Development of an Immunohistochemical Assay to Detect the Ataxia-Telangiectasia Mutated (ATM) Protein in Gastric Carcinoma

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Abstract: Ataxia-telangiectasia mutated (ATM), a key activator of DNA damage response mechanisms, represents a potential biomarker for targeted gastric carcinoma therapies. A phase II study (Study 39; NCT01063517) designed to investigate the combination olaparib plus paclitaxel in patients with recurrent or metastatic gastric cancer did not meet its primary endpoint of progression-free survival; however, an improvement in the secondary endpoint of overall survival was recorded with a greater overall survival benefit noted in patients with ATM-negative tumors. An ATM immunohistochemical (IHC) diagnostic assay was developed to identify patients who may respond favorably to targeted therapies and deployed in the confirmatory phase III GOLD trial (NCT01924533). The VENTANA ATM (Y170) assay was developed for investigational use in formalin-fixed, paraffin-embedded gastric carcinoma samples using an anti-ATM rabbit monoclonal antibody (clone Y170) and was optimized with OptiView DAB IHC Detection Kit on a BenchMark ULTRA instrument. The assay was deployed in studies assessing sensitivity, specificity, robustness, precision, and determining optimal ATM staining cutoff to define ATM-deficiency (ATM-low). The ATM (Y170) assay met all predefined product development acceptance criteria. Multiple parameters were characterized, including repeatability, reproducibility, analytical sensitivity, specificity, robustness, and product stability. The scoring algorithm was defined; gastric carcinoma samples were considered ATM-negative or ATM-positive when <25% or ≥25%, respectively, of tumor cell nuclei expressed ATM at any IHC stain intensity and nuclei of immune and/or endothelial cells expressed ATM at a moderate stain intensity (internal positive control). Results highlight reproducibility of the assay, supporting suitability for investigational use for evaluation of gastric carcinoma samples using tumor cell staining cutoff of <25% to define ATM-deficiency. Using this ATM assay, phase III GOLD trial (NCT01924533) clinical trial did not meet its primary endpoint, only suggesting, but not demonstrating, that assessment of ATM levels by IHC could possibly be useful in assessing the degree of benefit that may be achieved by adding olaparib to paclitaxel when treating gastric carcinoma. The utility of ATM (Y170) assay as a companion diagnostic requires further clinical validation.

Key Words: ATM, assay, immunohistochemistry, Ventana, olaparib, gastric cancer

Gastric carcinoma is the fifth most common malignancy worldwide, and the third leading cause of cancer-related deaths.1 It is difficult to diagnose because of the complex nature of the disease, leaving radical surgery as the only curative option for most patients presenting at later stages (stage II to IV).2,3 There have been recent improvements with molecular-directed treatments combined with standard chemotherapy as second-line therapies; overall survival (OS) has significantly longer.4 Gastric carcinoma patients expressing high levels of human epidermal growth factor receptor-2 when treated with cisplatin/flourouracil plus trastuzumab versus cisplatin/ fluorouracil alone.5 Methods to identify potential biomarkers as diagnostic tools to identify patients with gastric cancer who may benefit from earlier and targeted therapies is of clinical importance.

Ataxia-telangiectasia mutated protein (ATM) is a protein kinase involved in genome stability, cellular responses to DNA damage and cell-cycle control. Defects in ATM contribute to various B-cell and T-cell leukemias, and mutations in the ATM gene have been identified in gastric carcinoma, leading to loss of expression in tumor cells.6 Low or undetectable ATM expression has been observed in up to 22% of metastatic gastric carcinoma tumors from patients of Eastern origin.6 Gastric cancer cell lines, particularly those with low ATM, have been shown to be sensitive to the poly(ADP-ribose) polymerase inhibitor olaparib.7-10 A phase II study (Study 39; NCT01063517)11 was designed to investigate the combination
olaparib plus paclitaxel in patients with recurrent or metastatic gastric cancer. The study did not meet its primary endpoint of progression-free survival; however, an improvement in the secondary endpoint of OS was recorded with a greater OS benefit noted in patients with ATM-negative tumors.

A phase III study [GOLD trial (NCT01924533)] clinical trial was then designed to confirm the benefit in OS in patients with ATM-low tumor and an investigational-use-only immunohistochemical (IHC) assay was developed, validated and deployed to assess the ATM status of enrolled patients.

