Detecting clinically relevant ALK rearrangements: Comparing the HTG EdgeSeq ALK*Plus* Assay EU (CE-IVD) to a commercially available ALK FISH assay

Introduction

Numerous mutations have been shown to drive oncogenesis in non-small cell lung cancer (NSCLC). Measuring these events with conventional methods is difficult given the small amount of material typically collected in the standard needle core biopsy or similar tissue collection method.

The purpose of the study described in this white paper was to use the HTG EdgeSeq ALK*Plus* Assay EU to retrospectively determine ALK status in formalin-fixed, paraffin-embedded (FFPE) lung samples of patients diagnosed with NSCLC and whose ALK status had been previously characterized by a FISH DNA probe assay. A finding of equivalent or superior performance to the FISH assay supports the utility of the HTG EdgeSeq ALK*Plus* Assay EU to direct therapeutic decisions for tyrosine kinase inhibitors in NSCLC.

The HTG EdgeSeq ALK*Plus* Assay EU is a sensitive, accurate, and reproducible next-generation sequencing (NGS)-based assay that measures gene rearrangement events in the ALK gene at the mRNA level. Using only a single section of FFPE lung tumor tissue, the HTG EdgeSeq ALK*Plus* Assay EU detects expressed *ALK* gene fusion events. The assay uses a small input amount of tissue for a single assay, preserving valuable tissue for other uses. The *ALK* gene expression data are assessed by an integrated classification algorithm and the sample determined to be ALK-rearrangement positive or ALK-rearrangement negative. The HTG EdgeSeq ALK*Plus* Assay EU is CE-IVD marked and commercially available in the EU.

The extraction-free, mRNA-based assay is automated using the HTG EdgeSeq system. Laboratories then access the tested samples' ALK status by logging into the host software in IVD mode. This all-in-one test is available for use on the Illumina MiSeqDx and Illumina MiSeq sequencers.

Background

Gene rearrangements stem from chromosomal changes that essentially merge parts of two distinct genes. Expression of such a hybrid gene is often mis-regulated and the resultant mutated protein may possess some, all, or none of the characteristics of the two original genes. Gene fusions have been described throughout the scientific literature, and many clinically relevant examples form transformative proteins associated with human oncogenesis.

A well-known example of an oncogenic fusion gene event is the ALK rearrangement family in NSCLC [1-6]. This particular gene fusion will stimulate cell proliferation through the JAK/STAT pathway, which in turn flows through the mTOR pathway of cell proliferation stimulation [7].

The wild-type *ALK* gene encodes a kinase domain at its 3' end and is not expressed in most cell types. *ALK* gene expression is especially rare in non-hematopoietic lineages (such as lung). A DNA-level rearrangement may cause the ALK 3' kinase-encoding domain to be fused to all or part of another gene that is more highly expressed, which effectively bypasses the tight regulation of *ALK* gene expression and leads to overexpression of the *ALK* kinase domain[7]. This overexpression is believed to cause over-activation of the JAK/STAT pathway [8], in turn causing uncontrolled cell proliferation, a hallmark of cancer.

In the case of ALK, several targeted drugs that inhibits ALK kinase activity have been developed, offering a highly-specific treatment option to NSCLC patients with tumors driven by ALK rearrangement (see Figure 1 schematic). For example, the drug XALKORI®, known generically as crizotinib [16-18], is administered to the 3-5% of NSCLC patients whose tumors test positive for ALK gene rearrangement. This targeted therapy has proven much more effective on ALK rearrangement-positive tumors than standard therapies.

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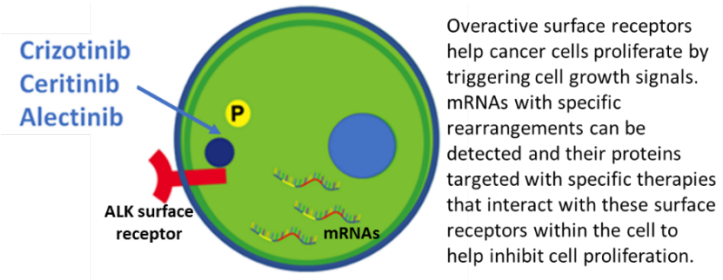

Figure 1: Tumors driven by ALK over-expression may respond to specific therapies.

