Supplementary Materials for “Gastroesophageal junction adenocarcinoma with germline ATM mutations: clinical descriptors, molecular characteristics and potential therapeutic implications”

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Supplementary Methods

Histopathology and Immunohistochemistry (IHC)

The Tumor Regression Score used is as follows: The complete absence of cancer cells (complete response) is scored 0. The presence of single or rare small groups of cancer cells (near complete response) is scored 1. The presence of residual cancer with an evidence of tumor regression (partial response) is scored 2. An extensive residual cancer without evidence of tumor regression (poor or no response) is scored 3.

Cell culture

ESO26 and OACM51C cells were purchased from Millipore-Sigma and SNU601 cells were obtained from the Korean Cell Line Bank. All cell lines were cultured in RPMI-1640 media (Corning 10-040-CV) supplemented with 10% fetal bovine serum (FBS; VWR 97068-085), 100U/ml penicillin and 100μg/ml streptomycin (Pen/Strep, Corning 30-001-Cl) at 37°C and 5% CO₂.

Absence of mycoplasma contamination was confirmed with a MycoAlert assay (Lonza LT07-118).

siRNA transfection and cell viability assays

siRNA SmartPools targeting ATM and ATR were purchased from Dharmacon (M-003201-04-0005 and M-003202-05-0005, respectively), along with a non-targeting siRNA pool (siCTRL #2, D-001206). 1x 10^6 cells were reverse-transfected with siRNAs at a final concentration of 12.5 nM (12.5 nM siCTRL, 7.5 nM siATM + 5 nM siCTRL, 7.5 nM siCTRL + 5 nM siATR, or 7.5 nM siATM + 5 nM siATR) using a Lipofectamine RNAiMAX reagent (Thermo Fisher 13778150) according to manufacturer’s protocol. Cells were collected 48 h post transfection and re-seeded on 96-well plates (Corning 3603) for cell viability assays. Remaining cells were pelleted and used for immunoblotting. Once siCTRL-transfected cells reached near-confluence, cell viability was analyzed by a CellTiter Glo assay (Promega) according to manufacturer’s instructions.
Luminescence was read on a FlexStation 3 plate reader and % viability was calculated by normalizing raw luminescence values to siCTRL-transfected cells.

**Immunoblotting**

Cell pellets were lysed in 2x Sample Buffer (Novex Tris-Glycine SDS Sample Buffer, ThermoFisher LC2676, supplemented with 200 mM DTT) at a concentration of 1x 10^7 cells/ml. Lysates were boiled at 95°C for 5 min, 20 ml of each sample was loaded on 4-12% Novex Tris-Glycine SDS-PAGE gels (Thermo Fisher) and ran at 75-120V in 1x Novex Tris-Glycine Running Buffer (ThermoFisher, LC2675) followed by blotting on nitrocellulose membranes (ThermoFisher, PB7220) in 1x Novex Tris-Glycine Transfer Buffer (ThermoFisher, LC3675) containing 20% methanol and 0.04% SDS at 90V for 2.5 h. Membranes were blocked in 5% milk / TBST (1x TBS + 0.1% Tween-20) and incubated with primary antibodies (rabbit anti-ATM Novus NB100-104 1:1000; rabbit anti-ATR CST 2790S 1:1000; rabbit anti-GAPDH Millipore-Sigma G9545; 1:20000) diluted in 5% milk / TBST overnight at 4°C. Membranes were then washed 3x 5 min with TBST and incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit IgG, Jackson ImmunoResearch 111-035-144) diluted 1:5000 in 5% milk / TBST. Membranes were washed as above, developed with a SuperSignal West Femto chemiluminescence reagent (ThermoFisher, PI34095) for 2 min and scanned on a ChemiDoc Touch imager (Bio-Rad).

