mutant alleles for both TaqMan and DxS assays. An assay was considered valid when the $C_T$ of wild-type assay was ≤30, and invalid or ‘no call’ when the $C_T$ was >30. For Therascreen KRAS assays, samples were determined to be mutant if $\Delta C_T$ was above the pre-specified cut-off for each assay. For *NRAS*, *BRAF* and *PIK3CA* mutation detection assays, samples were considered mutant when the $\Delta C_T$ values were ≤6 and mutation not detected (MND) when $\Delta C_T$ values were >6.

**Results**

The amount of gDNA obtained from IHC-stained sections and their unstained counterparts are summarized in Figure 1. In general, IHC-stained FFPE sections yielded less gDNA compared to unstained sections. The yield of DNA from the IHC-stained samples was up to 48-fold less than their unstained counterparts but in most cases it was still sufficient to carry out mutation analysis.

The quality of DNA obtained from unstained and IHC-stained FFPE sections was assessed to determine whether the quality was adequate for qRT-PCR. Multiplexed PCR analysis followed by gel electrophoresis showed that the quality of DNA obtained from stained and unstained sections was comparable (Figure 2). Despite lower yields, when equal amounts (25 ng) of gDNA from IHC-stained and unstained FFPE sections were amplified by real time PCR, similar $C_T$ values were obtained for target genes GAPDH, Beta Actin, and LINE1. These results are summarized in Figure 3 suggesting that DNA integrity is maintained through the IHC-staining and subsequent storage.

**Figure 1**: gDNA yield from IHC-stained tissue sections and their unstained counterparts.

**Figure 2**: Quality assessment of gDNA isolated from IHC-stained tissue sections and their unstained counterparts by multiplex PCR and gel electrophoresis.

The mutation detection method was validated using patient samples from clinical studies harboring known oncogenic mutations. Thirty-one FFPE samples with known mutations were analyzed and 100% concordance was observed in their mutational status (Table 1). Further, in order to confirm the reproducibility and consistency of data obtained, two independent IHC-stained sections from the same patient were processed separately for 9 samples, gDNA isolated and mutation analysis was performed (Table 2). Finally, we applied this method to perform mutation analysis on 68 additional patient samples where unstained sections were not available, and found that we were able to reliably assess their mutation status (wild-type $C_T$ ≤ 30, Table 3). The quantity of gDNA obtained was too low for 11 samples (16%) to make a reliable assessment (Wild-type $C_T$ >30).
Figure 3: qRT-PCR using 25 ng of DNA from IHC-stained and unstained sections (A) GAPDH, (B) Beta Actin, and (C) LINE1.