An interesting feature of crizotinib and other ALK kinase inhibitors (*e.g.* ceritinib, alectinib) is that they also inhibit the function of kinases of similar structure to ALK, including the kinase activity of ROS1 [10] and RET [11-12]. Crizotinib was recently approved for use in NSCLC patients with ROS1 rearrangements. Recent NCCN guideline changes include assessing a NSCLC patient's rearrangement status for these genes as well.

A common laboratory test for ALK, and several other clinically relevant gene rearrangements, is a two-color, break-apart FISH test. This method examines genomic DNA using paired fluorescent probes specific for the 5' and 3' ends of the target gene (*e.g.*, ALK, ROS1, RET, NTRK1). In the presence of such a rearrangement, the 5' and 3' probes "break apart," which can be detected *in situ* under the microscope. Such testing requires a significant amount of tissue - up to five sections to assess fusion events (usually one 5 µm FFPE section per gene rearrangement tested) - and involves a highly variable, manual interpretation of the stained tissue.

The HTG EdgeSeq ALK*Plus* Assay EU is an *in vitro* diagnostic assay for use by laboratory professionals, intended to measure and analyze mRNA *ALK* gene fusion events in FFPE lung tumor specimens from patients previously diagnosed with NSCLC. The HTG EdgeSeq ALK*Plus* Assay EU is indicated as an aid in identifying patients eligible for treatment with ALK inhibitors. In research-use only (RUO) mode, the assay also can detect certain ROS, RET and NTRK1 gene fusion events.

Using only a single section of FFPE lung tumor tissue, the HTG EdgeSeq ALK*Plus* Assay EU consolidates assessment of these rearrangements into a single assay, preserving valuable tissue for future research and evaluation. This use of only a single section of FFPE tissue to quantitatively assess all the markers described above is a strong advantage, especially given the small size of typical lung tumor biopsies in the clinic.

Study design

Purpose

This study utilized FFPE lung tumor specimens and the HTG EdgeSeq ALK*Plus* Assay EU to retrospectively determine ALK rearrangement status in patients diagnosed with NSCLC whose ALK status had been previously characterized by FISH DNA probe assay. The aim was to establish comparative performance, and to establish the utility of the HTG EdgeSeq ALK*Plus* Assay EU to direct crizotinib therapeutic decisions.

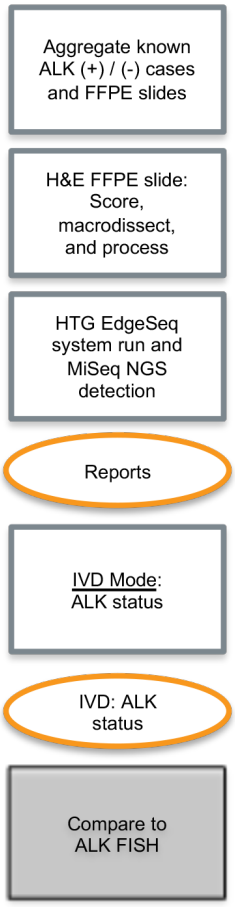

Figure 2: Method comparison study design.

Patient population

Samples used in the study were derived from patients diagnosed with NSCLC, either at initial diagnosis or after first line therapy had failed. Both H&E stained slides and unstained FFPE slides were available as part of the study design.

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FFPE samples

One hundred and forty-one FFPE lung tumor specimens from archived FFPE slides were obtained and analyzed at the University Hospital Göttingen (with institutional consent, courtesy of the University Hospital Göttingen, Göttingen, Germany). The specimens were from patients diagnosed with NSCLC either at initial diagnosis or after failing first-line therapy. All specimens were previously tested for ALK gene rearrangement using a commercially available FISH DNA probe test (ZytoLight SPEC ALK Dual Color Break Apart Probe, from ZytoVision, referred to as "FISH DNA probe assay" or "FISH ALK"). The 141 samples contained a mix of ALK-rearrangement positive and negative samples, as well as ROS1-rearrangement and RET-rearrangement positive samples. FFPE tissue samples included in the study were between 2 and 10 µm thick and provided at least 5 mm^2 of total surface area (for 5 µm; at least 8 mm^2 was used for 2 µm sections). Tumor content within the tissue ranged from 5% to 100%.