We report the development of the VENTANA ATM (Y170) assay [hereafter referred to as ATM (Y170) assay; Ventana Medical Systems Inc., Tucson, AZ], an investigational-use-only IHC assay (for use on a BenchMark ULTRA IHC/ISH automated instrument) to detect ATM protein in gastric tumor samples, as well as the development of scoring criteria to classify samples as ATM-negative.

MATERIALS AND METHODS

Antibody Clone Selection

Three commercial antibodies for ATM were evaluated for staining performance by VMSI; 2 rabbit monoclonal antibodies, Y170 [Abcam, Cambridge, UK (formerly Epitomics)] and SP224 (Spring Bioscience, Pleasanton, CA), and a mouse monoclonal antibody, 2C1 (Abcam) (Supplemental Table 1, Supplemental Digital Content 1, http://links.lww.com/AIMM/A238).12-14

The clonal selection was a necessary first step to identify suitable reagents, technologies, detection chemistries, platforms, and control tissues for assay optimization (Supplemental Material, Supplemental Digital Content 1, http://links.lww.com/AIMM/A238). The screening was performed on cell line controls and gastric carcinoma tissue specimens (Supplemental Material, Supplemental Digital Content 1, http://links.lww.com/AIMM/A238). Overall staining performance was assessed by specificity, sensitivity, stability, and robustness.

ATM (Y170) Assay Design

The assay was developed with a scoring algorithm to determine ATM protein expression in formalin-fixed, paraffin-embedded (FFPE) gastric tissue sections and is intended for use on the automated BenchMark ULTRA instrument (VMSI) with OptiView DAB IHC Detection Kit. The assay consists of one 5 mL dispenser of ~12-μg ATM (Y170) antibody diluted in phosphate buffer with a proprietary carrier protein and 0.05% of ProClin 300 (Sigma-Aldrich, St Louis, MO) preservative. The total protein concentration of the reagent is ~16.4 mg/mL (Supplemental Material; Supplemental Table 2, Supplemental Digital Content 1, http://links.lww.com/AIMM/A238).

Samples

Commercially sourced (Asterand Bioscience, Hertfordshire, UK) FFPE samples (n = 133) from Vietnamese gastric resections were used to define the assay cutoff for ATM-deficient gastric tumors. Verification studies included ~400 FFPE gastric carcinoma cases from US Biomax Inc. (Derwood, MD), PrecisionMed (Solana Beach, CA), and Conversant Bio (Huntsville, AL). All FFPE tissue samples were deidentified samples with no associated identifiable patient information.

Sensitivity and specificity of staining were assessed on 3 unique normal bladder and 3 unique normal parathyroid resection cases, and on 3 multitissue arrays (US Biomax, Inc.) of normal non-neoplastic and neoplastic tissues were assessed using 3 multitissue arrays (US Biomax Inc.):

1. Tour of body, p/n FDA999f, encompassing a range of human non-neoplastic tissues.
2. Tour of tumor, p/n FDA808e2, encompassing a range of human neoplastic tissues.
3. Stomach carcinoma tissue microarray, p/n STC961.

Antibody Peptide Inhibition

For the assessment of peptide inhibition, an FFPE gastric carcinoma ATM-high case was used. The scoring algorithm was defined [see ATM (Y170) assay cutoff determination] where samples of gastric carcinoma cancer were considered ATM-negative when <25% of tumor cell nuclei expressed ATM at any IHC stain intensity and nuclei of immune and/or endothelial cells expressed ATM at a moderate stain intensity. Samples of gastric carcinoma cancer were considered ATM-positive when ≥25% of tumor cell nuclei expressed ATM at any IHC stain intensity and nuclei of immune and/or endothelial cells expressed ATM at a moderate stain intensity (internal positive control). For additional information regarding internal control evaluation and IHC scoring, see the ATM IHC Scoring section. Antibody peptide inhibition analyses are described in the Supplemental Material (Supplemental Digital Content 1, http://links.lww.com/AIMM/A238).

Western Blot

Whole cell lysates from HEK293 (with moderate ATM staining intensity) and GM1526 (created from a patient with known ATM tumor deficiency and confirmed absence of ATM gene expression) cell lines were used. The Western blot also included denatured endogenous and recombinant ATM proteins and recombinant progesterone receptor protein as a negative control.

