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(54) Title: "IMMUNE CHECKPOINT INTERVENTION" IN CANCER

(57) Abstract: The present invention relates to methods for identifying a subject with cancer who is suitable for treatment with an immune checkpoint intervention, and to methods of treatment of such subjects. The invention further relates to a method for predicting or determining the prognosis of a subject with cancer.

"IMMUNE CHECKPOINT INTERVENTION" IN CANCER

FIELD OF THE INVENTION

The present invention relates to methods for identifying a subject with cancer who is suitable for treatment with an immune checkpoint intervention, and to methods of treatment of such subjects. The invention further relates to a method for predicting or determining the prognosis of a subject with cancer.

BACKGROUND TO THE INVENTION

Among the most promising approaches to activating therapeutic antitumour immunity is the blockade of immune checkpoints. Immune checkpoints are inhibitory pathways in the immune system that are crucial for maintaining self-tolerance and modulating the duration and amplitude of physiological immune responses in peripheral tissues in order to minimize collateral tissue damage. It is now clear that tumours co-opt certain immune-checkpoint pathways as a major mechanism of immune resistance, particularly against T cells that are specific for tumour antigens. Because many of the immune checkpoints are initiated by ligand–receptor interactions, they can be readily blocked by antibodies or modulated by recombinant forms of ligands or receptors.

Current approaches to immune checkpoint regulation in cancers involve a level of guesswork and serendipity based mostly in the order these compounds have been made available. CTLA4, PD-1 and PDL1 were discovered and produced in this order, and that is how they have been administered so far. Initial trials were carried out with CTLA-4, as this was the first to be approved by the FDA. Subsequently, PD-1/PDL1 treatments were approved and used.

WO 2015/103037 provides a method for identifying a subject as likely to respond to treatment with an immune checkpoint modulator, based on the discovery that cancer cells may harbour somatic mutations that result in neoepitopes that are recognisable by a patient's immune system as non-self. The identification of one or more neoepitopes in a cancer sample may be useful for determining which cancer patients are likely to respond favourably to treatment with an immune checkpoint modulator.

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SUMMARY OF THE INVENTION

The present inventors have made the important and surprising determination that cancer patients with higher numbers of clonal neo-antigens, and/or a higher ratio of clonal:sub-clonal neoantigens or a low sub-clonal neo-antigen fraction, are more likely to respond to treatment with an immune checkpoint intervention.

As demonstrated in the present examples, patients with tumours with a high clonal neo-antigen burden and/or a low subclonal neo-antigen burden have a better response to immunotherapy with checkpoint blockade (e.g. anti-PD1 therapy). This represents an important contribution to the art, in that it opens up the potential for improved and more directed treatments and preventative modalities for treating and preventing cancer. In this regard, therapeutic and preventative interventions can be targeted to the individual and to the particular context of the cancer.

Furthermore, the present inventors have found that, surprisingly, tumour cells with high numbers of clonal neo-antigens exhibit similar expression profiles of immune checkpoint molecules, that is they exhibit a common expression profile of immune checkpoint molecules. This is an important contribution to the art, as it has not previously been demonstrated that cancers of specific types exhibit particular expression profiles of immune checkpoint molecules. The present inventors have shown this for the first time, and this finding facilitates more directed approaches to treating or preventing particular cancers.

The present inventors have also surprisingly found that patients with higher numbers of clonal mutations, and a higher ratio of clonal:sub-clonal mutations, have improved prognosis.

The present invention therefore addresses a need in the art for new, alternative and/or more effective ways of treating and preventing cancer.

Accordingly, the present invention provides a method for identifying a subject with cancer who is suitable for treatment with an immune checkpoint modulator, said method comprising:

(i) determining the number of clonal neo-antigens in one or more cancer cells from said subject; and/or

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(ii) determining the ratio of clonal: sub-clonal neo-antigens and/or sub-clonal neo-antigen fraction in more than one cancer cell from said subject;

wherein a higher number of clonal neo-antigens, and/or a higher ratio of clonal:sub-clonal neo-antigens, or lower (or low) sub-clonal neo-antigen fraction in comparison to a reference sample is indicative of response to an immune checkpoint modulator.

In another aspect, the invention provides a method for predicting or determining the prognosis of a subject with cancer, the method comprising:

(i) determining the number of clonal neo-antigens in one or more cancer cells from said subject; and/or

(ii) determining the ratio of clonal: sub-clonal neo-antigens and/or sub-clonal neo-antigen fraction in more than one cancer cell from said subject,

wherein a higher number of clonal neo-antigens and/or a higher ratio of clonal:sub-clonal neo-antigens, or lower (or low) sub-clonal neo-antigen fraction, is indicative of improved prognosis.

In a further aspect, the invention provides a method of treating or preventing cancer in a subject, wherein said method comprises the following steps:

i) identifying a subject with cancer who is suitable for treatment with an immune checkpoint intervention according to the method of the invention; and

ii) treating said subject with an immune checkpoint intervention.

In a yet further aspect, the invention provides a method of treating or preventing cancer in a subject which comprises treating a subject with cancer with an immune checkpoint modulator, wherein the subject has been determined to have:

(i) a higher number of clonal neo-antigens; and/or

(ii) a higher ratio of clonal:sub-clonal neo-antigens, or lower (or low) sub-clonal neo-antigen fraction;

in comparison to a reference sample.

The invention also provides an immune checkpoint intervention for use in a method of treatment or prevention of cancer in a subject, the method comprising:

i) identifying a subject with cancer who is suitable for treatment with an immune checkpoint intervention according to the method of the invention; and

ii) treating said subject with an immune checkpoint intervention.

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The invention further provides an immune checkpoint modulator when used in the treatment or prevention of cancer in a subject, wherein the subject has:

(i) a higher number of clonal neo-antigens; and/or

(ii) a higher ratio of clonal:sub-clonal neo-antigens, or lower (or low) sub-clonal neo-antigen fraction

in comparison to a reference sample.

The invention further provides use of an immune checkpoint modulator in preparation of a medicament for treatment or prevention of cancer in a subject, wherein the subject has:

(i) a higher number of clonal neo-antigens; and/or

(ii) a higher ratio of clonal:sub-clonal neo-antigens, or lower (or low) sub-clonal neo-antigen fraction;

in comparison to a reference sample.

DESCRIPTION OF THE FIGURES

Figure 1: (A) Total putative neo-antigen burden in cohort of TCGA LUAD (LUng ADenocarcinoma) tumours. Proportion of neo-antigens arising from clonal (blue) or subclonal (red) mutations or those of undetermined (grey) clonality is shown. (B) Overall survival curves for patients with tumours exhibiting high neo-antigen burden, defined as the upper quartile of the cohort, (n = 30) compared to remainder of cohort (n = 86) (log-rank P = 0.011), (C) high clonal neo-antigen burden, defined as the upper quartile of the cohort, (n = 29) compared to remainder of cohort (n = 87) (log-rank P = 0.0077), and (D) high subclonal neo-antigen burden, defined as the upper quartile of the cohort (n = 30) compared to remainder of cohort (n = 86) (log-rank P = 0.12). (E) Differentially expressed genes between the tumours with high clonal neo-antigen burden and low clonal neo-antigen burden, defined as the bottom quartile of the cohort, clustered on co-expression. Clusters of immune genes highlighted in the text are boxed.

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Figure 2: A) Phylogenetic trees for L011 and L012, with trunk and branch lengths proportional to number of non-silent mutations. B) Putative neo-antigens predicted for all missense mutations in L011. The MTFR2D326Y neo-antigen (FAFQEYDSF) is highlighted. C) Putative neo-antigens predicted for all missense mutations in L012. The CHTF18 L769V neo-antigen (LLLDIVAPK) and MYADMR30W neo-antigen (SPMIVGSPW) are indicated. D, E) MHC-multimer analysis of in vitro expanded CD8+ T lymphocytes deriving from three tumour regions and normal tissues for L011 (D) and L012 (E). In both cases, frequency of CD3+CD8+ T lymphocytes reactive to mutant peptides are indicated.

