OPEN

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

Rachel M. Miller, MS, Chinedu Nworu, PhD,* Laurel McKee, MS,* Denis Balcerzak, PhD,† Linh Pham, BS,* Judith Pugh, MD,* Yu-Zhen Liu, MD, PhD,‡ Heather Gustafson, PhD,* Ekta Marwah, MPH,* Tiffany Lamb, PhD,* and June Clements, MD**

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 paxitaxel 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

(*Appl Immunohistochem Mol Morphol* 2020;28:303–310)

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) was significantly longer for gastric carcinoma patients expressing high levels of human epidermal growth factor receptor-2 when treated with cisplatin/fluorouracil plus trastuzumab versus cisplatin/fluorouracil alone.[4] 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.[5] 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

Received for publication November 28, 2018; accepted May 15, 2019.
From the *Ventana Medical Systems Inc., a Member of the Roche Group, Tucson, AZ; †Precision Medicine Laboratories, Precision Medicine and Genomics, IMED Biotech Unit; and ‡Precision Medicine and Genomics, IMED Biotech Unit, AstraZeneca, Cambridge, UK.
C.N., H.G., and E.M. were employees of Ventana Medical Systems Inc. when the study was undertaken.
The authors declare no conflict of interest.
Reprints: Rachel M. Miller, MS, Ventana Medical Systems Inc., a Member of the Roche Group, 1910 E Innovation Park Dr, Tucson, AZ 85755 (e-mail: rachel.miller.rm3@roche.com).
Supplemental Digital Content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's website, www.appliedimmunohist.com.

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

(Y170) assay were evaluated in HT-29 xenograft tumor samples and tonsil specimens (Supplemental Figs. 6 and 7, Supplemental Digital Content 1, http://links.lww.com/AIMM/A238).

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).

RESULTS

Antibody Clone Selection

Staining specificity, sensitivity, and stability studies were conducted on the 3 antibody candidate clones: Y170, SP224, and 2C1. Results are reported in the Supplemental Material (Supplemental Figs. 1–4, Supplemental Digital Content 1, http://links.lww.com/AIMM/A238). Robustness of the Y170 clone was demonstrated by the repeatability of staining performance across a variety of gastric tissue samples on a tissue multiarray encompassing neoplastic and non-neoplastic tissues (Supplemental Fig. 5, Supplemental Digital Content 1, http://links.lww.com/AIMM/A238). On the basis of combined results of the clone selection analyses, the Y170 antibody clone was selected for development in the IHC assay.

ATM (Y170) Assay Verification

Preanalytics: Effect of Fixation Time, Fixation Type, Cold Ischemia, and Tissue Thickness

Results from the analysis of fixation type and time, cold ischemia and tissue thickness are reported in the Supplemental Material (Supplemental Digital Content 1, http://links.lww.com/AIMM/A238). The recommendation for ATM (Y170) antigen preservation is to fix samples in 10% neutral-buffered formalin, zinc formalin, or Z-5 fixative for 6 to 72 hours; a delay in fixation (cold ischemia) of >2 hours should be avoided. Specimens should be cut at $\sim$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).

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.[15] 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.

Although ATM has primarily been considered a nuclear protein, cytoplasmic reactivity of the ATM (Y170) assay was documented in 9.2% of the ATM-stained normal tissue cores across 35 tissue categories and 3.1% of the assessable ATM-stained cores from the neoplastic tissue arrays across 48 diagnostic categories.