Assay Staining

The staining procedure for design verification studies is summarized (Supplemental Table 3, Supplemental Digital Content 1, http://links.lww.com/AIMM/A238). The ATM (Y170) assay required 3 serial tissue sections per case; (1) hematoxylin and eosin staining, (2) negative reagent control antibody staining, and (3) ATM (Y170) assay staining. The assay was evaluated based on nuclear DAB signal, with a staining intensity score of 0 to 3, scaled in 0.25-point increments (0, absence of specific signal within the nucleus; 0.5 to 1.25, weak specific staining with light tan to light brown nuclear staining; 1.5 to 2.25, moderate specific staining with moderate brown nuclear staining; 2.5 to 3, strong specific signal with dark brown nuclear staining).

Preanalytical Characterization

Effects of specific preanalytical factors (fixation time, type, and cold ischemia) on staining with the ATM
Heterogeneity Characterization

The ATM (Y170) assay was tested on a cohort of 15 gastric carcinoma FFPE specimens to determine potential variation in ATM status due to ATM expression heterogeneity (Supplemental Material, Supplemental Digital Content 1, http://links.lww.com/AIMM/A238).

Assay Sensitivity and Specificity

ATM (Y170) assay specificity was determined across multiple ATM-positive and ATM-negative cell lines (Table 1), as well as in neoplastic (sensitivity, Table 2A) and normal tissues (specificity, Table 2B). Assessment of the immunoreactivity of the ATM (Y170) assay on normal and neoplastic tissue arrays was performed in accordance with US Food and Drug Administration guidelines. From a total of 272 assessable test samples from the non-neoplastic multiple organ tissue array (tour of body), the neoplastic multiple organ tissue array (tour of tumor), the stomach cancer array, the human tissue microarrays, and 3 normal bladder and normal parathyroid cases, nearly all had acceptable morphology (99.3%, 270/272) and all had acceptable ATM background (100%, 272/272). Of the additional individual non-neoplastic resection tissues assessed, all 9 cases were reactive to the ATM (Y170) assay. These 9 cases replaced the following tissue cores: pancreas (n=1), kidney (n=3), testicle (n=1), and tonsil (n=4). One tonsil case could not be assessed due to a failed negative control slide; 8 cases were included in the analysis.

Neoplastic gastric and lymphoid aggregate cells from the stomach cancer array showed consistent nuclear staining with the ATM (Y170) assay across each stomach cancer tissue (Table 2C). Two ATM-stained medullary carcinoma cores exhibited cytoplasmic blush staining.

At a delay in fixation (cold ischemia) of >2 hours should be avoided. Specimens should be cut at ~4 μm to maximize the staining performance of the ATM (Y170) assay.

Antibody Specificity

Results of Western blot analyses to determine the specificity of the ATM (Y170) antibody to bind to its target biomarker protein and potential anti-ATM (Y170) cross-reactivity to other proteins along with peptide inhibition analysis are reported in the Supplemental Material (Supplemental Figs. 8 and 9, Supplemental Digital Content 1, http://links.lww.com/AIMM/A238).

**TABLE 1. ATM (Y170) Assay Cell Line Testing For Specificity**

| Cell line | Cell Type | Genotype | Predicted Protein | AT4BE Status | AT5BIVA Status | MRC5 Status | 1BR3 Status | AT22IE+pEBST-YZ5 Status | AT22IE+pEBST7 Status | GM01526 Status | GM14680 Status |
|-----------|-----------|----------|-------------------|--------------|---------------|-------------|-------------|-------------------------|----------------------|---------------|---------------|
| AT4BE     | Fibroblast| A>T/T1141 ins4 | 2756×/381× | --/-- | NEG (0) | POS (2+) 95% | POS (1.5+ 40%) | NA | POS (1-3+) very few positive cells (<1%) | POS (2+) |
| AT5BIVA   | Fibroblast| 3207 del6/7278 del6 | 1069 del2/2426 del2 | --/-- | NEG (0) | POS (2+) 95% | POS (1.5+ 40%) | NA | POS (1-3+) very few positive cells (<1%) | POS (2+) |
| MRC5      | Fibroblast| Wild-type | Full length | ++/++ | POS (2+) 95% | POS (1.5+ 40%) | NA | POS (1-3+) very few positive cells (<1%) | POS (2+) |
| 1BR3      | Fibroblast| Wild-type | Full length | ++/++ | POS (2+) 95% | POS (1.5+ 40%) | NA | POS (1-3+) very few positive cells (<1%) | POS (2+) |
| AT22IE+pEBST-YZ5 | Fibroblast | ATM complement | Full length | ++/++ | POS (2+) 95% | POS (1.5+ 40%) | NA | POS (1-3+) very few positive cells (<1%) | POS (2+) |
| AT22IE+pEBST7 | Fibroblast | Full length | ++/++ | POS (2+) 95% | POS (1.5+ 40%) | NA | POS (1-3+) very few positive cells (<1%) | POS (2+) |
| GM01526   | B-lymphocyte | 2 C>T/634 del7 | X/229× | --/-- | NEG (0) | POS (2+) 95% | POS (1.5+ 40%) | NA | POS (1-3+) very few positive cells (<1%) | POS (2+) |
| GM14680   | B-lymphocyte | Wild-type | Full length | ++/++ | POS (2+) 95% | POS (1.5+ 40%) | NA | POS (1-3+) very few positive cells (<1%) | POS (2+) |