Figure 3: A) MHC-multimer analysis of non-expanded CD8+ T cells from tumour regions 1-3, adjacent normal lung tissue and PBMCs from patient L011 (upper panel) and L012 (lower panel). Frequency of MHC-multimer positive cells out of the CD3+CD8+ compartment is

indicated. B) Immunophenotype of tumour-infiltrating CD8+ T cells from patient L011, comparing MTFR2-reactive CD8+ T cells (MTFR2+) with MHC-multimer negative CD8+ T cells (MTFR2-) in the same tumour region, in normal tissue and in PBMCs. Data shown is from tumour Region 3 and representative of all regions. Percentage of cells expressing CTLA-4, PD-1, LAG-3, Ki-67 and GzmB is shown. C) Co-expression of PD-1, Ki67 and GzmB on MTFR2- reactive (MTFR2+) and non-reactive CD8+ T cells (MTFR2-) D) Upper panel: Multi-color IHC of primary tumour from L011 and L012. CD8 (red), Granzyme B (blue) and LAG-3 (brown) are shown. Lower panel: PD-L1 staining in L011 region 3 versus adjacent normal tissue.

Figure 4: For discovery (A-C) and validation cohort (D-F), number of clonal neo-antigens and fraction of subclonal neo-antigens is shown for patients with a durable clinical benefit (DCB), or non-durable benefit (NDB). Progression free survival in tumours with a higher number of neo-antigens and low subclonal fraction compared to those with a lower number of neo-antigens or high subclonal fraction is shown for discovery (C) and validation (F) cohorts. G) Clonal architecture for each sequenced tumour. PFS are reported under barplot and those with ongoing progression-free survival are labeled with +. PD-L1 is indicated below barplot: Strong (+) 50% membranous staining; Weak (+/-), 1-49% membranous staining; Negative (-),<1% membranous staining; Unknown (?). (H) Progression free survival in combined tumour cohort comparing tumours with a higher number of neo-antigens and low subclonal fraction with those with a lower number of neo-antigens or high subclonal fraction. I) Clonal architecture of CA9903 tumour sample, with *HERC1* mutation highlighted and with subclones indicated. J) Putative neo-antigens predicted for all missense mutations in CA9903. The *HERC1*P3278S neo-antigen (ASNASSAAK) is highlighted.

Figure 5: Quartile Breakdown of LUAD Survival. Overall survival curves showing all four quartiles comparing patients on total neo-antigen load (A), clonal neo-antigen load (B), and subclonal neo-antigen load (C). Associated log-rank p-values between each quartile is given to the right of the plots.

Figure 6: Survival by number of SNVs in LUAD. (B) Overall survival curves of patients harboring tumours with high SNV burden (n = 30) compared to remainder of cohort (n = 86) (log-rank P = 0.01), (C) high clonal SNV burden (n = 30) compared to remainder of cohort (n = 86) (log-rank P = 0.014), and (D) high subclonal SNV burden (n = 30) compared to remainder of cohort (n = 86) (log-rank P = 0.14).

Figure 7: LUSC (Lung Squamous cell carcinoma) cohort summary. (A) Total putative neo-antigen burden of TCGA LUSCpatients. Columns coloured to show proportion of neo-antigens arising from clonal blue) or subclonal (red) mutations or arising from mutations of undetermined (grey) clonality. (B) Overall survival curves of patients with high neo-antigen burden (n = 30) compared to those with a low neo-antigen burden (n = 91) (log-rank P = 0.84), (C) high clonal neo-antigen burden (n = 29) compared to those with a low clonal neo-antigen burden (n = 92) (log-rank P = 0.99), and (D) high subclonal neo-antigen burden (n = 30) compared to those with a low subclonal neo-antigen burden (n = 91) (log-rank P = 0.32). (E) Overall survival curves of patients with high SNV burden (n = 30) compared to remainder of cohort (n = 90) (log-rank P = 0.52), (F) high clonal SNV burden (n = 30) compared to remainder of cohort (n = 91) (log-rank P = 0.89), and (G) high subclonal SNV burden (n = 30) compared to remainder of cohort (n = 92) (log-rank P = 0.28).

Figure 8: Differential Gene Expression Analysis. Differentially expressed genes between the high clonal neo-antigen burden patients and remainder of cohort, clustered on coexpression.

Figure 9: Immunophenotype of tumour-infiltrating CD8+ T cells from patient L012 A) Activation and functional phenotype of tumour-infiltrating CD8+ CHTF18-reactive (CHTF18+) and MYADM-reactive (MYADM+) T cells versus MHC-multimer negative CD8+ T cells in tumour (Multimer-), normal tissue and PBMCs. Percentage of cells expressing CTLA-4, PD-1, LAG-3, Ki-67 and GzmB is shown. Histograms are generated from L012, region 2 and findings representative of all tumour regions. B) Co-expression of PD-1, Ki67 and granzyme B on tumour-infiltrating CD8+ CHTF18-reactive (CHTF18+) and MYADM-reactive (MYADM+) T cells compared to tumour infiltrating MHC-multimer negative CD8+ T cells (Multimer-). C) In vitro expanded tumour-infiltrating CD8+ T cells were stained with MHC-multimers loaded with either mutant or wild type peptides and analyzed by flow cytometry. Percentage of MHC multimer positive cells of the CD3+CD8+ gate is shown. L011 (Top panel): Expanded CD8+ T cells from tumour region 1 recognize mutant but not wild type MTFR2. L012 (middle panel): Expanded CD8+ T cells from tumour region 2 recognize mutant but not wild type CHTF18. L012 (bottom panel): Expanded CD8+ T cells from tumour region 2 recognize both mutant and wild type MYADM. The mutation in MYADM is on the anchor residue, primary affecting HLA binding and not T cell recognition. Whilst the data suggest that T cells in this patient can recognize both mutant and wildtype peptides (when stabilized in our MHC-multimer system), the very low affinity of the wild type peptide would prevent adequate presentation in vivo. (D) Validation of BV650 and PE-Cy7 MHC-multimer binding to expanded tumour-infiltrating lymphocytes from L011 and L012. To validate the quality of the reagents used to characterize MTFR2-, MYADM- and CHTF18-reactive T cells

in non-expanded tumour samples, we used the same reagents to stain a larger number of expanded TILs. Data from L011 (left panel), and L012 (right panel) show clear and defined populations of MTFR2-, MYADM- and CHTF18-reactive T cells in the expanded TILs.

Figure 10: Mutational burden and clonal architecture of (A) discovery and (B) validation cohort tumours.

Figure 11: PD-L1 expression for two groups of tumours. PD-L1 exhibits significantly stronger expression in tumours harboring a high clonal neo-antigen burden and a low subclonal neo-antigen fraction compared to tumours harboring a low clonal neo-antigen burden or high subclonal neo-antigen fraction.

Figure 12: A) Number of predicted clonal mutations in the discovery cohort tumours from patients with a durable clinical benefit (DCB) or with non durable benefit (NDB). B) Subclonal fraction in tumours from patients with a DCB or NDB C) Progression free survival in discovery tumours with a higher number of clonal mutations and low subclonal fraction compared to those with a lower number of clonal mutations or high subclonal fraction. D) Number of predicted clonal mutations in the validation cohort tumours from patients with a DCB or with NDB. E) Subclonal fraction in tumours from validation patients with a DCB or NDB F) Progression free survival in validation tumours with a higher number of clonal mutations and low subclonal fraction compared to those with a lower number of clonal mutations or high subclonal fraction. G) Number of clonal and subclonal mutations for each sequenced tumour with clonal (dark shading) and subclonal (light shading) displayed in the barplot. Bars are shaded to indicate clinical benefit status: DCB, green; NDB, red. PFS are reported under the barplot and those with ongoing progression-free survival are labelled with +. PD-L1 is indicated below barplot: Strong (+) 50% membraneous staining; Weak (+/-), 1-49% membraneous staining; Negative (-),1% membraneous staining; Unknown (?), unassessable. H) Progression free survival in combined tumour cohort comparing tumours with a higher number of clonal mutations and low subclonal fraction with those with a lower number of clonal mutations or high subclonal fraction.