ATM IHC Scoring

In gastric carcinoma, neoplastic cells labeled with the ATM (Y170) assay were evaluated for percent positivity based on the nuclear DAB signal. A dynamic range of nuclear immunostaining was observed, from the complete absence of staining to weak and/or strong staining denoting positive ATM protein expression. Loss of staining of

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

Cell line	Cell Type	Genotype	Predicted Protein	ATM Status	ATM Status Determined by ATM (Y170) Assay
AT4BE	Fibroblast	8266 A > T/1141 ins4	2756×/381×	–/–	NEG (0)
AT5BIVA	Fibroblast	3207 del6/7278 del6	1069 del2/2426 del2	–/–	NEG (0)
MRC5	Fibroblast	Wild-type	Full length	+/+	POS (2+) 95%
1BR3	Fibroblast	Wild-type	Full length	+/+	POS (1.5+) 40%
AT22IE+pEBS7-YZ5	Fibroblast	2284 delCT	762×	–/–	NEG (0) 40%
AT22IE+pEBS7	Fibroblast	ATM complement	Full length	+/NA	POS (1-3+) very few positive cells (< 1%)
GM01526	B-lymphocyte	2 C > T/634 del7	X/229×	–/–	NEG (0)
GM14680	B-lymphocyte	Wild-type	Full length	+/+	POS (2+)

ATM indicates ataxia-telangiectasia mutated protein; NA, not available; NEG, negative; POS, positive.

TABLE 2. (A) Sensitivity, (B) Specificity and (C) Reactivity of ATM (Y170) Assay For Stomach Cancer Array

(A) Sensitivity

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		

(B) Sensitivity

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	1/3
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 adenocarcinoma	8/8
Mucinous adenocarcinoma	10/10
Adenocarcinoma	42/44
Intestinal metaplasia	4/4
Normal gastric	6/6
Chronic gastritis	5/6
Undifferentiated carcinoma	3/4
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.

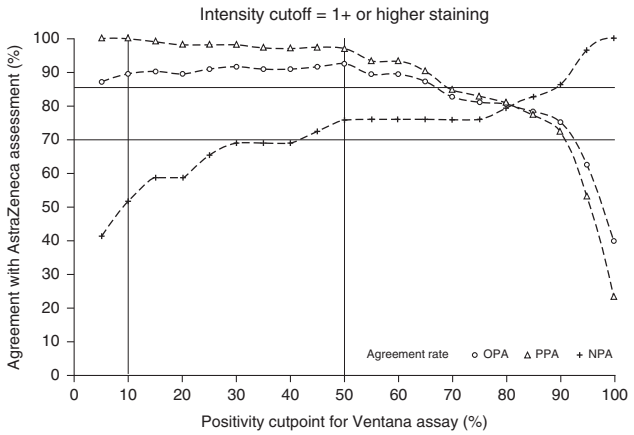

FIGURE 1. Assessment of OPA, PPA, and NPA for the Study 39 assay[6] at different ATM-deficient cutoffs for the ATM (Y170) assay (Vietnamese gastric resection sample panel). ATM indicates ataxia-telangiectasia mutated protein; NPA, negative percentage agreement; OPA, overall percentage agreement; PPA, positive percentage agreement. Note: % Positivity assessed by separate pathologist for each assay.

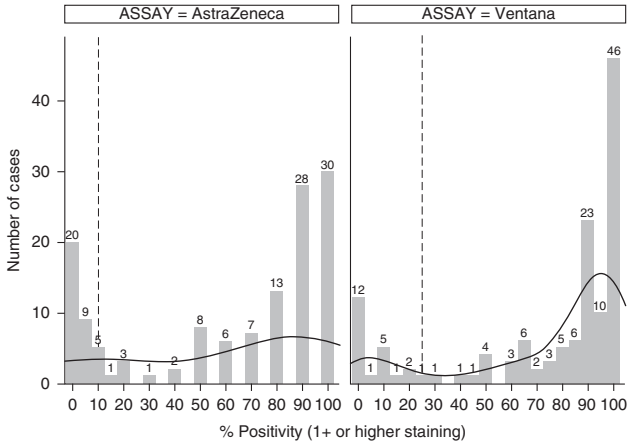

FIGURE 2. The percentage distribution of positive ATM staining (1+ or higher) across the Vietnamese panel samples for the ATM (Y170) assay and the Study 39 assay. Two distinct subpopulations (ATM-low and ATM-high) were observed with the ATM (Y170) assay. The dotted vertical line represents the cutoff for identification of ATM-low patients [10% in Study 39 and <25% for the ATM (Y170) assay]. ATM indicates ataxia-telangiectasia mutated protein.