Results

The comparative performance of the HTG EdgeSeq ALK*Plus* Assay EU was tested against a group of NSCLC samples that had been previously evaluated for ALK gene rearrangements by FISH DNA probe assay (methods are shown schematically in Figure 2 and described in more detail in the Appendix). These FFPE samples were retrospectively analyzed in conformance with the HTG EdgeSeq ALK*Plus* Assay EU package insert. The resulting overall agreement between the two methodologies was 93.6% (see Table 1).

HTG EdgeSeq ALK*Plus* AssayEU	FISH POS	FISH NEG
POS	41	1
NEG	8	91

Table 1: HTG EdgeSeq ALK*Plus* Assay EU agreement with FISH results.

Discrepant analysis

The study identified nine discordant results. For eight of these discordant results, additional slides were tested by IHC and RNAseq analysis. Eight of the discordant samples were FISH ALK positive and HTG EdgeSeq ALK*Plus* Assay EU negative, and one was FISH ALK negative and HTG EdgeSeq ALK*Plus* Assay EU positive. In six of the eight cases, IHC and RNAseq analysis agreed with the HTG EdgeSeq ALK*Plus* Assay EU results.

HTG EdgeSeq ALK*Plus* AssayEU	Consensus FISH / IHC / RNAseq POS	Consensus FISH / IHC / RNAseq NEG
POS	42	0
NEG	2	97

Table 2: HTG EdgeSeq ALK*Plus* Assay EU agreement with consensus results based on FISH, IHC, and RNASeq testing.

Therefore, as summarized in Table 2, overall agreement of the HTG EdgeSeq ALK*Plus* Assay EU with the consensus methods is 98.6% (negative and positive predictive values of 98% and 100% respectively).

Discussion

Overall agreement between the HTG EdgeSeq ALK*Plus* Assay EU and the FISH DNA probe assay was 93.6%. Eight of the nine discrepant samples were further analyzed by IHC and RNAseq. As shown in Table 3, five of the re-tested samples had borderline FISH ALK results (using a cutoff of 15 positive cells of a total of 100 evaluable cells). The other three re-tested samples did not have available percentage values at time of writing.

Sample	FISH (+) cells (%)	FISH Result	HTG EdgeSeq ALK*Plus* AssayEU	IHC / RNASeq
1	10	Negative	Positive	Positive
2	31	Positive	Negative	Negative
3	18	Positive	Negative	Negative
4	15	Positive	Negative	Negative
5	22	Positive	Negative	Negative

Table 3: Discrepant analysis of five samples.

While a very small sampling, the data in Table 3 suggest that NSCLC lung biopsy samples with borderline FISH results might be more accurately called by the HTG EdgeSeq ALK*Plus* Assay EU than by ALK FISH (analysis of the remaining discordant samples pending).

Conclusion

The HTG EdgeSeq instrumentation and simplified NGS workflow was easily adopted in the clinical molecular lab. The lab was able to generate ALK rearrangement results after only a brief training period.

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The HTG EdgeSeq ALK*Plus* Assay EU and the HTG EdgeSeq system offer clinical laboratories a unique automation and simplified NGS workflow solution to support personalize medicine in NSCLC patient populations.

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APPENDIX

Methods

The HTG EdgeSeq System

To provide researchers with a sensitive, accurate, and reliable platform for tumor profiling, HTG developed and optimized the HTG EdgeSeq system to automate multiplex analysis of small FFPE samples. The extraction-free HTG EdgeSeq chemistry utilizes nuclease protection to produce an untagged library of probes which are further modified to prepare them for NGS sequencing enabling identification of mRNA expression patterns indicative of gene fusions.

Figure 3: The HTG EdgeSeq system.

Sample preparation reagents and materials are included in the HTG EdgeSeq assay kit. The heart of the HTG EdgeSeq system is the automated processor which uses proprietary reagents optimized for FFPE samples and can yield reproducible results using a 5 µm tissue section. The HTG EdgeSeq system can process 96 samples per day and hundreds or thousands of mRNAs can be assessed in each sample. The total process from tissue dissection to report generation takes approximately two days and approximately 3.5 hours of hands-on time.

Sample preparation: Lysis buffer and proteinase K are added to prepare the FFPE tissue sample, making the RNA available to hybridize to corresponding target-specific nuclease protection probes (NPPs). The lysed samples are transferred to the HTG EdgeSeq system in standard 96-well microplates.