DETAILED DESCRIPTION OF THE INVENTION

A “neo-antigen” is a tumour-specific antigen which arises as a consequence of a mutation within a cancer cell. Thus, a neo-antigen is not expressed by healthy cells in a subject.

The neo-antigen described herein may be caused by any non-silent mutation which alters a protein expressed by a cancer cell compared to the non-mutated protein expressed by a wild-type, healthy cell. For example, the mutated protein may be a translocation or fusion.

A "mutation" refers to a difference in a nucleotide sequence (e.g. DNA or RNA) in a tumour cell compared to a healthy cell from the same individual. The difference in the nucleotide sequence can result in the expression of a protein which is not expressed by a healthy cell from the same individual.

For example, the mutation may be a single nucleotide variant (SNV), multiple nucleotide variants, a deletion mutation, an insertion mutation, a translocation, a missense mutation or a splice site mutation resulting in a change in the amino acid sequence (coding mutation).

The mutations may be identified by Exome sequencing, RNA-seq, whole genome sequencing and/or targeted gene panel sequencing and or routine Sanger sequencing of single genes. Suitable methods are known in the art.

Descriptions of Exome sequencing and RNA-seq are provided by Boa *et al.* (Cancer Informatics. 2014;13(Suppl 2):67-82.) and Ares *et al.* (Cold Spring Harb Protoc. 2014 Nov 3;2014(11):1139-48); respectively. Descriptions of targeted gene panel sequencing can be found in, for example, Kammermeier *et al.* (J Med Genet. 2014 Nov; 51(11):748-55) and Yap KL *et al.* (Clin Cancer Res. 2014. 20:6605). See also Meyerson *et al.*, Nat. Rev. Genetics, 2010 and Mardis, Annu Rev Anal Chem, 2013. Targeted gene sequencing panels are also commercially available (e.g. as summarised by Biocompare ((http://www.biocompare.com/Editorial-Articles/161194-Build-Your-Own-Gene-Panels-with-These-Custom-NGS-Targeting-Tools/)).

Sequence alignment to identify nucleotide differences (e.g. SNVs) in DNA and/or RNA from a tumour sample compared to DNA and/or RNA from a non-tumour sample may be performed using methods which are known in the art. For example, nucleotide differences compared to a reference sample may be performed using the method described by Koboldt *et al.* (Genome Res. 2012; 22: 568-576). The reference sample may be the germline DNA and/or RNA sequence.

CLONAL NEO-ANTIGENS

The present inventors have determined that intratumour heterogeneity (ITH) can cause variation between the neo-antigens expressed in different regions of a tumour and between different cells in a tumour. In particular, the inventors have determined that, within a tumour, certain neo-antigens are expressed in all regions and essentially all cells of the tumour whilst other neo-antigens are only expressed in a subset of tumour regions and cells.

As such, a "clonal" or "truncal" neo-antigen is a neo-antigen which is expressed effectively throughout a tumour and encoded within essentially every tumour cell. A "sub-clonal" or "branched" neo-antigen is a neo-antigen which is expressed in a subset or a proportion of cells or regions in a tumour.

References herein to "essentially all" are intended to encompass the majority of tumour cells in a subject. For example, this may comprise 60-100% of cells, e.g. 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% of tumour cells in a subject.

"Present throughout a tumour", "expressed effectively throughout a tumour" and "encoded within essentially every tumour cell" may mean that the clonal neo-antigen is expressed in all regions of the tumour from which samples are analysed.

It will be appreciated that a determination that a mutation is "encoded within essentially every tumour cell" refers to a statistical calculation and is therefore subject to statistical analysis and thresholds.

Likewise, a determination that a clonal neo-antigen is "expressed effectively throughout a tumour" refers to a statistical calculation and is therefore subject to statistical analysis and thresholds.

"Expressed effectively in essentially every tumour cell or essentially all tumour cells" may mean that the mutation is present all tumour cells analysed in a sample, as determined using appropriate statistical methods.

By way of example, the cancer cell fraction (CCF), describing the proportion of cancer cells that harbour a mutation may be used to determine whether mutations are clonal or branched. For example, the cancer cell fraction may be determined by integrating variant allele frequencies with copy numbers and purity estimates as described by Landau *et al.* (Cell. 2013 Feb 14;152(4):714-26).

In brief, CCF values are calculated for all mutations identified within each and every tumour region analysed. If only one region is used (i.e. only a single sample), only one set of CCF values will be obtained. This will provide information as to which mutations are present in all tumour cells within that tumour region, and will thereby provide an indication if the mutation is clonal or branched. All sub clonal mutations (i.e. CCF<1) in a tumour region are determined as branched, whilst clonal mutations with a CCF=1 are determined to be clonal.

As stated, determining a clonal mutation is subject to statistical analysis and threshold. As such, a mutation may be identified as clonal if it is determined to have a CCF 95% confidence interval >= 0.60, for example 0.65, 0.70, 0.75, 0.80, 0.85, 0.90, 0.95, 1.00 or >1.00. Conversely, a mutation may be identified as branched if it is determined to have a CCF 95% confidence interval <= 0.60, for example 0.55, 0.50, 0.45, 0.40, 0.35, 0.30, 0.25, 0.20, 0.15, 0.10. 0.05 or 0.01, in any sample analysed.

It will be appreciated that the accuracy of a method for identifying clonal mutations is increased by identifying clonal mutations for more than one sample isolated from the tumour.

TUMOUR SAMPLES

Isolation of biopsies and samples from tumours is common practice in the art and may be performed according to any suitable method, and such methods will be known to one skilled in the art.

The method of this aspect may comprise, for example, determining the mutations present in cancer cells from one or more tumour regions isolated from a tumour. For example, the mutations present in a single biopsy, or alternatively, at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine or at least ten or more biopsies isolated from a tumour may be determined.

The individual tumour samples may be isolated from different regions located throughout a tumour within a primary site or between primary and metastases or within a metastasis or between metastases. For example, determining the mutations present in tumours which are known to display morphological disparate histology in different regions may involve determining the mutations present in a number of individual samples isolated from morphologically disparate regions.

The sample may be a blood sample. For example, the blood sample may comprise circulating tumour DNA, circulating tumour cells or exosomes comprising tumour DNA.

SUBJECT SUITABLE FOR TREATMENT

The invention provides a method for identifying a subject with cancer who is suitable for treatment with an immune checkpoint intervention, said method comprising determining the number of clonal neo-antigens in one or more cancer cells from said subject, wherein a high number of clonal neo-antigens is indicative of response to an immune checkpoint intervention.

As used herein, the term “suitable for treatment” may refer to a subject who is more likely to respond to treatment with an immune checkpoint intervention, or who is a candidate for treatment with an immune checkpoint intervention. A subject suitable for treatment may be more likely to respond to said treatment than a subject who is determined not to be suitable using the present invention. A subject who is determined to be suitable for treatment according to the present invention may demonstrate a durable clinical benefit (DCB), which may be defined as a partial response or stable disease lasting for at least 6 months, in response to treatment with an immune checkpoint intervention.

The number of clonal neo-antigens identified or predicted in the cancer cells obtained from the subject may be compared to one or more pre-determined thresholds. Using such thresholds, subjects may be stratified into categories which are indicative of the degree of response to treatment.

A threshold may be determined in relation to a reference cohort of cancer patients. The cohort may comprise 10, 25, 50, 75, 100, 150, 200, 250, 500 or more cancer patients. The cohort may be any cancer cohort. Alternatively the patients may all have the relevant or specific cancer type of the subject in question.

In one embodiment, a “high” number of clonal neo-antigens means a number greater than the median number of clonal neo-antigens predicted in a reference cohort of cancer patients, such as the minimum number of clonal neo-antigens predicted to be in the upper quartile of the reference cohort.