ATM indicates ataxia-telangiectasia mutated protein; NA, not available; NEG, negative; POS, positive.
| Neoplastic Tissue (TOT) | ATM Expression Positive Samples/Total Cases (n/N) | Neoplastic Tissue (TOT) | ATM Expression Positive Samples/Total Cases (n/N) |
|------------------------|--------------------------------------------------|------------------------|--------------------------------------------------|
| Abdominal cavity       | 1/1                                              | Pancreas               | 1/2                                              |
| Back (neurofibroma)    | 0/1                                              | Pelvic cavity          | 1/1                                              |
| Bladder                | 2/2                                              | Prostate               | 1/2                                              |
| Bone                   | 1/1                                              | Rectum                 | 1/3                                              |
| Breast                 | 3/3                                              | Retroperitoneum        | 1/3                                              |
| Cerebrum               | 4/4                                              | Skeletal muscle        | 0/3                                              |
| Colon                  | 0/2                                              | Skin                   | 3/5                                              |
| Esophagus              | 1/2                                              | Smooth muscle          | 1/1                                              |
| Intestine              | 1/2                                              | Spleen                 | 1/1                                              |
| Kidney                 | 1/1                                              | Stomach                | 16/22                                            |
| Liver                  | 0/2                                              | Striated muscle        | 1/1                                              |
| Lung                   | 3/6                                              | Testis                 | 1/2                                              |
| Lymph node             | 2/2                                              | Thyroid                | 2/2                                              |
| Mediastinum            | 1/1                                              | Uterine cervix         | 1/2                                              |
| Nerve                  | 3/3                                              | Uterus                 | 1/6                                              |
| Ovary                  | 2/2                                              |                        |                                                  |

| Non-neoplastic tissue (TOB) | ATM expression positive samples/total cases (n/N) | Non-neoplastic tissue (TOB) | ATM expression positive samples/total cases (n/N) |
|----------------------------|--------------------------------------------------|-----------------------------|--------------------------------------------------|
| Cerebrum                   | 3/3                                              | Thymus                      | 2/3                                              |
| Cerebellum                 | 1/3                                              | Myeloid (bone marrow)      | 3/3                                              |
| Adrenal gland              | 3/3                                              | Lung                        | 1/3                                              |
| Ovary                      | 0/1                                              | Heart                       | 0/3                                              |
| Pancreas                   | 3/4                                              | Esophagus                   | 1/3                                              |
| Parathyroid gland          | 3/3                                              | Stomach                     | 2/3                                              |
| Hypophysis                 | 3/3                                              | Small intestine             | 3/3                                              |
| Testis                     | 3/4                                              | Colon                       | 1/5                                              |
| Thyroid                    | 3/3                                              | Liver                       | 1/3                                              |
| Breast                     | 3/3                                              | Uterine/cervix              | 2/3                                              |
| Spleen                     | 3/3                                              | Kidney                      | 4/6                                              |
| Tonsil                     | 4/4                                              | Prostate                    | 0/3                                              |
| Endometrium                | 2/3                                              | Cervix                      | 3/3                                              |
| Skeletal muscle            | 0/3                                              | Skin                        | 1/3                                              |
| Thyroid gland              | 3/3                                              | Larynx                      | 2/3                                              |
| Nerve (sparse)             | 2/3                                              | Mesothelium and lung        | 0/3                                              |
| Tongue                     | 3/3                                              | Bladder                     | 3/3                                              |