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, nontargeted 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.

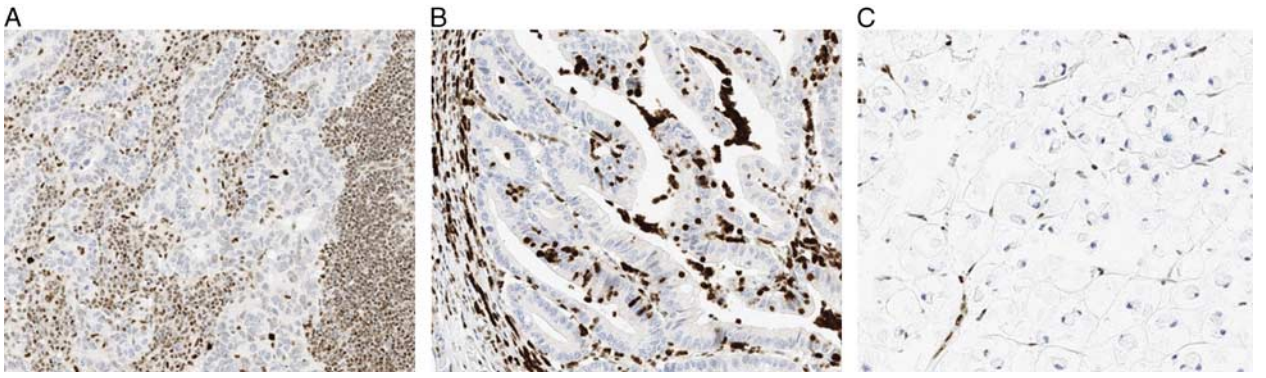

FIGURE 3. Representative images (A–C, ×20) from 3 ATM-low cases: <25% of neoplastic cells demonstrate specific nuclear ATM staining with the ATM (Y170) assay. ATM indicates ataxia-telangiectasia mutated protein.

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.

TABLE 3. Inter-reader and Intra-reader Precision of the ATM (Y170) Assay Scoring Algorithm to Assess ATM-stained Gastric Carcinoma Tissue

Study	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)[11] 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 $\leq$10% ATM tumor cell nuclear staining.[11] 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.[11] 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.

ACKNOWLEDGMENTS

The authors acknowledge the contribution of Margaret Smith, Penny Towne, Deboleena Sarkar, Samina Jafri, Craig Barker, Carl Barrett, Gillian Dalgliesh, Ed Del Valle, Rebecca Fish, Mark Fidock, Thorsten Gutjahr, Darren Hodgson, Helen Jones, Maria Orr, Alan Sharpe, Magnus Söderberg, Vickie Williams, Chris Womark and Lucy Yin. This study was sponsored by Ventana Medical Systems (Roche Tissue Diagnostics) and AstraZeneca. Medical writing support during the development of this manuscript was provided by Martin Goulding, DPhil from Mudskipper Business Ltd and was funded by AstraZeneca.

REFERENCES

1. World Health Organization and International Agency for Research on Cancer. Globocan 2012: stomach cancer; 2012. Available at: http://gco iarc fr/today/fact-sheets-cancers?cancer=5&type=0&sex=0 2012. Accessed December 16, 2012.
2. Howlader N, Noone AM, Krapcho M, et al. *SEER Cancer Statistics Review, 1975–2012*. Bethesda, MD: National Cancer Institute; 2015.
3. Parkin DM. The global health burden of infection-associated cancers in the year 2002. *Int J Cancer*. 2006;118:3030–3044.
4. Bang YJ, Van Cutsem E, Feyereislova A, et al. Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of HER2-positive advanced gastric or gastro-oesophageal junction cancer (ToGA): a phase 3, open-label, randomised controlled trial. *Lancet*. 2010;376:687–697.
5. Schaffner C, Idler I, Stilgenbauer S, et al. Mantle cell lymphoma is characterized by inactivation of the ATM gene. *Proc Natl Acad Sci USA*. 2000;97:2773–2778.
6. Kim HS, Kim MA, Hodgson D, et al. Concordance of ATM (ataxia telangiectasia mutated) immunohistochemistry between biopsy or