**Gamma-H2AX Immunofluorescence**

ESO26 cells were transfected with the ATM-targeting siRNA pool or a control siRNA pool and 24 h later plated on poly-D-lysine coated high-content microscopy-compatible 96-well plates (CellCarrier-96 Ultra PDL, Perkin Elmer 6055500). On the next day, cells were treated with increasing concentrations of the ATR inhibitor RP-3500 (prepared as a 0.1 mM DMSO stock solution) using a Tecan D300E automatic dispenser. 72 h later media was removed, cells were
rinsed with PBS and fixed with 4% paraformaldehyde/PBS for 10 min at room temperature (RT). Cells were then permeabilized for 30 min at RT with 0.3% Triton X-100/PBS and subsequently blocked with PBG (0.2% cold water fish gelatin / 0.5% bovine serum albumin in PBS) for 30 min at RT. After blocking, cells were incubated for 2h at RT with a mouse anti-gamma-H2AX primary antibody (JBW.301, Millipore Sigma 05-636) diluted at 1:1000 in PBG. Plates were subsequently rinsed with PBS and incubated with an Alexa Fluor 555-conjugated goat anti-mouse secondary antibody (ThermoFisher A32727) diluted 1:1000 in PBG containing 0.5 μg/ml 4’,6-diamidino-2-phenylindole (DAPI). Finally, plates were rinsed 2x with PBS and imaged on an Operetta automated high-content confocal microscope (Perkin Elmer). Image analysis (quantification of nuclear gamma-H2AX intensity) was performed using the Harmony software (Perkin Elmer) using built-in algorithms. The nuclear gamma-H2AX intensity in untreated samples was used as a reference to select a threshold for quantification of Gamma-H2AX-positive cells in cells treated with RP-3500.
### SUPPLEMENTARY TABLES

#### Supplementary Table 1. Summary of P/LP ATM variants identified in GEJ adenocarcinoma cases.

| Case ID      | ATM Variant (NM_000051.4)                                                                 | ATM Variant Type  | Overall allele frequency in gnomAD                      | Ethnicity-specific allele frequency in gnomAD | dbSNP ID         | ClinVar Variant ID | ClinGen Canonical Allele Identifier | ACMG Variant Classification (Applicable Evidence Categories) |
|--------------|------------------------------------------------------------------------------------------|-------------------|--------------------------------------------------------|----------------------------------------------|-----------------|--------------------|-------------------|-----------------------------------------------------------------------------------|
| MSK_GEJ_07   | c.8418+5_8418+8delGTGA                                                                     | Intronic deletion | 2/282394 (0.0007%)                                     | 2/128848 (0.0016%)                          | rs769139997     | 181866             | CA298009          | Pathogenic (PVS1, PM2, PM3)                                                     |
| MSK_GEJ_08   | c.8977C>T (p.Arg2993*)                                                                     | Truncating SNV    | 3/251400 (0.0012%)                                     | 3/113686 (0.0026%)                         | rs770641163     | 186330             | CA194505          | Pathogenic (PVS1, PM2, PM3)                                                     |
| MSK_GEJ_09   | c.3802delG (p.Val1268*)                                                                    | Truncating SNV    | 11/282542 (0.0039%)                                    | 8/128978 (0.0062%)                         | rs587779834     | 127347             | CA286815          | Pathogenic (PVS1, PM2, PM3)                                                     |
| MSK_GEJ_10   | c.1027_1030delGAA (p.Glu343Ilefs*2)                                                        | Truncating frameshift | 8/281172 (0.0007%)                                   | 1/30352(0.0033%)                           | rs762089971     | 569567             | NA                | Pathogenic (PVS1, PM2, PM3)                                                     |
| MSK_GEJ_11   | c.217_218delGA (p.Glu73Metfs*26)                                                           | Truncating frameshift | 2/251274 (0.0008%)                                   | 1/113592 (0.0009%)                         | rs587781823     | 141534             | CA165711          | Pathogenic (PVS1, PM2, PM3)                                                     |
| MSK_GEJ_13   | c.3894dupT (p.Ala1299Cysfs*3)                                                             | Truncating frameshift | 2/251274 (0.0008%)                                   | 1/30352(0.0033%)                           | rs762089971     | 569567             | NA                | Pathogenic (PVS1, PM2, PM3)                                                     |