| Patient ID | Tissues     | Gene/Mutation    | Stained         | Unstained        |
|------------|-------------|------------------|-----------------|------------------|
|            |             |                  | Wild-type C<sub>T</sub> | Mutant C<sub>T</sub> | Wild-type C<sub>T</sub> | Mutant C<sub>T</sub> |
| 6012       | Colorectal  | MND              | 25.1            | 26.3             | 25.1              | 26.3            |
| 6165       | Colon       | MND              | 25.2            | 24.8             | 25.2              | 24.8            |
| 6172       | Rectum      | MND              | 24.8            | 24.1             | 24.8              | 24.1            |
| 6173       | Colon       | MND              | 24.8            | 24.2             | 24.8              | 24.2            |
| 1703       | Breast      | PIK3CA, 1047R    | 26.7            | 30.3             | 19.0              | 23.4            |
| 1740       | Breast      | MND              | 24.8            | 21.6             | 24.8              | 21.6            |
| 1743       | Breast      | PIK3CA, E545K    | 26.7            | 30.1             | 18.8              | 22.6            |
| 1786       | Breast      | MND              | 25.2            | 19.9             | 25.2              | 19.9            |
| 2282       | Breast      | PIK3CA, E545K    | 19.8            | 21.0             | 16.8              | 21.7            |
| 2286       | Breast      | MND              | 27.5            | 18.4             | 27.5              | 18.4            |
| 2317       | Breast      | MND              | 22.9            | 18.7             | 22.9              | 18.7            |
| 2485       | Breast      | MND              | 18.8            | 19.0             | 18.8              | 19.0            |
| 2643       | Breast      | MND              | 25.6            | 18.2             | 25.6              | 18.2            |
| 2765       | Breast      | MND              | 24.1            | 19.3             | 24.1              | 19.3            |
| 3064       | Breast      | MND              | 27.6            | 17.6             | 27.6              | 17.6            |
| 3528       | Breast      | MND              | 29.2            | 18.3             | 29.2              | 18.3            |
| 3565       | Breast      | PIK3CA, H1047R   | 23.3            | 28.3             | 18.2              | 23.3            |
| 3582       | Breast      | MND              | 24.8            | 18.9             | 24.8              | 18.9            |
| 3659       | Breast      | MND              | 26.1            | 21.1             | 26.1              | 21.1            |
| 3849       | Breast      | MND              | 21.9            | 18.5             | 21.9              | 18.5            |
| 3920       | Breast      | MND              | 28.7            | 18.8             | 28.7              | 18.8            |
| 025-A074   | Colorectal  | Kras, G13D       | 28.2            | 33.2             | 24.5              | 26.2            |
| 025-A090   | Colorectal  | Kras, G12A       | 28.0            | 28.2             | 24.9              | 24.0            |
| 025-A027   | Colorectal  | Kras, G12A       | 28.0            | 29.9             | 24.8              | 26.2            |
**Table 1:** Correlation between mutation calls made using unstained and IHC-stained sections. Mutation analysis was done using TaqMan (BRAF, NRAS) and Therascreen® KRAS and PI3K Mutation kits.

| Patient ID | Tissues     | Gene/Mutation | Stained Section | Wild-type C<sub>T</sub> | Mutant C<sub>T</sub> |
|------------|-------------|---------------|-----------------|--------------------------|----------------------|
| 025-A020   | Colorectal  | MND           | 21.5            | 32.3                     | 21.0                 |
| 025-A020   | Colorectal  | MND           | 24.2            | 37.6                     | 23.1                 |
| 025-A072   | Colorectal  | Kras, G13D    | 22.3            | 26.0                     | 24.1                 |
| 025-A061   | Colorectal  | Nras, Q61R    | 21.3            | 22.5                     | 19.8                 |
| 025-A121   | Colorectal  | Nras, Q61K    | 25.2            | 25.7                     | 21.4                 |
| 025-A028   | Colorectal  | BRAF, V600E   | 16.2            | 19.0                     | 15.7                 |
| 025-A028   | Colorectal  | BRAF, V600E   | 18.1            | 21.1                     | 16.8                 |

C<sub>T</sub>: Cycle Threshold; MND: Mutation not detected

**Table 2:** Reproducibility of two consecutive IHC-stained sections. Mutation analysis was done using TaqMan (BRAF, NRAS) and Therascreen® KRAS and PI3K Mutation kits.