In another embodiment, a “high” number of clonal neo-antigens may be defined as 10, 20, 30, 40, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190 or 200 or more clonal neo-antigens.

A skilled person will appreciated that references to “high” or “higher” numbers of clonal neo-antigens may be context specific, and could carry out the appropriate analysis accordingly.

The invention further provides a method for identifying a subject with cancer who is suitable for treatment with an immune checkpoint intervention, said method comprising determining the ratio of clonal:sub-clonal neo-antigens and/or sub-clonal neo-antigen fraction in more than one cancer cell subject, wherein a high ratio of clonal:sub-clonal neo-antigens or lower/low sub-clonal neo-antigen fraction is indicative of to response to an immune checkpoint intervention.

As above, the clonal:sub-clonal ratio may be within the context of a cohort of subjects, either with any cancer or with the relevant/specific cancer. Accordingly, the clonal:sub-clonal neo-antigen ratio may be determined by applying methods discussed above to a reference cohort. A “high” or “higher” clonal:sub-clonal ratio may therefore correspond to a number greater than the median clonal:sub-clonal ratio predicted in a reference cohort of cancer patients, such as the minimum clonal:sub-clonal ratio predicted to be in the upper quartile of the reference cohort.

In another embodiment, a “high” or “higher” clonal:sub-clonal ratio means a ratio in the range of 3:1 to 100:1, such as a ratio of at least 3:1, 5:1, 10:1, 15:1, 20:1, 25:1, 50:1, 75:1 or 100:1. One skilled in the art will appreciate that the values may depend on the cohort in question.

The fraction of subclonal neo-antigens may also be defined in relation to a reference cohort, as discussed above. For example, a “lower” or “low” fraction of subclonal neo-antigens may correspond to a fraction smaller than the median fraction of subclonal neo-antigens predicted in a reference cohort of cancer patients, such as the maximum number predicted to be in the bottom quartile of the cohort.

Alternatively, one skilled in the art will appreciate that a sub-clonal neo-antigen fraction can be determined (for example for each patient) by dividing the number of subclonal neoantigens (for example that are predicted in the one or more cancer cells from said subject) by the number of total neoantigens (for example that are predicted in the one or more cancer cells from said subject).

In one embodiment, a “lower” or “low” fraction of subclonal neo-antigens may mean a fraction of 25% or less, such as a fraction of 20, 15, 10, 5, 3, 2 or 1% or less.

In a preferred embodiment, the method may comprise determining both the number of clonal neo-antigens and the ratio of clonal:sub-clonal neo-antigens or the fraction of of sub-clonal neo-antigens. As shown in the Example, combining measures of both neo-antigen burden and neo-antigen sub-clonal fraction was able to predict sensitivity to pembrolizumab better than either measure alone (see Fig. 4C), and outcome could be predicted in almost all cases (Fig 4G-H).

According the invention provides a method method for identifying a subject with cancer who is suitable for treatment with an immune checkpoint intervention, said method comprising:

(i) determining the number of clonal neo-antigens in one or more cancer cells from said subject; and

(ii) determining the ratio of clonal: sub-clonal neo-antigens and/or sub-clonal neo-antigen fraction in more than one cancer cell from said subject;

wherein a higher number of clonal neo-antigens and a higher ratio of clonal:sub-clonal neo-antigens, or lower (or low) sub-clonal neo-antigen fraction, is indicative of response to an immune checkpoint intervention.

Furthermore, the present inventors have found that, surprisingly, tumour cells with high numbers of clonal neo-antigens exhibit similar expression profiles of immune checkpoint molecules, that is they exhibit a common expression profile of immune checkpoint molecules. As such, approaches to identify particular immune checkpoint molecules whose expression is increased or decreased relative to non-cancerous cells can also be used to identify patients likely to respond to checkpoint blockade therapies.

Therefore, in one aspect the invention provides a method for identifying subjects who have cancer who are more likely to respond to immune checkpoint interventions, comprising determining the expression profile of immune checkpoint molecules in cancer cells from said subject, or tumour type.

In one aspect the method comprises determining the expression profile of immune checkpoint molecules in the tumour, for example by identifying differentially expressed genes, e.g. relative to a suitable reference sample. The reference sample in respect of

differential immune checkpoint molecule expression may be a non-cancerous cell or tumour, (e.g. with low clonal neoantigen burden) or peripheral blood lymphocytes.

For example, the expression profile of the immune checkpoint molecules may be determined by:

(i) determining the RNA sequence of a sample isolated from the tumour; and/or

(ii) performing a transcriptome-wide differential gene expression analysis to identify differential expression of immune checkpoint-related genes (e.g. adjusted to $p<0.05$). Non-cancer cell data may be used as a comparison, for example from the same patient or from a standard reference.

The invention further provides a method for determining the expression profile of immune checkpoint molecules in a particular cancer type comprising the steps of:

(i) obtaining RNA-sequencing data from the Cancer Genome Atlas (TCGA) data portal for a cohort of patients with the cancer of interest;

(ii) obtaining Level_3 gene-level data from each patient;

(iii) inputting the raw read counts into the package DESeq2 for analysis; and

(iv) performing a transcriptome-wide differential gene expression analysis to identify significantly differentially expressed (adjusted $p < 0.05$) immune checkpoint-related genes.

The invention thus provides a method for identifying subjects who have cancer who are more likely to respond to immune checkpoint interventions, comprising determining the expression profile of immune checkpoint molecules in cancer cells from said subject, or tumour type, using said method.

In a preferred aspect, differentially expressed genes between tumours with high clonal neo-antigen burden and low clonal neo-antigen burden are identified (see e.g. Figure 1E). Thus, information regarding the number of clonal neo-antigens is informative and facilitates the combining of the two approaches, namely identifying and targeting subjects/tumours with a high number of clonal neo-antigens, and further investigating the gene expression of immune checkpoint molecules in those subjects/tumours with a high level of clonal neo-antigens. This facilitates a “double-pronged” therapeutic attack.

In one aspect, said differential immune expression is upregulation or high expression of an immune checkpoint molecule which is an inhibitory receptor or costimulatory receptor compared to a suitable reference sample, wherein such upregulation or high expression is

indicative of a response to immune checkpoint interventions targeting the immune checkpoint molecule that has been upregulated or shown high expression.

Gene expression profiles may, for example, be determined by a method as described in present Example 1.

In a preferred embodiment the immune checkpoint molecule is PD-1 and/or LAG-3. In a particularly preferred embodiment the subject has lung cancer, preferably non small-cell lung cancer.

In an alternative embodiment, the immune checkpoint molecule is CTLA4.

In a preferred embodiment the cancer is lung cancer or melanoma, preferably non small-cell lung cancer or melanoma.

This method may also be used in combination with the previously described methods for identifying a subject with cancer who is likely to respond to treatment with an immune checkpoint intervention.

Accordingly the invention provides a method for identifying a subject with cancer who is suitable for treatment with an immune checkpoint intervention, said method comprising:

(i) determining the number of clonal neo-antigens in one or more cancer cells from said subject; and

(ii) determining the expression profile of immune checkpoint molecules in cancer cells and/or tumour infiltrating immune cells from said subject, or tumour type,

wherein a higher number of clonal neo-antigens and differential immune checkpoint molecule expression in comparison to a reference sample is indicative of response to an immune checkpoint intervention.

METHOD OF PROGNOSIS

The present inventors have made the important and surprising determination that cancer patients with higher numbers of clonal neo-antigens, and/or a higher ratio of clonal:sub-clonal neoantigens or a low sub-clonal neo-antigen fraction, have improved prognosis.

One skilled in the art would appreciate in the context of the present invention that subjects with high or higher numbers of clonal neo-antigens, for example within a cohort of subjects

or within a range identified using a number of different subjects or cohorts, may have improved survival relative to subjects with lower numbers of clonal neo-antigens.

A reference value for the number of clonal neo-antigens could be determined using the following method, with a "high number" or "higher number" being anything above that.