| MSK_GEJ_15   | c.8147T>C (p.Arg2443*)                                                                    | Missense          | 1/251068 (0.0004%)                                     | 1/113450 (0.0009%)                         | rs121434220     | 3036               | CA325513          | Pathogenic (PVS1, PM2, PM3)                                                     |
| MSK_GEJ_108  | c.3154-2A>G                                                                               | Essential splice site SNV | Absent                                                |Absent                                      | rs730881357     | 181940             | CA298200          | Pathogenic (PVS1, PM2, PM3)                                                     |
| MSK_GEJ_109  | c.9022C>T (p.Arg3008Cys)                                                                   | Missense          | 4/251428 (0.0016%)                                     | Absent                                      | rs587782292     | 142187             | CA294307          | Pathogenic (PVS1, PM2, PM3)                                                     |
| MSK_GEJ_110  | c.5932G>T (p.Glu1978*)                                                                    | Truncating SNV    | 11/251182 (0.0044%)                                    | 10/113596 (0.0088%)                        | rs587779852     | 127414             | CA286910          | Pathogenic (PVS1, PM2, PM3)                                                     |
| MSK_GEJ_111  | c.5228C>T (p.Glu7143lle)                                                                  | Missense          | 5/282558 (0.0018%)                                     | 4/128908 (0.0031%)                         | rs587779844     | 127403             | CA286882          | Likely pathogenic (PS3, PM2, PM3)                                               |
| MSK_GEJ_112  | c.5712dupA (p.Ser1905Ilefs*25)                                                            | Truncating frameshift | 2/251146 (0.0008%)                                   | 2/113578 (0.0018%)                         | rs587781730     | 141146             | CA273879          | Pathogenic (PVS1, PM2, PM3)                                                     |
| MSK_GEJ_113  | c.1A>C (p.Met(?)                                                                           | Start loss        | Absent                                                | NA                                          | rs730881359     | 187213             | CA197028          | Likely pathogenic (PVS1, PM2, PM5)                                              |
| MSK_GEJ_114  | exons 17-63 deletion; c.2467_9171_?_del (p.Ala823_Vali3056delins28)                      | Multi-exon deletion | NA                                                     | NA                                          | rs1555055356    | 487450             | CA382521837        | Likely pathogenic (PVS1, PM2, PM3)                                              |
| MSK_GEJ_115  | c.331+1G>A                                                                               | Essential splice site SNV | Absent                                                | NA                                          | rs1591128179    | 642095             | NA                | Likely pathogenic (PVS1, PM2, PM3)                                              |
| MSK_GEJ_116  | c.6573-9G>A                                                                              | Intrinsic SNV     | Absent                                                | NA                                          | rs587776547     | 3019               | CA115924          | Pathogenic (PS3, PM2, PM3)                                                     |
| MSK_GEJ_117  | c.7638_7646delTATAATTTTC (p.Arg2547_Ser2549del)                                           | In-frame deletion | 7/251148 (0.0028%)                                    | 6/113524 (0.0053%)                         | rs587776547     | 3019               | CA115924          | Pathogenic (PS3, PM2, PM3)                                                     |
a P/LP = pathogenic/likely pathogenic; GEJ = gastroesophageal junction; gnomAD = Genome Aggregation Database; dbSNP = Single Nucleotide Polymorphism Database; ACMG = American College of Medical Genetics; SNV = single nucleotide variant; PVS = pathogenic very strong; PS = pathogenic strong; PM = pathogenic moderate; PP = pathogenic supporting
Supplementary Table 2: Burden analysis: comparison of P/LP ATM variant prevalence in GEJ adenocarcinoma cohort to the Genome Aggregation Database.

