

# Simultaneous identification of clinically relevant single nucleotide variants, copy number alterations and gene fusions in solid tumors by targeted next-generation sequencing

## SUPPLEMENTARY MATERIALS

### Patients

Median age of patients diagnosed of NSCLC was 69 years (range 57–80) 19 out of 28 were current or ex-smokers. Women were 7 and males were 21. Stage was known for 21 patients: 15 patients had stage IA–IB disease, 5 patients had IIA–IIB and 1 patients had stage III disease. Grade was known for 15 patients: 11 cases were G1–G2, 4 cases were G3.

Additional clinical-pathological information is reported in Supplementary Table 1. As to GC patients, archive material was retrieved from 22 patients. Median age of patients was 70 years (range 51–89). Women were 5 and males were 17. Stage was known for all patients: 5 patients had stage IA–IB disease, 6 patients had IIA–IIB and 4 patients had stage IIIA–IIIB disease and 7 patients and stage IVA–IVB disease. Grade was also known all patients: 9 cases were G1–G2 and 13 cases were G3. Additional clinical-pathological information is reported in Supplementary Table 2.

Archive material was retrieved from 31 CC patients. Median age of patients was 70 years (range 28–86). Women were 14 and males were 19. Grade was known for 29 patients: 21 cases were G1–G2 and 8 cases were G3. Additional clinical-pathological information is reported in Supplementary Table 3.

Archive material was retrieved from 25 RC patients. Median age of patients was 69 years (range 29–81). Women were 8 and males were 17. Grade was known for 19 patients: 11 cases were G1–G2 and 8 cases were G3. Additional clinical-pathological information is reported in Supplementary Table 4.

### DNA sequencing

The OncoPrint™ Focus Assay (ThermoFisher.com) is a next-generation sequencing panel that includes 52 genes associated with solid tumors that can be targeted by drugs.

Genes analysed for the presence of SNVs include: AKT1, ALK, AR, BRAF, Cdk4, CTNNB1, DDR2, EGFR, ERBB2, ERBB3, ERBB4, ESR1, FGFR2, FGFR3, GNA11, GNAQ, HRAS, IDH1, IDH2, JAK1, JAK2, JAK3, KIT, KRAS, MAP2K1, MAP2K2, MET, MTOR, NRAS, PDGFRA, PIK3CA, RAF1, RET, ROS1, SMO.

Gene potentially subjected to CNAs include: ALK, AR, BRAF, CCND1, Cdk4, Cdk6, EGFR, ERBB2, FGFR1, FGFR2, FGFR3, FGFR4, KIT, KRAS, MET, MYC, MYCN, PDGFRA, PIK3CA.

Genes candidate for fusions include: ALK, RET, ROS1, NTRK1, NTRK2, NTRK3, FGFR1, FGFR2, FGFR3, MET, BRAF, RAF1, ERG, ETV1, ETV4, ETV5, ABL1, AKT3, AXL, EGFR, ERBB2, PDGFRA, PPARG.

### NGS data analysis

For the identification of SNVs and small indels, the IR 5.0 integrated the Torrent Variant Caller (TVC) algorithm optimized for sequencing data obtained on the Ion Torrent platform. The automated pipeline of IR 5.0 filters SNVs for quality, coverage, strand bias and signal shift. All SNVs called as positive were covered at least 400X.

The CNV calling Algorithm in IR 5.0 makes use of the Median of the Absolute values of all Pairwise Differences (MAPD) between log<sub>2</sub> ratios per tile for a given run to give a measurement of noise and/or other errors. The observed difference is due to CNVs (all CNV reported presented a MAPD value < 0.4).

The fusion analysis pipeline processes 3 input files (an uBAM file, a fusion reference Fasta file comprising chimeric sequences for targeted fusion transcripts and control targets, a target bed file that includes information about fusion breakpoint, expression controls and target assays). The algorithm processes the input files in order to extract read counts for fusion and expression controls. Parameters set to obtain accurate imbalance determinations were: a threshold of 40,000 total reads/sample, presence of ≥4 out of 5 expression controls and >20 reads for a specific fusion.