Said method may involve determining the number of clonal neo-antigens predicted in a cohort of cancer subjects and either:

(i) determining the median number of clonal neo-antigens predicted in that cohort; wherein that median number is the reference value; or

(ii) determining the minimum number of clonal neo-antigens predicted to be in the upper quartile of that cohort, wherein that minimum number is the reference value. (See e.g. TCGA data analysis in the present Examples.)

Such a "median number" or "minimum number to be in the upper quartile" could be determined in any cancer cohort *per se*, or alternatively in the relevant / specific cancer types.

Alternatively, a "high" or "higher" number of clonal neo-antigens may be defined as 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190 or 200 or more clonal neo-antigens.

One skilled in the art would appreciate that references to "high" or "higher" numbers of clonal neo-antigens may be context specific, and could carry out the appropriate analysis accordingly.

As such, the present invention also provides a method for predicting or determining the prognosis of a subject with cancer, comprising determining the number of clonal neo-antigens in one or more cancer cells from the subject, wherein a higher number of clonal neo-antigens, for example relative to a cohort as discussed above, is indicative of improved prognosis. In a preferred embodiment the cancer is lung cancer or melanoma, preferably non small-cell lung cancer or melanoma.

In an alternative embodiment the invention comprises a method for predicting or determining the prognosis of a subject with cancer, the method comprising determining the clonal:sub-clonal ratio and/or sub-clonal neo-antigen fraction in more than one cancer cell from said subject, wherein a higher clonal:sub-clonal ratio and/or a lower/low sub-clonal neo-antigen

fraction, for example relative to a cohort as discussed above, is indicative of improved prognosis. In a preferred embodiment the cancer is melanoma or lung cancer, preferably melanoma or non small-cell lung cancer.

TREATMENT OF CANCER

The present invention also provides a method of treating or preventing cancer in a subject, wherein said method comprises the following steps:

i) identifying a subject with cancer who is suitable for treatment with an immune checkpoint intervention according to the method of the invention; and

ii) treating said subject with an immune checkpoint intervention.

As defined herein "treatment" refers to reducing, alleviating or eliminating one or more symptoms of the disease, disorder or infection which is being treated, relative to the symptoms prior to treatment.

"Prevention" (or prophylaxis) refers to delaying or preventing the onset of the symptoms of the disease, disorder or infection. Prevention may be absolute (such that no disease occurs) or may be effective only in some individuals or for a limited amount of time.

The term “immune checkpoint intervention” is used herein to refer to any therapy which interacts with or modulates an immune checkpoint molecule. For example, an immune checkpoint intervention may also be referred to herein as a “checkpoint blockade therapy”, “checkpoint modulator” or “checkpoint inhibitor”.

By “inhibitor” is meant any means to prevent inhibition of T cell activity by these pathways. This can be achieved by antibodies or molecules that block receptor ligand interaction, inhibitors of intracellular signalling pathways, and compounds preventing the expression of immune checkpoint molecules on the T cell surface.

Checkpoint inhibitors include, but are not limited to, CTLA-4 inhibitors, PD-1 inhibitors, PD-L1 inhibitors, Lag-3 inhibitors, Tim-3 inhibitors, TIGIT inhibitors and BTLA inhibitors, for example. Co-stimulatory antibodies deliver positive signals through immune-regulatory receptors including but not limited to ICOS, CD137, CD27 OX-40 and GITR.

Examples of suitable immune checkpoint interventions include pembrolizumab, nivolumab, atezolizumab and ipilimumab.

As shown in Example 1 (see Figures 5 and 7), lung tumours with a high number of clonal neoantigens express high levels of PD-1 and Lag-3, and in keeping, T cells reactive to clonal neoantigens in lung cancer subjects also express high levels of PD-1 and LAG-3. The co-expression of PD-1 and Lag-3 in tumours with high clonal neo-antigen burden versus low clonal burden suggests that simultaneous targeting of both pathways may generate maximal benefit.

Hence, in one aspect the invention relates to co-targeting PD-1 and Lag-3 pathways, for example in lung cancer, either by co-administration of inhibitors targeting each pathway or by administration of a single reagent targeting both pathways. As an example of the latter, bispecific antibodies are able to bind to PD-1 and Lag-3, or PD-L1 and Lag-3.

In a preferred embodiment of the present invention, the subject is a mammal, preferably a cat, dog, horse, donkey, sheep, pig, goat, cow, mouse, rat, rabbit or guinea pig, but most preferably the subject is a human.

In one aspect the method of treatment or prevention of cancer according to the invention comprises the step of identifying a patient in need of said treatment or therapy.

The cancer may be selected from, for example, bladder cancer, gastric cancer, oesophageal cancer, breast cancer, colorectal cancer, cervical cancer, ovarian cancer, endometrial cancer, kidney cancer (renal cell), lung cancer (small cell, non-small cell and mesothelioma), brain cancer (e.g. gliomas, astrocytomas, glioblastomas), melanoma, lymphoma, small bowel cancers (duodenal and jejunal), leukemia, pancreatic cancer, hepatobiliary tumours, germ cell cancers, prostate cancer, head and neck cancers, thyroid cancer and sarcomas.

In a preferred embodiment of the invention the cancer is lung cancer. In a particularly preferred embodiment the lung cancer is non-small cell lung cancer.

In one embodiment of the invention the cancer is melanoma.

In one aspect of the invention, the subject has pre-invasive disease, or is a subject who has had their primary disease resected who might require or benefit from adjuvant therapy, such as that provided by the present invention.

Treatment using the methods of the present invention may also encompass targeting circulating tumour cells and/or metastases derived from the tumour.

The methods and uses for treating cancer according to the present invention may be performed in combination with additional cancer therapies. In particular, the immune checkpoint interventions according to the present invention may be administered in combination with co-stimulatory antibodies, chemotherapy and/or radiotherapy, targeted therapy or monoclonal antibody therapy.

The invention will now be further described by way of Examples, which are meant to serve to assist one of ordinary skill in the art in carrying out the invention and are not intended in any way to limit the scope of the invention.

EXAMPLES

Example 1

The clinical relevance of neo-antigens and immune modulation within the context of NSCLC ITH, and the identity of neo-antigen-reactive tumour-infiltrating T cells was investigated.

Materials and Methods

Description of Patient Cohorts

Samples for sequencing (L011 and L012) were obtained from patients diagnosed with non-small cell lung cancer (NSCLC) who underwent definitive surgical resection prior to receiving any form of adjuvant therapy, such as chemotherapy or radiotherapy. Informed consent allowing for genome sequencing had been obtained. Both samples were collected from University College London Hospital, London (UCLHRTB 10/H1306/42) and were subjected to pathology review to establish the histological subtype: one tumour was classified with CK7+/TTF1+ adenocarcinoma (L011) and one tumour (L012) with squamous cell carcinoma histology. Detailed clinical characteristics are provided in table S1.

Samples obtained from (*1*) reflected a patient cohort of stage IV NSCLC, and a detailed description of this patient cohort, including tumour processing, can be found in supplementary material of (*1*). Detailed clinical characteristics of this cohort are provided in table S3.

Clinical efficacy analysis

Clinical efficacy analysis was performed as in (*1*). In brief, objective response to pembrolizumab was assessed by investigator-assessed immunerelated response criteria (irRC) by a study radiologist. As outlined in protocol, CT scans were performed every nine weeks. Partial and complete responses were confirmed by a repeat imaging occurring a minimum of 4 weeks after the initial identification of response; unconfirmed responses were considered stable or progressive disease dependent on results of the second CT scan. Durable clinical benefit (DCB) was defined as stable disease or partial response lasting longer than 6 months (week 27, the time of third protocol-scheduled response assessment). No durable benefit (NDB) was defined as progression of disease ≤ 6 months of beginning therapy. For patients with ongoing response to study therapy, progression-free survival was censored at the date of the most recent imaging evaluation. For alive patients, overall survival was censored at the date of last known contact. Details regarding response for each patient can be found in table S2.