| GEJ Cohort | gnomAD Cohort | Comparative Statistics |
|------------|---------------|------------------------|
| Individuals with ATM germline pathogenic variant, No. | Total individuals, No. | Carrier frequency, % | Individuals with ATM germline pathogenic variant, No. | Total individuals in gnomAD, No. | Carrier frequency, % | Chi-Square | \( P^a \) | OR (95%CI) | \( P^b \) |
| 18 | 312 | 5.8 | 500 | 125,695 | 0.40 | 219.3 | <.001 | 15.33 (9.45-24.87) | <.001 |

\( P^a \) values were calculated using a two-sided chi-squared test. P/LP = pathogenic/likely pathogenic; GEJ = gastroesophageal junction; gnomAD = the Genome Aggregation Database; OR = odds ratio.

\( P^b \) values were calculated using a two-sided Fisher's exact test.
Supplementary Table 3: Clinical and pathological characteristics of individuals with GEJ adenocarcinoma.

| Patient and disease characteristics according to gATM mutational status | Germline ATM-mut (n = 18) | Germline ATM-wt (n = 294) | P |
|---------------------------------------------------------------|--------------------------|--------------------------|---|
| Median age at diagnosis (range), years                      | 60.2 (23-68)            | 61.4 (20-86)            | .35<sup>a</sup> |
| Sex, No. (%)                                                 |                          |                          |    |
| Male                                                         | 13 (72.2)                | 249 (84.7)               | .77<sup>b</sup> |
| Female                                                       | 5 (27.8)                 | 45 (15.3)                |    |
| Race, No. (%)                                                |                          |                          |    |
| Asian                                                        | 1 (5.6)                  | 17 (5.8)                 | .97<sup>c</sup> |
| Black                                                        | 0                        | 9 (3.1)                  |    |
| White                                                        | 17 (94.4)                | 233 (79.3)               |    |
| Unknown                                                      | 0                        | 35 (11.9)                |    |
| History of prior malignancy, No. (%)                         |                          |                          |    |
| Yes                                                          | 4 (22.2)                 | 36 (12.2)                | .27<sup>b</sup> |
| No                                                           | 14 (77.8)                | 258 (87.8)               |    |
| 1st degree family cancer history, No. (%)                   |                          |                          |    |
| Yes                                                          | 14 (77.8)                | 127 (43.2)               | .006<sup>b</sup> |
| No                                                           | 4 (22.3)                 | 167 (56.8)               |    |
| Poorly differentiated, No. (%)                               |                          |                          |    |
| Yes                                                          | 7 (38.9)                 | 124 (42.2)               | .99<sup>b</sup> |
| No                                                           | 11 (61.1)                | 170 (57.8)               |    |
| HER2 positive, No. (%)                                       |                          |                          |    |
| Yes                                                          | 1 (7.7)                  | 73 (28.6)                | <.001<sup>c</sup> |
| No                                                           | 12 (92.3)                | 182 (71.4)               |    |
| Unknown                                                      | 5                        | 39                       |    |
| PDL1 positive (>1), No. (%)                                  |                          |                          |    |
| Yes                                                          | 6 (50.0)                 | 71 (64.0)                | .61<sup>c</sup> |
| No                                                           | 6 (50.0)                 | 40 (36.0)                |    |
| Unknown                                                      | 6                        | 168                      |    |
| Localized at diagnosis, No. (%)                              |                          |                          |    |
| Yes                                                          | 10 (55.6)                | 106 (36.1)               | .13<sup>b</sup> |
| No                                                           | 8 (44.4)                 | 188 (63.9)               |    |
| Resected when presented with locally advanced disease, No. (%) |                          |                          |    |
| Yes                                                          | 10 (100.0)               | 92 (86.8)                | .04<sup>b</sup> |
| No                                                           | 0 (0.0)                  | 14 (13.2)                |    |
| Preoperative therapy, No. (%) |       |       |       |       |
|-------------------------------|-------|-------|-------|-------|
| Chemoradiation                | 6 (60.0) | 70 (83.3) |       | .09b |
| Chemotherapy                  | 4 (40.0) | 14 (16.7) |       |       |
| Clinical T staging, No. (%)   |       |       |       |       |
| Tx                            | 1 (10.0) | 13 (15.5) |       | .87c |
| T1                            | 0      | 2 (2.4) |       |       |
| T2                            | 0      | 8 (9.5) |       |       |
| T3                            | 9 (90.0) | 58 (69.0) |       |       |
| T4                            | 0      | 3 (3.6) |       |       |
| Clinical N staging, No. (%)   |       |       |       |       |
| Nx                            | 2 (20.0) | 19 (22.6) |       |       |
| N0                            | 2 (20.0) | 41 (48.8) |       | .66c |
| N1                            | 5 (50.0) | 9 (10.7) |       |       |
| N2                            | 1 (10.0) | 3 (3.6) |       |       |
| N3                            | 0      | 12 (14.3) |       |       |
| Pathologic complete response, No. (%) |       |       |       |       |
| Yes                           | 3 (30.0) | 12 (14.3) |       | .20b |
| No                            | 7 (30.0) | 72 (85.7) |       |       |
| Median percentage treatment response | 80.0 | 77.5 |       | .03a |
| Pathologic T stage, No. (%)   |       |       |       |       |
| T0                            | 3 (30.0) | 13 (15.5) |       |       |
| T1                            | 1 (10.0) | 17 (20.2) |       | .89c |
| T2                            | 1 (10.0) | 9 (10.7) |       |       |
| T3                            | 5 (50.0) | 44 (52.4) |       |       |
| T4                            | 0      | 1 (1.2) |       |       |
| Pathologic N stage, No. (%)   |       |       |       |       |
| N0                            | 4 (40.0) | 46 (54.8) |       |       |
| N1                            | 2 (20.0) | 22 (26.2) |       | .34c |
| N2                            | 3 (30.0) | 12 (14.3) |       |       |
| N3                            | 1 (10.0) | 4 (4.8) |       |       |
| Lympho-vascular invasion present, No. (%) |       |       |       |       |
| Yes                           | 4 (40.0) | 30 (35.8) |       | .72b |
| No                            | 6 (60.0) | 54 (64.2) |       |       |
| Perineural invasion present, No. (%) |       |       |       |       |
| Yes                           | 4 (40.0) | 35 (41.7) |       | .99b |
| No                            | 6 (60.0) | 49 (58.3) |       |       |
Pathologic Siewert classification, No. (%)