metastatic tumor samples and primary tumors in gastric cancer patients. *Pathobiology*. 2013;80:127–137.
7. Hodgson D, Mason H, Oplustilova L, et al. Activity of the PARP inhibitor olaparib in ATM-deficient gastric cancer: from preclinical models to the clinic. *Cancer Res*. 2014;19(suppl):2398.
8. Williamson CT, Muzik H, Turhan AG, et al. ATM deficiency sensitizes mantle cell lymphoma cells to poly(ADP-ribose) polymerase-1 inhibitors. *Mol Cancer Ther*. 2010;9:347–357.
9. Guo X, Shen D, Cheng W, et al. ATM deficiency sensitizes gastric cancer cells to the PARP inhibitior olaparib. *Mol Cancer Ther*. 2009;8(suppl):B42.
10. Kubota E, Williamson CT, Ye R, et al. Low ATM protein expression and depletion of p53 correlates with olaparib sensitivity in gastric cancer cell lines. *Cell Cycle*. 2014;13:2129–2137.
11. Bang YJ, Im SA, Lee KW, et al. Randomized, double-blind phase II trial with prospective classification by ATM protein level to evaluate the efficacy and tolerability of olaparib plus paclitaxel in patients with recurrent or metastatic gastric cancer. *J Clin Oncol*. 2015;33:3858–3865.
12. Abcam. Anti-ATM antibody [Y170] (ab32420); 2018. Available at: www abcam com/atm-antibody-y170-ab32420 html 2018. Accessed February 15, 2018.
13. Spring Bioscience. Rabbit anti-human ATM monoclonal antibody (clone SP224); 2018. Available at: www diagomicscom/media/file/bd/f6/bdd85fe19f098f8b6cb12c7565b4 pdf 2018. Accessed February 15, 2018.
14. Abcam. Anti-ATM antibody [2C1 (1A1)] (ab78); 2018. Available at: www abcam com/atm-antibody-2c1-1a1-ab78-references html 2018. Accessed February 15, 2018.
15. Food and Drug Administration. Guidance for sponsors, institutional review boards, clinical investigators, and Food and Drug Administration staff; Guidance on informed consent for in vitro diagnostic device studies using leftover human specimens that are not individually Identifiable; 2006. Available at: www.fda gov/downloads/medicaldevices/deviceregulationandguidance/guidancedocuments/ucm071265 pdf 2006. Accessed February 15, 2018.
16. Yang DQ, Kastan MB. Participation of ATM in insulin signalling through phosphorylation of eIF-4E-binding protein 1. *Nat Cell Biol*. 2000;2:893–898.
17. Yang DQ, Halaby MJ, Li Y, et al. Cytoplasmic ATM protein kinase: an emerging therapeutic target for diabetes, cancer and neuronal degeneration. *Drug Discov Today*. 2011;16:332–338.
18. Lim DS, Kirsch DG, Canman CE, et al. ATM binds to beta-adaptin in cytoplasmic vesicles. *Proc Natl Acad Sci USA*. 1998;95:10146–10151.
19. Hewitt SM, Robinowitz M, Bogen SA, et al. *Quality Assurance for Design Control and Implementation of Immunohistochemistry Assays; Approved Guideline—CLSI document I/LA28-A2*, 2nd ed. Wayne, PA: Clinical and Laboratory Standards Institute; 2011.
20. Bang YJ, Xu RH, Chin K, et al. Olaparib in combination with paclitaxel in patients with advanced gastric cancer who have progressed following first-line therapy (GOLD): a double-blind, randomised, placebo-controlled, phase 3 trial. *Lancet Oncol*. 2017;18:1637–1651.
21. Bang Y-J. Use of olaparib in patients with advanced gastric cancer—authors' reply. *Lancet Oncol*. 2018;19:e76.