TCGA exome data sets

Tumour samples, with mutation calls and HLA typing described below, were obtained from the Cancer Genome Atlas (TCGA) for a cohort of lung adenocarcinoma (LUAD, n = 124) and lung squamous cell carcinoma (LUSC, n = 124). SNV data was obtained from TumourPortal (*2*) for the LUAD and LUSC TCGA cohorts (http://www.tumourportal.org/tumour_types?ttype=LUAD | LUSC). One LUAD patient, TCGA-05-4396, was excluded for having over 7000 low quality mutations called, mostly in a C[C>G]G context. A LUSC patient, TCGA-18-3409, was excluded for bearing a strong UV signature, uncharacteristic of a LUSC tumour.

Tumour Processing

For both L011 and L012 four primary tumour regions from a single tumour mass, separated by 1cm intervals, and adjacent normal tissue were selected by a pathologist, documented by photography, and snap-frozen. For the brain metastasis in L011, four tumour regions as determined by hematoxylin and eosin (H&E) staining, were selected by a pathologist in the form of formalin-fixed, paraffin-embedded (FFPE) tissue blocks. Peripheral blood was collected at the time of surgery from all patients and snap-frozen. Approximately 5x5x5mm snap-frozen tumour tissue and 500µl of blood was used for genomic DNA extraction, using the DNeasy kit (Qiagen) according to manufacturer's protocol. For the FFPE tissue, manual blade macrodissection was used to remove tumour-rich areas of tissue from 10-40µm unstained slides, aand DNA was extracted from this using the DNeasy Blood and Tissue kit (Qiagen) DNA was quantified by Qubit (Invitrogen) and DNA integrity was examined

by agarose gel eletrophoresis. Details regarding processing of validation and discovery cohort can be found in supplementary material of (*1*).

Multi-region Whole-Exome Sequencing and variant calling

L012

For each tumour region and matched germ-line from patient L012, exome capture was performed on 1-2 µg DNA using the Illumina Nextera kit according to the manufacturer's protocol (Illumina). Samples were paired-end multiplex sequenced on the Illumina HiSeq 2500 at the Advanced Sequencing Facility at the LRI, as described previously (*3, 4*). Each captured library was loaded on the Illumina platform and paired-end sequenced to the desired average sequencing depth (mean across exomes = 392.75). Raw paired end reads (100bp) in FastQ format generated by the Illumina pipeline were aligned to the full hg19 genomic assembly (including unknown contigs) obtained from GATK bundle 2.8 (*5*), using bwa mem (bwa-0.7.7) (*6*). Picard tools v1.107 was used to clean, sort and merge files from the same patient region and to remove duplicate reads (http://broadinstitute.github.io/picard). Quality control metrics were obtained using a combination of picard tools (1.107), GATK (2.8.1) and FastQC (0.10.1) (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/).

SAMtools mpileup (0.1.16) (*7*) was used to locate non-reference positions in tumour and germ-line samples. Bases with a phred score of <20 or reads with a mapping-quality <20 were skipped. BAQ computation was disabled and the coefficient for downgrading mapping quality was set to 50. Somatic variants between tumour and matched germ-line were determined using VarScan2 somatic (v2.3.6) (*8*) utilizing the output from SAMtools mpileup. Default parameters were used with the exception of minimum coverage for the germ-line sample that was set to 10, minimum variant frequency was changed to 0.01 and tumour purity was set to 0.5. VarScan2 processSomatic was used to extract the somatic variants. The resulting SNV calls were filtered for false positives using Varscan2's associated fpfilter.pl script, having first run the data through bam-readcount (0.5.1). Only INDEL calls classed as 'high confidence' by VarScan2 processSomatic were kept for further analysis.

All variants were manually reviewed using Integrated Genomics Viewers (IGV) (*9*), and those showing an Illumina specific error profile (*10*) were removed. Remaining variants were sequenced on Ion Torrent PGM sequencer (Life Technologies) to a median depth of 1513. For this an Ion AmpliSeqTM custom panel (Life Technologies) was designed using the online designer (www.ampliseq.com). Multiplex PCRs were performed on DNA from each region according to the manufacturer's protocol. Barcoded sequencing libraries were TM constructed, which were sequenced with 200 bp read length on the Ion Torrent PGM

sequencer (Life Technologies). Sequence alignment to target regions from the hg19 genome was performed using the IonTorrent TorrentSuiteTM software. Variants for which the coverage was 50 in at least one region were selected. A variant was considered to be present in a region if the variant frequency was ≥ 0.01 for SNVs and ≥ 0.02 for INDELS. Again manual review in IGV was performed and variants that passed this stage were used for subsequent analyses. All variants were annotated using ANNOVAR (*11*) and potential driver mutations were defined as described in (*12*).

L011

The sequencing and analysis of the germline, and primary tumour regions have previously been described in (*13*). Sequencing of the metastatic regions was performed by BGI Tech following the protocols described in (*13*). Computational processing of the metastatic regions was performed using the methods described for L012 above, with an average median depth across the samples of 93.7. The non-silent variants were manually reviewed using IGV as for L012.

Variant calling from Rizvi data

BAM files representing both the germline and tumour regions from (i) 16 samples representing the discovery cohort and 18 samples representing a validation cohort (Rizvi data), were obtained and converted to FASTQ format using picard tools (1.107) SamToFastq Alignment and variant calling was performed as described for L012 above.

Clonal analysis

For TCGA samples, the clonal status of each mutation was estimated by integrating the wild-type and mutant allele counts, absolute major and minor copy numbers, and tumour purity estimates as previously described (*14*). For L011 and L012 clonal status of each mutation was estimated based on multiregion sequencing analysis. In brief, each mutation was classified as clonal if identified and present in each and every tumour region sequenced within the tumour. Conversely, any mutations not ubiquitously present in every tumour region was classified as subclonal.

For discovery and validation cohort tumour, encompassing data obtained from (*1*), the cancer cell fraction of each mutation was estimated by integrating the local copy number (obtained from ASCAT, see below), tumour purity (also obtained from ASCAT), and variant allele frequency. In brief, for a given mutation we first calculated the observed mutation copy number, *nmut*, describing the fraction of tumour cells carrying a given mutation multiplied by the number of chromosomal copies at that locus using the following formula:

$$n_{mut} = \frac{VAF}{p}[pCN_t + CN_n(1-p)]$$

where *VAF* corresponds to the variant allele frequency at the mutated base, and p, CN_t, CN_n are respectively the tumour purity, the tumour locus specific copy number, and the normal locus specific copy number. We then calculated the expected mutation copy number, n_{chr}, using the *VAF* and assigning a mutation to one of the possible copy numbers using maximum likelihood. We also assessed whether mutation copy number could be better explained by subclonal copy numbers when applicable. Ultimately, this allowed us to obtain modified variant and reference counts for every mutation, corrected for both copy number and tumour purity. All mutations were then clustered using the PyClone Dirichlet process clustering (*15*). Given that copy number and purity had already been corrected, we set integer copy numbers to 1 and purity to 1; allowing clustering to simply group clonal and subclonal mutations. We ran PyClone with 10,000 iterations and a burn-in of 1000, and default parameters. Notably, for assessing mutation clonal status, mutations were first further filtered to ensure reliable clustering. In brief, only mutations with a read depth of at least 10 in both germline and tumour were used, a Varscan2 somatic p-value threshold of 0.01. A minimum of 5 alternate reads was required for each variant, as well as a minimum tumour variant allele frequency of 1%. Mutations were also filtered such that a maximum of 2 germline reads, and 2% germline variant allele frequency was permitted.