|     |     |     |
|-----|-----|-----|
| I   | 2 (20.0) | 28 (30.4) | .49<sup>c</sup> |
| II  | 3 (30.0) | 50 (54.3) |     |
| III | 5 (50.0) | 14 (15.3) |     |

Recurrence, No. (%)

|     |     |     |
|-----|-----|-----|
| Yes | 1 (10.0) | 52 (56.5) | .006<sup>b</sup> |
| No  | 9 (90.0) | 40 (43.5) |     |

Median recurrence-free survival, mo

| N/A (median f/u 25 months) | 24.6 | HR = 0.21 (95% CI = 0.03 to 1.54; P = .13)<sup>d</sup> |

<sup>a</sup>P values were calculated using 2-sided Mann-Whitney U test. T = tumor; N = node; GEJ = gastroesophageal junction; mut = mutated; wt = wild type; f/u = follow-up

<sup>b</sup>P values were calculated using 2-sided Fisher’s exact test.

<sup>c</sup>P values were calculated using 2-sided Chi-squared test.

<sup>d</sup>P values were calculated using 2-sided Log-rank test.
Supplementary Table 4: Immunohistochemistry analysis of ATM expression in GEJ adenocarcinomas.\textsuperscript{a}

| IHC staining | ATM status (germline or somatic) |  
|--------------|----------------------------------|
|              | ATM mutated. | ATM wild-type. |
| Present      | 0             | 95             |
| Absent       | 12            | 1              |

\textsuperscript{a}GEJ = gastroesophageal junction; IHC = immunohistochemical
Supplementary Table 5: Immunohistochemistry analysis of ATM expression in GEJ adenocarcinomas with supporting somatic genomic data.

| IHC staining | ATM status (germline only) |   |   |
|--------------|-----------------------------|---|---|
|              | ATM mutated                 | ATM wild-type |
| Present      | 0                           | 95            |
| Absent       | 10                          | 3             |

\(^{a}\) GEJ = gastroesophageal junction; IHC = immunohistochemical.
SUPPLEMENTARY FIGURES

Supplementary Figure 1: Copy number alteration pattern in GEJ adenocarcinomas. Patterns of copy number alterations in germline ATM wild type (top) and germline ATM-mutated (middle) GEJ tumors from MSK-IMPACT sequencing. Bottom, −log10(P) by two-sided Fisher’s exact tests, corrected for multiple testing through the Benjamini-Hochberg method. The frequencies of amplifications (green bars) and homozygous deletions (purple bars) are plotted above and below the x axis, respectively.
Supplementary Figure 2: Histologic features of the ATM-mutated gastroesophageal junction adenocarcinomas. (A-I): Microscopically, all nine cases were characterized by a glandular or glandular-like morphology and hence graded as moderately differentiated. Note the common feature of increased fibrosis in between the malignant glands (known as desmoplasia) and the polymorphic inflammatory infiltrate in the stroma. (H&E X100). Scale bar represents 200uM.
Supplementary Figure 3: H2AX phosphorylation on serine 139 (g-H2AX), a well-defined marker of DNA double strand breaks, upon treatment with a potent and selective ATR inhibitor RP-3500. A) ATR inhibition induces DNA damage in ATM-depleted GEJ cancer cells. Quantification of g-H2AX-positive ESO26 cells transfected with non-targeting siRNAs (siCTRL) or siRNAs targeting ATM (siATM). Cells were either left untreated or treated with indicated concentrations of the ATR inhibitor RP-3500 for 72h and processed for immunofluorescence with a g-H2AX-specific antibody. Data from N = 2 independent biological replicates (open symbols) with mean (solid lines). B) Representative micrographs of siCTRL or siATM-transfected ESO26 cells with or without RP-3500 treatment from experiments shown in A). g-H2AX is shown in red, DAPI (blue) is a nuclear counterstain.