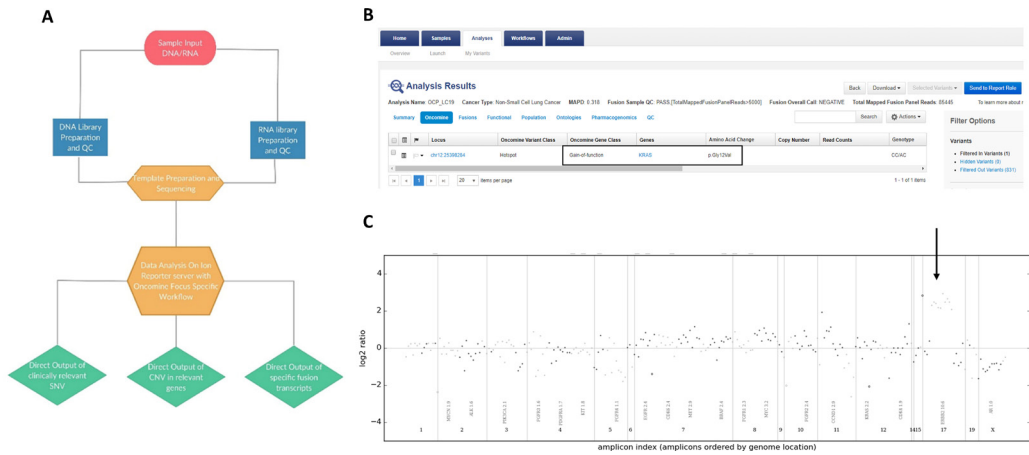
### Primers used for fusion validation

The primers for RT-PCR in validation of MET exon 14 skipping were as follows:

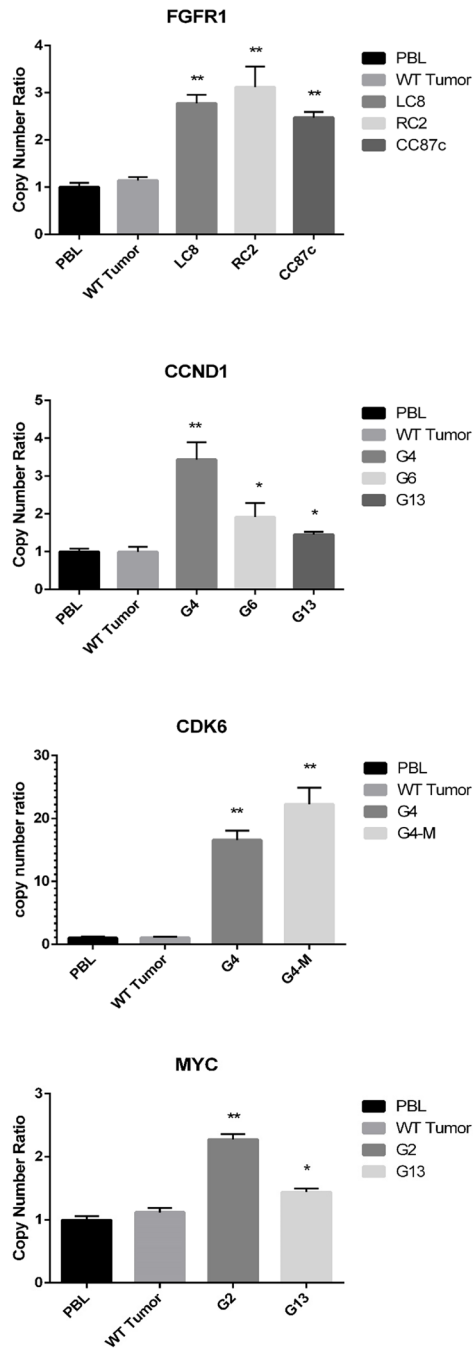
FW, 5'- CTT CAA CCG TCC TTG GAA AA -3';  
REV, 5'- CCTATGACTTCATTGAAATGCAC -3'.