### (C) Reactivity of AMT (Y170) Assay for Stomach Cancer Array

| Gastric tissue            | ATM expression positive samples/total cases (n/N) |
|---------------------------|--------------------------------------------------|
| Gastric stromal tumor     | 2/6                                              |
| Gastric ulcer             | 1/2                                              |
| Signet-ring cell          | 8/8                                              |
| adenocarcinoma            |                                                  |
| Mucinous adenocarcinoma   | 10/10                                             |
| Adenocarcinoma            | 42/44                                             |
| Intestinal metaplasia     | 4/4                                              |
| Normal gastric            | 6/6                                              |
| Chronic gastritis         | 5/6                                              |
| Undifferentiated          | 3/4                                              |
| carcinoma                 |                                                  |
| Medullary carcinoma       | 2/4*                                             |
| Non-Hodgkin lymphoma      | 0/2                                              |

Determined by testing ATM expression of formalin-fixed, paraffin-embedded (A) neoplastic TOT (B) TOB non-neoplastic tissues from the multiple organ tissue array and (C) reactivity of ATM (Y170) Assay for stomach cancer array.

*Both cases possessed cytoplasmic blush staining.

ATM indicates ataxia-telangiectasia mutated protein; TOB, tour of body; TOT, tour of tumor.
the ATM antigen with the ATM (Y170) assay was observed in tumor cells for a subset of patients with gastric cancer. In these cases, nuclear staining of non-neoplastic gastric epithelial tissue and lymphoid aggregates and the endothelium of blood vessels (all of which exhibit moderate to strong ATM-positivity) served as important internal positive controls (Supplemental Fig. 10, Supplemental Digital Content 1, http://links.lww.com/AIMM/A238). Histiocytes and neutrophils may show strong nuclear and nonspecific cytoplasmic staining and were therefore not useful as internal positive controls. Specific, blush-like, non-targeted ATM staining was sometimes detected in smooth muscle tissue (if present; Supplemental Fig. 10, Supplemental Digital Content 1, http://links.lww.com/AIMM/A238); however, this did not affect the ability of a qualified reader to determine ATM status.

**ATM (Y170) Assay Cutoff Determination**

The ATM assay in Study 39\(^6\) defined ATM-low gastric tumors as those with a cutoff of ≤10% ATM tumor cell nuclear staining.\(^11\) Although the Study 39 assay (a laboratory-developed test [LDT]) and the newly developed assay reported here use the same ATM (Y170) antibody clone, differences in assay configurations and reagents required a redefinition of the cutoff for ATM-deficient tumors. Because of the lack of availability of Study 39 samples, and to ensure both assays identified the same ATM-low patient population, an alternative cohort from Vietnamese gastric resection samples were stained using the newly developed ATM (Y170) assay and the Study 39 LDT ATM assay.\(^6\) The cutoff for ATM-low patients in this population was defined based on the ≤10% cutoff in Study 39. The distribution of ATM expression was examined to (1) determine the correlation between the assays and (2) define a cutoff to separate the ATM-low and ATM-high expressers. The cutoff was determined to achieve the maximum agreement with assessment by the Study 39 assay and identify the ATM-low subpopulation, as well as minimize the rate of misclassifications (ie, enhancing the robustness of pathologist reading) that may be expected when applied in clinical practice. The cutoff was further evaluated using ATM gene mutation data from a subset of the cohort.\(^7\) Figure 1 shows the overall percentage agreement (OPA), negative percentage agreement (NPA) and positive percentage agreement (PPA) for the Study 39 assay\(^6\) at different percentage positivity ATM cutoffs for the ATM (Y170) assay.

The distributions of ATM status for the cohort from Vietnamese gastric resection samples stained with the ATM (Y170) assay and the Study 39 assay are in Figure 2. Combining the OPA, PPA, NPA, and distribution results, the ATM (Y170) assay with a <25% cutoff clearly identified ATM-low expressers.

A <25% cutoff was selected to identify ATM-low tumors for the ATM (Y170) assay within the positive ATM staining distribution range (between 25% and 50%). The cutoff yielded acceptable levels of concordance with the Study 39 assay (OPA, 91%; NPA, 66%; PPA, 98%). In addition, there were 7 samples with loss-of-function ATM mutations in the Vietnamese panel, all of which were defined as ATM-low by the ATM (Y170) assay (cutoff <25%) and the Study 39 assay (cutoff ≤10%).