Automated target capture with the HTG EdgeSeq system: Target capture is automatically performed by the HTG EdgeSeq processor. NPPs are added to the lysed samples to hybridize and capture the target mRNA. Then S1 nuclease is added to digest non-hybridized mRNA and excess NPPs producing a stoichiometric amount of target-mRNA and NPP heteroduplexes. After S1 digestion, samples are transferred to a new 96-well microplate and the reaction is terminated by the addition of a termination solution followed by heat denaturation.

Adding barcodes and adapters: Processed samples are modified with specially designed tags and Illumina MiSeq sequence adapters that are added via PCR. Each tag contains a unique tag that is used for sample identification and multiplexing. Sequencer specific adapters are required for cluster generation during sequencing. After PCR, individual samples are cleaned-up to remove excess tag primers.

Library quantitation and sequencing: The concentrations of the per-sample libraries are balanced and adjusted to ensure appropriate cluster generation then sequencing is performed on Illumina MiSeq platform. Sequencing data for target genes are imported into HTG Edge Parser software, where the ALK rearrangement status is reported to the laboratorian in a concise, patient-centric format.

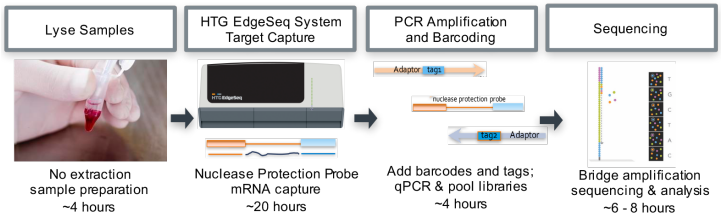

Figure 4: Workflow for the HTG EdgeSeq ALK*Plus* Assay EU.

Data analysis with the HTG Edge parser: The data can be accessed by logging into the HTG Edge host computer in one of two modes: IVD mode or research use only (RUO) mode. When logged into IVD mode, laboratories can assess patients' ALK status. When logged into RUO mode, laboratories can access additional biomarker data

including rearrangement events in the *ALK*, *ROS1*, *RET*, and *NTRK1* genes, activating mutations in HER2 and over-expression of *cMET*. As previously stated, an interesting feature of ALK inhibitors is that they may also inhibit the function of kinases similar in structure to ALK, including the kinase activity of ROS1 [10] and RET [11-12]. Therefore, RUO-mode data from the HTG EdgeSeq ALK*Plus* Assay EU may provide valuable research insight into ALK, ROS1, RET, NTRK1, HER2 and cMET biomarkers and possibly provide clinically relevant data regarding patient status.

The ZytoVision ALK FISH Probe test

The archived FFPE samples utilized in this study were previously analyzed using the ZytoLight SPEC ALK Dual Color Break Apart probe assay (p/n Z-2124). The assay is intended for the detection of genetic aberrations, *e.g.*, translocations, deletions, amplifications, and chromosomal aneuploidies, in formalin-fixed, paraffin-embedded specimens by fluorescence *in situ* hybridization (FISH).

FISH allows for the detection and visualization of specific nucleic acid sequences in cell preparations. Fluorescently-labeled DNA fragments (FISH probes) and their complementary target DNA strands in the preparations are co-denatured and subsequently allowed to anneal during a hybridization step. Unbound probe fragments are then removed, and the remaining FISH probes are counterstained and visualized using a fluorescence microscope. The total process from tissue dissection to fluorescent microscopy takes approximately 2 – 3 days, with significant hands-on time required. Additional time is required for reading the slides and report generation by a qualified pathologist.

Example of the procedure: FFPE tissue cut from blocks were processed and analyzed according to the manufacturer's recommendations [24-25]. Fixation was performed with neutral buffered formalin and samples were embedded in paraffin. Four to six µm microtome sections were attached to positively charged slides and then heat treated and washed with a series of ethanol and wash buffers to remove paraffin. Slides were then deproteinized in a pepsin solution followed by additional washes to remove pepsin activity and to dehydrate the tissue. Following removal of paraffin and pepsin treatment, ZytoLight FISH probes are applied to each slide, sealed under a coverslip, heat denatured, and then allowed to hybridize overnight. The next day, the cover slip is removed followed by a series of washes to remove unhybridized probe. Detection solution is added to the slides and following an dark incubation period, slides are imaged with a fluorescent microscope.

Results interpretation: Refer to the manufacturer's package insert for guidance on the detection and interpretation of FISH probes visualized under fluorescent microscopy.

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