The primers used for RT-PCR in validation of FGFR3-TACC3 fusion were as follows:

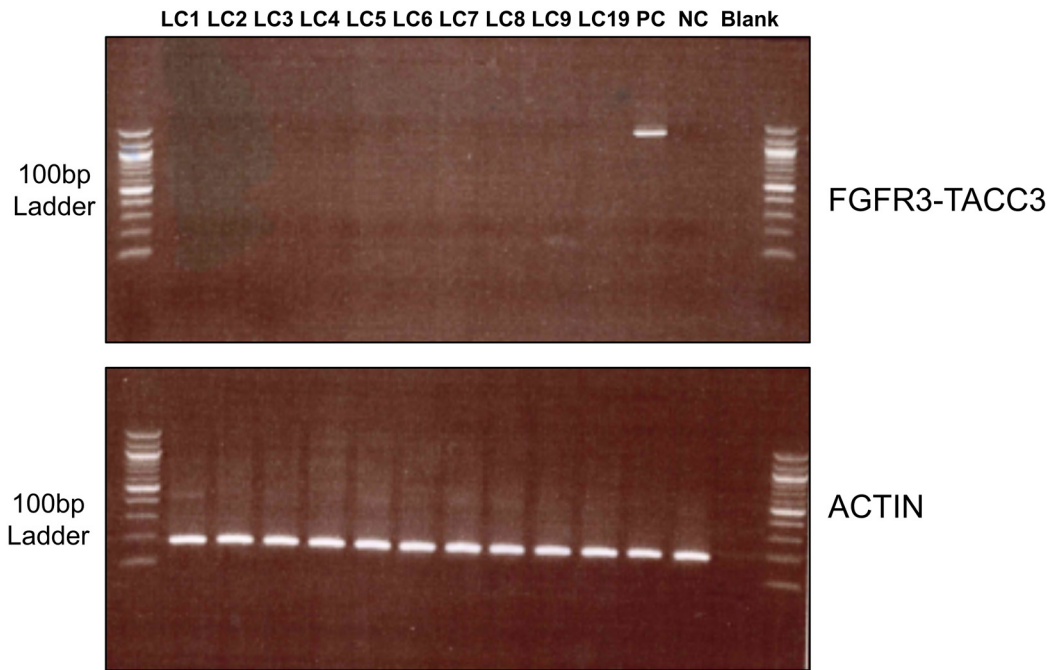
FW: 5'- CGTGAAGATGCTGAAAGACGATG - 3'  
REV: 5' - AAACGCTTGAAGAGGTCGGAG - 3'