For two tumours, ZA6965 and GR0134, reliable copy number, mutation and purity estimations could not be extracted, rendering clonal architecture analysis intractable and these tumours were omitted from the analysis

Copy Number Analysis

For data obtained from (*1*) processed sample exome SNP and copy number data from paired tumour-normal was generated using VarScan2 (v2.3.6). Varscan2 copy number was run using default parameters with the exception of min-coverage (21221095)and data-ratio. The data-ratio was calculated on a per-sample basis as described in (22300766). The output from Varscan was processed using the ASCAT v2.3 (20837533) to provide segmented copy number data and cellularity and ploidy estimates for all samples based on the exome sequence data. The following setting was altered from its default value: Threshold for setting ACF to 1 was adjusted from 0.2 to 0.15 and the package was run with gamma setting of 1. For TCGA samples, SNP6.0 data was processed to yield copy number information, as described in McGranahan, 2015.

Phylogenetic Tree Construction

The phylogenetic trees were built using binary presence/absence matrices built from the regional distribution of variants within the tumour, as described in (*12*). For tumour L011, the primary tumour data was reanalyzed using the method described for L012 and the L011 metastatic regions, allowing for a combined tree featuring both primary and metastatic regions.

HLA Typing of Patient Samples

For all TCGA patients, the 4-digit HLA type was determined using POLYSOLVER (POLYmorphic loci reSOLVER)(*16*). Patients L011 and L012 were serotyped and simultaneously genotyped using Optitype (*17*), which produced concordant results.

Identification of Putative Neo-antigens

Identified non-silent mutations were used to generate a comprehensive list of peptides 9-11 amino acids in length with the mutated amino acid represented in each possible position. The binding affinity of every mutant peptide and its corresponding wild-type peptide to the patient's germline HLA alleles was predicted using netMHCpan-2.8 (*18, 19*). Candidate neo-antigens were identified as those with a predicted binding strength of < 500 nM.

TCGA Survival Analysis

Clinical data for the TCGA patients was accessed through the TCGA data portal and downloaded from https://tcgadata.nci.nih.gov/tcgafiles/ftp_auth/distro_ftpusers/anonymous/tumour/CANCER.TYPE /bcr/biotab/clin/. Survival analyses were performed in R using the survival package.

Differential Gene Expression Analysis

RNA-sequencing data was downloaded from the TCGA data portal. For each LUAD patient, all available 'Level_3' gene-level data was obtained. The raw read counts were used as input into the R package DESeq2 for analysis. A transcriptomewide differential gene expression analysis was performed and significantly differentially expressed (adjusted $p < 0.05$) immune related genes (listed in Table S1) were identified. These genes were clustered on their co-expression using the metric $1-r^2$.

Isolation of tumour-infiltrating lymphocytes (TILs) for L011 and L012

Tumours were taken directly from the operating theatre to the department of pathology where the sample was divided into regions. Samples were subsequently minced under sterile conditions followed by enzymatic digestion (RPMI-1640 (Sigma) with Liberase TL

research grade (Roche) and DNAse I (Roche)) at 37°C for 30 minutes before mechanical dissociation using gentleMACS (Miltenyi Biotech). Resulting single cell suspensions were enriched for leukocytes by passage through a Ficoll-paque (GE Healthcare) gradient. Live cells were counted and frozen in human AB serum (Sigma) with 10% dimethyl sulfoxide at -80°C before transfer to liquid nitrogen.

In-vitro expansion of tumour-infiltrating lymphocytes for L011 and L012

TILs were expanded using a rapid expansion protocol (REP) in T25 flasks containing EX-VIVO media (Lonza) supplemented with 10% human AB serum (Sigma), soluble anti-CD3 (OKT3, BioXCell), 6000IU/mL recombinant human (rhIL-2, PeproTech) and $2x10^7$ irradiated PBMCs (30Gy) pooled from 3 allogeneic healthy donors. Fresh media containing rhIL-2 at 3000IU/mL was added every three days as required. Following 2 weeks of expansion, TILs were counted, phenotyped by flow cytometry and frozen in human AB serum (Sigma) at -80°C before use in relevant assays or long-term storage in liquid nitrogen.

MHC multimer generation and combinatorial encoding-flow cytometry analysis

MHC-multimers holding the predicted neoepitopes were produced in-house (Technical University of Denmark, laboratory of SRH). Synthetic peptides were purchased at Pepscan Presto, NL. HLA molecules matching the HLA-expression of L011 (HLA-A1101, A2402, and B3501) and L012 (HLA-A1101, A2402, and B0702) were refolded with a UV-sensitive peptide, and exchanged to peptides of interest following UV exposure (*20-23*). Briefly, HLA complexes loaded with UV-sensitive peptide were subjected to 366-nm UV light (CAMAG) for one hour at 4°C in the presence of candidate neo-antigen peptide in a 384-well plate. Peptide-MHC multimers were generated using a total of 9 different fluorescent streptavidin (SA) conjugates: PE, APC, PE-Cy7, PE-CF594, Brilliant Violet (BV)421, BV510, BV605, BV650, Brilliant Ultraviolet (BUV)395 (BioLegend). MHC-multimers were generated with two different streptavidin-conjugates for each peptide-specificity to allow a combinatorial encoding of each antigen responsive T cells, enabling analyses for reactivity against up to 36 different peptides in parallel (*24, 25*).

Identification of neo-antigen-reactive CD8+ T cells

MHC-multimer analysis was performed on *in-vitro* expanded CD8+ T lymphocytes isolated from region-specific lung cancer samples and adjacent normal lung tissue. 290 and 355 candidate mutant peptides (with predicted HLA binding affinity <500nM, including multiple potential peptide variations from the same missense mutation) were synthesized and used to screen expanded L011 and L012 TILs respectively. For staining of expanded CD8+ T lymphocytes, samples were thawed, treated with DNAse for 10 min, washed and stained

with MHC multimer panels for 15 min at 37°C. Subsequently, cells were stained with LIVE/DEAD® Fixable Near-IR Dead Cell Stain Kit for 633 or 635 nm excitation (Invitrogen, Life Technologies), CD8-PerCP (Invitrogen, Life Technologies) and FITC coupled antibodies to a panel of CD4, CD14, CD16, CD19 (all from BD Pharmingen) and CD40 (AbD Serotec) for an additional 20 min at 4°C. Data acquisition was performed on an LSR II flow cytometer (Becton Dickinson) with FACSDiva 6 software. Cutoff values for the definition of positive responses were ≥0.005% of total CD8+ cells and ≥10 events.

For patient L011, HLA-B3501 MTFR2-derived multimers were found to bind the mutated sequence FAFQEYDSF (netMHC binding score: 22) but not the wild type sequence FAFQEDDSF (netMHC binding score: 10) (Fig 11B and D, Fig 9C). No responses were found against overlapping peptides AFQEYDSFEK and KFAFQEYDSF. For patient L012 HLA-A1101 CHTF18-derived multimers bound the mutated sequence LLLDIVAPK (netMHC binding score: 37) but not the wild type sequence: LLLDILAPK (netMHC binding score: 41) (Fig 11C and E, Fig 9C). No responses were found against overlapping peptides CLLLDIVAPK and IVAPKLRPV. Finally, HLA-B0702 MYADM-derived multimers bound the mutated sequence SPMIVGSPW (netMHC binding score: 15) as well as the wild type sequence SPMIVGSPR (netMHC binding score: 1329). No responses were found against overlapping peptides SPMIVGSPWA, SPMIVGSPWAL, SPWALTQPLGL and SPWALTQPL.

MHC-multimer analysis and multi-parametric flow cytometric phenotyping of baseline, non-expanded tumour samples for L011 and L012

Tumour samples were thawed, washed and first stained with custom-made MHCmultimers for 10-15 minutes at 37°C in the dark. Cells were thereafter transferred onto wet ice and stained for 30 minutes, in the dark, with a panel of surface antibodies used at the manufacturer's recommended dilution: CD8-V500, SK1 clone (BD Biosciences), PD-1-BV605, EH12.2H7 clone (Biolegend), CD3-BV785, OKT3 clone (Biolegend), LAG-3-PE, 3DS223H clone (eBioscience). Cells were permeablized for 20 minutes with use of the intracellular fixation and permeabilization buffer set from eBioscience. An intracellular staining panel was applied for 30 minutes, on ice, in the dark, and consisted of the following antibodies used at the manufacturers recommended dilution: granzyme B-V450, GB11 clone (BD Biosciences), FoxP3-PerCP-Cy5.5, PCH101 clone (eBioscience), Ki67-FITC, clone B56 (BD Biosciences)and CTLA-4 – APC, L3D10 clone (Biolegend). Data acquisition was performed on a BD FACSAria III flow cytometer (BD Biosciences) and analysed in Flowjo version 10.0.8 (Tree Star Inc.).