| Patient ID | Tissues | Gene/Mutation | Section | Wild-type C<sub>T</sub> | Mutant C<sub>T</sub> |
|------------|---------|---------------|---------|--------------------------|----------------------|
| 6169       | Colorectal | MND          | 24.6    |                          |                      |
| 4176       | Breast  | No call       | 30.1    | 32.7                     |                      |
| 4417       | Breast  | MND           | 28.3    |                          |                      |
| 4042       | Breast  | PIK3CA, E542K | 27.9    | 32.9                     |                      |
| 4152       | Breast  | MND           | 25.7    |                          |                      |
| 4126       | Breast  | No call       | 34.8    |                          |                      |
| 4396       | Breast  | No call       | 33.7    |                          |                      |
| 4092       | Breast  | No call       | 33.1    |                          |                      |
| 4367       | Breast  | MND           | 27.4    |                          |                      |
| 4146       | Breast  | No call       | 32.2    |                          |                      |
| 4340       | Breast  | MND           | 29.2    |                          |                      |
| 4332       | Breast  | PIK3CA, H1047R | 26.5   | 29.7                     |                      |
| 4331       | Breast  | MND           | 26.9    |                          |                      |
| 4098       | Breast  | MND           | 26.3    |                          |                      |
| 4288       | Breast  | MND           | 27.3    |                          |                      |
| 4289       | Breast  | MND           | 27.4    |                          |                      |
| 6203       | Colorectal | Kras, G12V   | 26.2    | 28.2                     |                      |
| 6014       | Colorectal | Kras, G12D   | 25.7    | 26.6                     |                      |
| 4081       | Colorectal | Kras, G12V   | 29.0    | 30.7                     |                      |
| 4002       | Colorectal | MND          | 24.3    |                          |                      |
| 4091       | Colorectal | Kras, G12D   | 25.6    | 28.5                     |                      |
| 4088       | Colorectal | MND          | 26.3    |                          |                      |
| 4087       | Colorectal | MND          | 26.0    |                          |                      |
| 2302       | Breast   | MND           | 27.1    |                          |                      |
| 3841       | Breast   | MND           | 29.7    |                          |                      |
| 2520       | Breast   | MND           | 29.3    |                          |                      |
2362  Breast  PIK3CA, E545K/D  27.6  29.65
3803  Breast  MND  27.3
2309  Breast  No call  31.0
3500  Breast  MND  26.5
2503  Breast  MND  25.3
3020  Breast  MND  26.0
3641  Breast  MND  26.6
2644  Breast  MND  25.4
4003  Breast  PIK3CA, H1047R  29.1  32.51
2363  Breast  No call  30.5
3069  Breast  No call  30.6
4005  Breast  MND  27.2
2342  Breast  MND  27.5
3700  Breast  MND  25.0
2314  Breast  No call  30.4
2529  Breast  MND  26.5
3022  Breast  MND  29.2
3940  Breast  MND  25.6
3703  Breast  PIK3CA, E545K/D  24.6  25.7
3701  Breast  MND  28.4
4041  Breast  PIK3CA, E545K/D  24.1  32.4
3704  Breast  No call  31.9
2367  Breast  MND  25.6
3560  Breast  PIK3CA, E545K/D  29.1  34.3
1961  Breast  MND  25.5
2480  Breast  MND  25.6
1620  Breast  MND  26.6
3605  Breast  MND  28.4
2985  Breast  MND  22.0
3655  Breast  MND  25.7
3653  Breast  MND  24.8
3942  Breast  MND  23.2
3052  Breast  MND  21.4
3658  Breast  MND  26.9
2661  Breast  MND  22.2
3567  Breast  MND  26.5
2767  Breast  No call  30.4
2160  Breast  MND  25.5
3853  Breast  MND  25.2
2946  Breast  MND  22.8
2145  Breast  MND  21.1
2947  Breast  MND  24.8

Table 3: Unstained sections not available for these samples, mutation analysis was done using IHC stained sections.

Discussion
We have developed a method for isolating gDNA from tissue sections initially used for IHC staining and subsequently stored at ambient temperature for up to 1 to 3 years. The data presented here indicate that the quality of DNA obtained from IHC-stained sections is comparable to those obtained from their unstained counterparts. We analyzed multiple IHC sections from the same tissue sample and were able to demonstrate the reproducibility of the entire process. The quantity of gDNA obtained from these sections may be much lower than unstained sections. This depends on various factors such as the duration of sample exposure to aqueous phase during IHC staining, number of washes performed during IHC staining, and incubation temperatures. In some instances as shown in Figure 1, the DNA obtained from such sections may be too little for making a reliable assessment. However, given sufficient quantity, we demonstrate that the integrity of gDNA is maintained through the process to enable mutation analysis. We also demonstrate that we were able to reliably discriminate between closely-related mutations such as G12A, G12D, and G12V for KRAS. Our results suggest that the gDNA obtained from these FFPE sections may also be suitable for other applications such as sequencing or mass spectrometry.

Thus, we have demonstrated that it is possible to perform IHC followed by genomic analysis using a single 5 µM section of FFPE tissue. Such an approach is extremely valuable in instances where the availability of tissue is limited. We believe that such multimodal analysis approaches will enable diagnostic testing for targeted therapies and personalized healthcare.

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