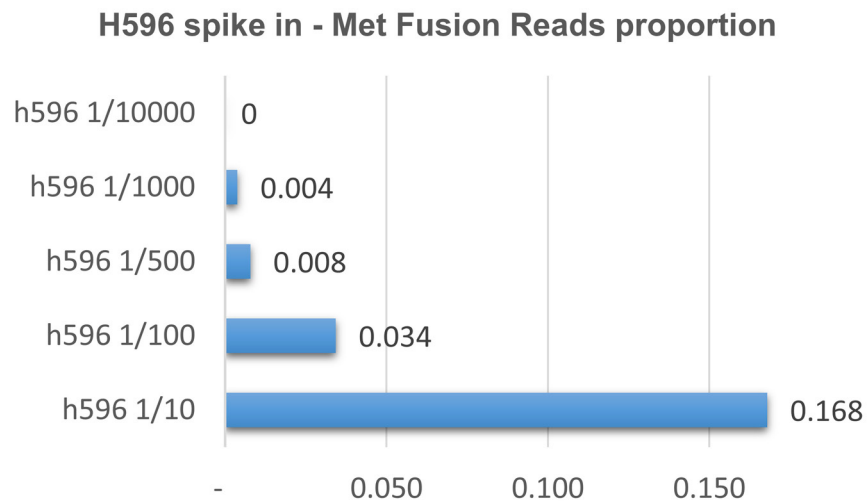
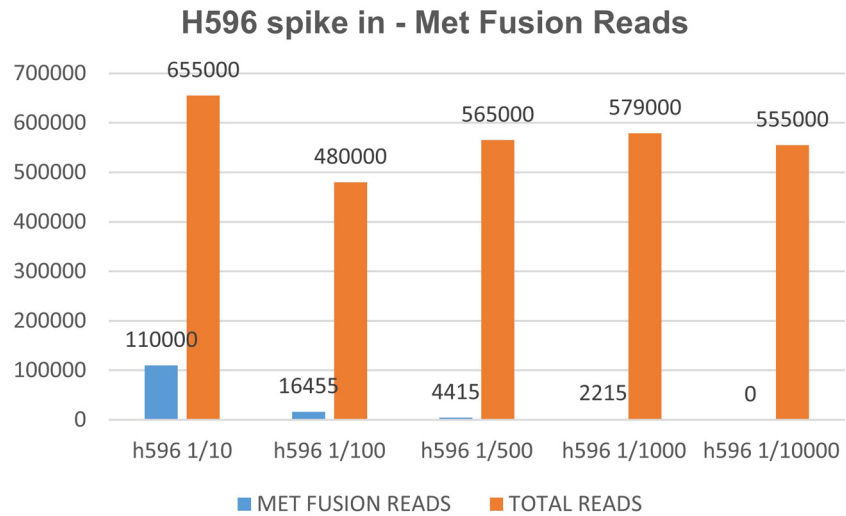
**Supplementary Figure 1: OncoPrint™ Focus and representative output. (A)** FFPE material was used to prepare RNA/DNA libraries, which were loaded onto an Ion Select 318 chip and run onto the Ion PGM machine. Automated analysis of data for detection of SNV, CNV, and gene fusions was performed with the Ion Reporter Workflow Software. **(B)** Representative output of the OncoPrint™ Focus – Ion Reporter Variant Detection, with the indication of the gene, nucleotide variant, type of alteration and the targeting drug. **(C)** Representative output of the OncoPrint™ Focus – Ion Reporter CNA Detection, with the indication of the gene, nucleotide variant, type of alteration and the targeting drug.



**Supplementary Figure 2: Validation of Copy number amplifications detected by OncoPrint™ Focus Assay.** Quantitative realtime PCR in patients with amplified genes encoding FGFR1 (patients CC4, LC8, RC2), CDK6 (G4, G4-M), CCND1 (G6, G13, G4) and MYC (patients G2, G13). PBL, peripheral blood lymphocytes; tumors showing noamplification of FGFR1, CDK6 or CCND1, respectively. Statistical significance indicated by number of stars in each patient when confronted with PBL.



**Supplementary Figure 3: Analysis of FGFR3-TACC3 fusion by semi-quantitative RT-PCR in LC Patients.** Representative RT-PCR analysis for detection of FGFR3-TACC3 fusion in LC samples included in the study. A positive control (PC), negative control (NC) and blank (Blank) were included. Actin was used as internal control of the RNA used.



**Supplementary Figure 4: Determination of OFA sensitivity in identification of specific MET fusions.** (A) Limiting dilution (from 1:10 to 1:10,000) of RNA from the MET-positive NCI-H596 cells into the RNA from the MET fusion negative A549 cells. (B) OncoPrint™ Focus Assay of RNA from NCI-H596 cells diluted (from 1:10 to 1:10,000) into the RNA from MET fusion-negative A549 cells. Blue bar, MET fusion specific reads; brown bar, total reads.