Figure 3 shows staining examples with the ATM (Y170) assay from ATM-low cases (cutoff <25%). Supplemental Figure 11 (Supplemental Digital Content 1, http://links.lww.com/AIMM/A238) and Supplemental Figure 12 (Supplemental Digital Content 1, http://links.lww.com/AIMM/A238) show staining examples from ATM-high and challenging cases, respectively. Staining can be heterogeneous, occurring at >1 intensity level and resulting in more challenging cases.
In these cases, assessment at multiple levels of magnification may be useful in estimating the relative percentages of tumor cell staining and differentiating tumor cells from normal stained cells (Supplemental Fig. 12, Supplemental Digital Content 1, http://links.lww.com/AIMM/A238).

Heterogeneity Characterization
Fifteen cases were included in the heterogeneity study. For the block-level heterogeneity, 5 cases were evaluated (2 ATM-low and 3 ATM-high); all showed consistent percentage tumor cell staining throughout the block and were deemed homogenous for ATM status (Supplemental Fig. 13, Supplemental Digital Content 1, http://links.lww.com/AIMM/A238). For the case-level heterogeneity, 10 cases were enrolled in the study (5 ATM-low, 4 ATM-high; 1 ATM-high case was excluded due to a lack of internal control staining). All 4 ATM-high cases were deemed homogenous for ATM status (Supplemental Fig. 14a, Supplemental Digital Content 1, http://links.lww.com/AIMM/A238). Four of the 5 ATM-low cases were deemed homogenous for ATM status. One case, enrolled with 15% ATM tumor cell staining and categorized as borderline, showed variable ATM tumor cell staining which impacted on ATM status and this case was deemed heterogeneous (Supplemental Fig. 14b, Supplemental Digital Content 1, http://links.lww.com/AIMM/A238).

Repeatability and Reproducibility
An analysis of the lot-to-lot reproducibility across 3 preproduction lots of the ATM (Y170) assay demonstrated equivalent staining performance, with 100% agreement for ATM-high and ATM-low cases. All lots demonstrated acceptable nonspecific ATM (Y170) assay background staining and acceptable morphology.

Analysis of intrareader (within individuals) and interreader (between individuals) precision when using the ATM (Y170) assay scoring algorithm to assess ATM-stained gastric carcinoma tissue are summarized in Table 3. The OPA by 3 trained readers (interreader agreement) was 93.3% [95% confidence interval (CI), 89.3-97.3], with an average PPA of 92.8% (95% CI, 87.7-96.8) and an average NPA of 93.8% (95% CI, 89.4-97.3). Similar results were observed with intrareader precision (Supplemental Results, Supplemental Digital Content 1, http://links.lww.com/AIMM/A238).

ATM (Y170) Assay and Epitope Shelf Life and Stability
An initial product dating of 24 months was established based on stability assessments of the ATM (Y170) assay antibody (Supplemental Material, Supplemental Fig. 15, Supplemental Digital Content 1, http://links.lww.com/AIMM/A238). Stability assessment of the ATM epitope suggests that cut slides of gastric carcinoma can be stored for 1 month at room temperature and up to 6 months under refrigerated conditions (2° to 8°C) (Supplemental Material, Supplemental Table 4, Supplemental Digital Content 1, http://links.lww.com/AIMM/A238). Storing slides beyond 1 month without temperature control may result in ATM epitope instability, thereby compromising ATM (Y170) assay interpretation.

![Figure 3](image-url)

**FIGURE 3.** Representative images (A–C, ×20) from 3 ATM-low cases: <25% of neoplastic cells demonstrate specific nuclear ATM staining with the ATM (Y170) assay. ATM indicates ataxia-telangiectasia mutated protein.

| Study Design | Average PPA [n/N (%) [95% CI] | Average NPA [n/N (%) [95% CI] | Average OPA [n/N (%) [95% CI] |
|--------------|-------------------------------|-------------------------------|-------------------------------|
| Intrareader precision: Average of all 3 readers’ comparisons | 272/286 (95.1) [91.6-97.8] | 300/314 (95.5) [92.4-98.0] | 286/300 (95.3) [92.3-98.0] |
| Interreader precision: Average of all 3 readers’ comparisons | 256/276 (92.8) [87.7-96.8] | 304/324 (93.8) [89.4-97.3] | 280/300 (93.3) [89.3-97.3] |