**Supplementary Table 1: Clinical-pathological characteristics of LC patients included in the study**

| <b>Patient</b> | <b>Gender</b> | <b>Age (years)</b> | <b>Smoking Status</b> | <b>WHO</b>                   | <b>Grade</b> | <b>Stage</b> |
|----------------|---------------|--------------------|-----------------------|------------------------------|--------------|--------------|
| LC1            | M             | 68                 |                       | Adenosquamous Carcinoma      |              |              |
| LC2            | M             | 74                 | smoker                | Adenocarcinoma               | G2           | IA           |
| LC3            | F             | 69                 | no smoker             | Other                        |              |              |
| LC4            | M             | 75                 | ex smoker             | Other                        | G2           | IB           |
| LC5            | M             | 67                 | ex smoker;            | Squamous cell carcinoma      | G2           | IIA          |
| LC6            | M             | 75                 | ex smoker             | Bronchioloalveolar carcinoma | G2           | IB           |
| LC7            | M             | 66                 | smoker                | Adenocarcinoma               | G2           | IB           |
| LC8            | M             | 80                 |                       | Squamous cell carcinoma      | G2           | IB           |
| LC9            | M             | 62                 | smoker                | Squamous cell carcinoma      | G2           | IB           |
| LC10           | M             | 68                 | smoker                | Squamous cell carcinoma      | G3           | IIB          |
| LC11           | M             | 69                 | smoker                | Large Cell Carcinoma         | G3           | IB           |
| LC12           | F             | 76                 | no smoker             | Adenocarcinoma               | G2           | IB           |
| LC13           | M             | 65                 | ex smoker             | Adenocarcinoma               | G2           | IB           |
| LC14           | F             | 66                 | ex smoker;            | Bronchioloalveolar carcinoma |              |              |
| LC15           | M             | 57                 | ex smoker             | Other                        | G2           | IB           |
| LC16           | M             | 44                 | ex smoker             | Adenocarcinoma               | G2           | IIB          |
| LC17           | M             | 75                 | smoker                | Bronchioloalveolar carcinoma | G2           | IB           |
| LC18           | M             | 54                 | ex smoker             | Large Cell Carcinoma         | G3           | IIB          |
| LC19           | M             | 74                 | ex smoker             | Squamous cell carcinoma      | G2           | IIB          |
| LC20           | M             | 63                 | smoker                | Adenocarcinoma               | G2           | IB           |
| LC21           | F             | 77                 | ex smoker             | Squamous cell carcinoma      |              |              |
| LC22           | M             | 59                 | ex smoker             | Squamous cell carcinoma      | G2           | IB           |
| LC23           | F             | 70                 | ex smoker             | Squamous cell carcinoma      | G2           | IB           |
| LC24           | F             | 65                 |                       | Adenocarcinoma               | G2           | IB           |
| LC25           | F             | 71                 | ex smoker             | Adenocarcinoma               | G2           | IB           |
| LC26           | M             | 75                 | no smoker             | Squamous cell carcinoma      | G3           | IIIA         |
| LC27           | M             | 73                 | ex smoker             | Squamous cell carcinoma      | G3           | IIB          |
| LC28           | M             | 70                 | no smoker             | Adenocarcinoma               |              |              |

**Supplementary Table 2: Clinical-pathological characteristics of GC patients included in the study**

| <b>Patient</b> | <b>Gender</b> | <b>Age (years)</b> | <b>T</b> | <b>N</b> | <b>M</b> | <b>Grade</b> | <b>WHO</b>                              | <b>Stage</b> |
|----------------|---------------|--------------------|----------|----------|----------|--------------|---|--------------|
| G1             | M             | 77                 | T3       | N1       | M1       | G2           | Intestinal                              | IV           |
| G2             | F             | 51                 | T4       | N1       | M0       | G3           | Signet ring cell carcinoma              | IV           |
| G3             | F             | 70                 | T3       | N2       | M0       | G3           | Signet ring cell carcinoma              | IIIB         |
| G4             | M             | 69                 | T2       | N1b      | M1       | G2           | Tubulo-papillary gastric adenocarcinoma | II           |
| G5             | M             | 57                 | T4a      | N3a      | M0       | G2           | Signet ring cell carcinoma              | IV           |
| G6             | M             | 79                 | T2b      | N1       | M0       | G2           | Signet ring cell carcinoma              | II           |
| G7             | M             | 77                 | T3       | N2       | M0       | G3           | Intestinal                              | III          |
| G8             | M             | 78                 | T2b      | N2       | M0       | G3           | Poorly Differentiated                   | III          |
| G9             | F             | 73                 | T2       | N1       | M0       | G3           | Intestinal                              | II           |
| G10            | F             | 89                 | T3       | N0       | M0       | G2           | Intestinal                              | II           |
| G11            | M             | 69                 | T3       | N1       | M0       | G3           | Intestinal                              | IIIA         |
| G12            | M             | 70                 | T2b      | N0       | M0       | G2           | Intestinal                              | I            |
| G13            | M             | 58                 | T3       | N3       | M1       | G3           | Signet ring cell carcinoma              | IV           |
| G14            | M             | 81                 | T1b      | N1       | M0       | G2           | Intestinal                              | IB           |
| G15            | M             | 48                 | T1       | N0       | M0       | G1           | Intestinal                              | I            |
| G16            | M             | 77                 | T2a      | N0       | M0       | G2           | Intestinal                              | I            |
| G17            | F             | 72                 | T1       | N0       | M0       | G3           | Diffuse                                 | I            |
| G18            | M             | 75                 | T3       | N3       | M0       | G3           | Poorly Differentiated                   | IV           |
| G19            | M             | 54                 | T2       | N1       | M0       | G3           | Intestinal                              | II           |
| G20            | M             | 72                 | T2       | N3       | M1       | G3           | Signet ring cell carcinoma              | IV           |
| G21            | M             | 64                 | T3       | N3       | M1       | G3           | Poorly Differentiated                   | IV           |
| G22            | M             | 82                 | T3       | N0       | M0       | G3           | Intestinal                              | II           |