ATM indicates ataxia-telangiectasia mutated protein; CI, confidence interval; NPA: negative percentage agreement; OPA, overall percentage agreement; PPA, positive percentage agreement.
**DISCUSSION**

The discovery of biomarkers in gastric carcinoma is important to aid our understanding of the pathogenesis and management of this difficult-to-treat disease. The phase II trial (Study 39)\(^1\) of patients with advanced gastric cancer which showed a significant improvement in OS for olaparib plus paclitaxel compared with paclitaxel alone, with the greatest benefits in patients with low or undetectable ATM levels strengthened interest in ATM as a potential biomarker for targeted therapy. We report here the successful development and verification of an automated IHC investigational-use-only assay using rabbit monoclonal Y170 to detect ATM levels in tumor cell nuclei in FFPE gastric carcinoma tissues.

Antibody clone selection is a critical step in the development of a specific, sensitive, and robust assay. Initial assessments of staining performance across 3 ATM-targeting antibody clones showed that the ATM Y170 clone demonstrated optimal staining performance relative to the 2C1 and SP224 clones, and was therefore selected for assay development. Multiple parameters were characterized for the ATM (Y170) assay, including repeatability, reproducibility, analytical sensitivity and specificity, robustness, product stability, and pre-analytic factors for gastric cancer FFPE samples. Assessment of assay sensitivity and specificity across multiple tissues supported its use in the detection of ATM levels in cell nuclei of gastric carcinoma tissue. Cytoplasmic reactivity of the assay was observed (9.2% of normal tissue cores and 3.1% of neoplastic tissue arrays), which although atypical for a nuclear protein is supported by data suggesting that ATM may potentially be involved in other cellular processes such as insulin signaling\(^16,17\) or vesicle trafficking.\(^18\)

The ATM assay in Study 39 defined ATM-low gastric tumors as those with a cutoff of \(\geq 10\%\) ATM tumor cell nuclear staining.\(^1\) Although the Study 39 LDT assay and the newly developed assay reported here used the same ATM (Y170) antibody clone, differences in assay configurations and reagents required a redefinition of the cutoff for ATM-low tumors. Using a cohort of Vietnamese gastric resection samples and through comparison with the \(\leq 10\%\) cutoff used in Study 39, ATM-low status was defined using a cutoff of \(<25\%\) of ATM tumor cell nuclear staining (Fig. 2). The \(<25\%\) cutoff separates a fitted bimodal distribution (Fig. 2) and is largely based on frequency distributions of gastric resection samples in the Vietnamese cohort population used within this study. This cutoff was also considered to have benefited from a pathology training perspective. Given the limitation of the human eye, a reader tends to default to ‘binning’ the percentage of tumor cell positive for marker staining (eg, an increment of quartiles or deciles). As highlighted in the Clinical and Laboratory Standards Institute guidance,\(^19\) manual reading of IHC assays is not continuous and is expressed by assigning values to different bins.

Study 39 did not meet its primary endpoint of progression-free survival; however, an improvement in the secondary endpoint of OS was recorded with a greater OS benefit noted in patients with ATM-negative tumors.\(^1\) The subsequent phase III GOLD trial (NCT01924533) did not meet its primary endpoint and did not confirm these findings from phase II, although a strong survival benefit trend was observed in the overall population with olaparib plus paclitaxel.\(^20\) Furthermore, the median OS in the ATM-low group (defined as ‘ATM-negative’) in GOLD was longer than in the overall population, suggesting ATM-low status might represent a prognostic factor in these patients. The different techniques used to determine ATM status in these 2 trials (Study 39 identified ATM-low patients with a single batch of anti-ATM antibody, whereas GOLD used the ATM (Y170) assay (described here) were not considered a contributory factor to the outcomes\(^21\) but highlight the importance of establishing a robust ATM diagnostic assay for universal use.

These clinical trials have suggested, but not demonstrated, that assessment of ATM levels by IHC might possibly be useful in assessing the degree of benefit that may be achieved by adding olaparib to paclitaxel when treating gastric carcinoma.

The results presented here demonstrate that the ATM (Y170) assay is sensitive, specific, robust, and precise for the detection of ATM protein in gastric carcinoma. The clinical utility of the ATM (Y170) assay as a companion diagnostic requires further validation in clinical studies. This assay has the potential to provide high-value clinical utility to identify patients who could benefit from targeted cancer treatment.

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