**Supplementary Table 3: Clinical-pathological characteristics of CC patients included in the study**

| <b>Patients</b> | <b>Gender</b> | <b>Age (years)</b> | <b>Segment</b>   | <b>T</b> | <b>N</b> | <b>M</b> | <b>Grade</b> |
|-----------------|---------------|--------------------|------------------|----------|----------|----------|--------------|
| CC1             | M             | 58                 | Transverse Colon | T3       | N0       | M0       | G2           |
| CC2             | F             | 50                 | Descending Colon |          |          |          |              |
| CC3             | M             |                    | Transverse Colon | T3       | N1a      | M0       | G2           |
| CC4             | M             | 79                 | Cecum            | T3       | N0       | MX       | G2           |
| CC5             | M             | 48                 | Descending Colon | T2       | N0       | M0       | G1           |
| CC6             | M             | 65                 | Transverse Colon | T3       | N2b      | Mx       | G2           |
| CC7             | F             | 78                 | Cecum            | T2       | N0       | Mx       | G2           |
| CC8             | F             | 70                 | Hepatic Flexure  | T4a      | N2b      | MX       | G3           |
| CC9             | M             | 68                 | Descending Colon | T4a      | N1b      | MX       | G2           |
| CC10            | M             | 78                 |                  | T3       | N0       | Mx       | G2           |
| CC11            | M             | 75                 |                  | T3       | N0       | Mx       | G2           |
| CC12            | M             | 59                 | Ascending Colon  | T3       | N2b      | Mx       | G2           |
| CC13            | M             | 75                 | Descending Colon | T3       | N0       | M0       | G2           |
| CC14            | M             | 67                 | Descending Colon | T3       | N2       | M0       | G3           |
| CC15            | F             | 84                 | Hepatic Flexure  |          |          |          |              |
| CC16            | M             | 75                 | Ascending Colon  | T4a      | N2a      | MX       | G2           |
| CC17            | F             | 84                 | Splenic Flexure  | T3       | N1       | M0       | G2           |
| CC18            | M             | 79                 | Descending Colon | T3       | N0       | M0       | G2           |
| CC19            | M             | 56                 | Descending Colon | T4       | N1a      | M0       | G3           |
| CC20            | F             | 86                 | Ascending Colon  | T3       | N0       | M0       | G2           |
| CC21            | M             | 71                 | Descending Colon | T3       | N0       | M0       | G2           |
| CC22            | F             | 67                 | Descending Colon | T3       | N1B      | MX       | G2           |
| CC23            | M             | 68                 | Descending Colon | T3       | N0       | M0       | G3           |
| CC24            | M             | 56                 | Descending Colon | T2       | N0       | MX       | G2           |
| CC25            | F             | 56                 | Descending Colon | T4       | N0       | M0       | G3           |
| CC26            | F             | 46                 | Descending Colon | T4       | N1b      | M0       | G3           |
| CC27            | F             | 89                 | Descending Colon | T3       | N1b      | M0       | G3           |
| CC28            | M             |                    | Ascending Colon  | T3       | N1b      | M1       | G2           |
| CC29            | F             | 84                 | Descending Colon | T4b      | N0       | M1       | G2           |
| CC30            | F             | 85                 |                  | T4a      | N1b      | M1       | G2           |
| CC31            | F             | 70                 | Descending Colon | T4       | N2a      | M1       | G3           |



**Supplementary Table 4: Clinical-pathological characteristics of RC patients included in the study**

| <b>Patient</b> | <b>Gender</b> | <b>Age (years)</b> | <b>T</b> | <b>N</b> | <b>M</b> | <b>Grade</b> |
|----------------|---------------|--------------------|----------|----------|----------|--------------|
| RC1            | M             | 72                 | T3       | N1B      | Mx       | G3           |
| RC2            | M             | 29                 | T4a      | N2b      | M0       | G3           |
| RC3            | M             | 76                 | T2       | N0       | M0       | G2           |
| RC4            | M             | 78                 | T2       | N0       | MX       | G2           |
| RC5            |               |                    |          |          |          |              |
| RC6            | M             | 64                 |          | N0       | M0       |              |
| RC7            | M             | 78                 | T3       | N0       | MX       | G2           |
| RC8            | M             | 79                 | T2       | N0       | M0       | G2           |
| RC9            | F             | 63                 | T4       | N1       | M0       | G3           |
| RC10           | M             | 76                 |          | N0       | M0       |              |
| RC11           | F             | 61                 | T4a      | N1       | MX       | G3           |
| RC12           | M             | 78                 | T3       | N0       | MX       | G2           |
| RC13           | M             | 80                 | T2       | N1       | M0       | G2           |
| RC14           | F             | 71                 | T2       | N0       | MX       | G3           |
| RC15           | M             | 73                 |          |          |          | G3           |
| RC16           | M             | 63                 | T3       | N1       | MX       | G2           |
| RC17           | M             | 73                 | T4a      | N2b      | MX       | G2           |
| RC18           | M             | 81                 | T3       | N0       | M0       | G3           |
| RC19           | F             | 81                 | T2       | N0       | Mx       | G2           |
| RC20           | M             | 59                 | T4b      | N1       | M0       | G3           |
| RC21           | F             | 69                 | T2       | N0       | Mx       | G2           |
| RC22           | F             | 60                 | NA       | NA       | NA       | NA           |
| RC23           |               |                    |          |          |          |              |
| RC24           | F             |                    |          |          |          |              |

**Supplementary Table 5: List of gene fusions detected by the oncomine focus assay**

See Supplementary File 1