Immunohistochemistry for L011 and L012

Samples from patients L011 and L012 and reactive human tonsils were fixed in buffered formalin and embedded in paraffin according to conventional histological protocols. 2-5 micrometer tissue sections from paraffin blocks were cut and transferred on electrically charged slides to subject to immunohistochemistry. Details of the primary used antibodies are listed in the below table. To establish optimal staining conditions (i.e. antibody dilution and incubation time, antigen retrieval protocols, suitable chromogen) each antibody was tested and optimized on sections of human reactive tonsil by conventional single immunohistochemistry using the automated platforms BenchMark Ultra (Ventana/Roche) and the Bond-III Autostainer (Leica Microsystems) according to a protocol described elsewhere (*26, 27*).

Where available, at least two distinct antibodies raised against the same protein were analyzed in tonsil to confirm the specificity of its staining pattern. For multiple staining a protocol previously described was carried out (*28*). For evaluation of protein co-expression in the cytoplasm or cell membrane, change of the single colour of the chromogen is noted i.e. blue and red gave rise to a purple and brown and blue to an almost black labelling. Immunohistochemistry and protein reactivity patterns were assessed by TM. Scoring of multiple immuno-staining was performed together with AF. Approval for this study was obtained from the National Research Ethics Service, Research Ethics Committee 4 (REC Reference number 09/H0715/64).

Molecule	Antibody type	Clone name	Dilution	Source
Anti-human CD8	Rabbit Monoclonal	SP239	1:100	Spring Biosciences Inc., Pleasanton, CA, US
Anti-human FoxP3	Mouse Monoclonal	236A/E7	1:100	Kind gift from Dr G Roncador, CNIO, Madrid (Spain)
Anti-human PD-L1	Rabbit Monoclonal	SP142	1:50	Spring Biosciences Inc., Pleasanton, CA, US
Anti-human LAG-3	Mouse Monoclonal	17B4	1:750	LifeSpan Biosciences Inc., Nottingham,UK
Anti-human Granzyme B	Mouse Monoclonal	11F1	RTU	Leica Microsystems Ltd., Newcastle-upon-Tyne, UK)

Results

A large tumour neo-antigen burden may increase tumour recognition by T cells, reducing the potential for immune-evasion (*12*). In support of the clinical relevance of tumour neo-antigens (*7*), it was found that high neo-antigen load (defined as the upper quartile of the number of neo-antigens predicted in the cohort) was associated with longer overall survival times in LUAD samples with matched clinical data (n=117) when compared to tumours in the remaining quartiles (Fig 1B, logrank p = 0.011; Fig 5A).

To determine whether neo-antigen clonal status (the presence of a neo-antigen in all tumour (clonal) compared to a subset of tumour cells (subclonal)) might influence the relationship with survival outcome, the cancer cell fraction (proportion of cancer cells harboring each mutation) was calculated and each putative neo-antigen was classified as either clonal or subclonal (*13*). Tumours harboring a high number of predicted clonal neo-antigens (defined as the upper quartile of the cohort) were associated with longer overall survival compared to all other tumours in the cohort (Fig 1C, log-rank p = 0.0077; Fig 5B). Conversely, the number of predicted subclonal neo-antigens was not significantly associated with overall survival (Fig 1D, log-rank p = 0.12; Fig 5C). Although neo-antigen burden was related to mutation burden, we observed a stronger relationship between overall survival and number of neo-antigens compared to number of mutations (Fig 6). These data suggest the presence of a high number of clonal neo-antigens in LUAD may favor effective immunosurveillance. The LUSC cohort had a narrower range of putative neo-antigens (Fig 7A), with a median absolute deviation of 50 and interquartile range of 71 and a statistically significant association between overall survival and neo-antigen load was not observed in this cohort (Fig 7 B-G). This might reflect difficulties in dissecting the clonal architecture of tumours from single samples (*14*).

Gene expression analysis revealed 27 immune-related genes differentially expressed between low (defined as the lower quartile of the number of clonal neo-antigens predicted in the cohort) and high clonal neo-antigen cohorts (Table S1). CD8A (p=0.005) and genes associated with antigen presentation (TAP-1 p=0.003, STAT-1 p<0.001), T cell infiltration (CXCL-10 p=0.005, CXCL-9 p = p<0.001) and effector T cell function (IFN-γ p<0.001, Granzymes B p<0.001 and H p=0.008) were up-regulated in the high clonal neo-antigen cohort and clustered together (Fig 1E). PD-1 (p=0.02) and lymphocyte activation gene 3 (LAG-3, p<0.001), negative regulators of T cell function (*15*), were also identified in this cluster. PD-L1 was also significantly up-regulated (p<0.001) in the high clonal cohort, clustering with PD-L2. When we compared the high clonal neo-antigen tumours to all other

tumours in the cohort, PD-L1 was identified as the most significantly differentially expressed immune gene (Fig 8, $p<0.001$).

These data suggest that a high clonal neo-antigen burden is associated with the presence ofactivated effector T cells potentially regulated by the expression of specific immune checkpoint proteins (PD-1, LAG-3, PD-L1/2).

It was next addressed whether CD8+ T cells reactive to clonal neo-antigens could be identified in primary NSCLC tumours. Two early stage tumours, L011 and L012, subjected to multi-region exome sequencing (*13*), permitted phylogenetic analysis and prediction of neo-antigens within each primary tumour region (Fig 2A). L011 included a brain metastasis, resected 14 months following primary surgery, subjected to multi-region sequencing. While both tumours were derived from female smokers (>40 pack-years), their mutation burden and extent of heterogeneity was distinct (Fig 2A). L011, an adenocarcinoma, exhibited a homogenous primary tumour and metastatic dissemination to the brain (M1-M4), likely originating from tumour region R3 (Fig 2A). A total of 313 neo-antigens were predicted within the primary tumour, 88% of which were clonal, identified in every region of the primary tumour (Fig 2B). Conversely, L012, a squamous cell carcinoma, exhibited a low mutation burden and extensive heterogeneity, with 75% of the predicted neo-antigens being subclonal (Fig 2A, C).

MHC-multimers loaded with predicted neo-antigens were used to screen CD8+ T cells expanded from different tumour regions and adjacent normal lung tissue (*13*). In L011, CD8+ T cells reactive to mutant MTFR2D326Y (FAFQE*Y*DSF), a clonal mutation with high predicted HLA binding in wild type (10nM) and mutant (22nM) forms (Fig 2B), were identified in all tumour regions (2.8-4.4%) and at lower frequency in normal regions (0.1%) (Fig 2D). In L012, CD8+ T cells reactive to mutant CHTF18L769V (LLDI*V*APK) and MYADMR30W (SPMIVGSP*W*) were identified in all tumour regions and at lower frequencies in normal tissue (Fig 2E). Both were clonal mutations, CHTF18 with high predicted HLA binding (<50nM) in mutant and wild type forms, and MYADM with lower predicted binding in wild type (>1000nM) compared to mutant form (<50nM) (Fig 2C).

In L011, MTFR2-reactive CD8+ T cells could also be detected in non-expanded TILs (tumour infiltrating lymphocytes) (Fig 3A) from all primary tumour regions (0.79-1.35%), and at lower frequencies in normal tissue (0.16%) and peripheral blood mononuclear cells (PBMCs) (0.02%). Similarly, CHTF18-reactive and MYADM-reactive CD8+ T cells were identified in non-expanded samples from all tumour regions in L012 (CHTF18 0.16-0.58%,

MYADM 2.25- 2.31%) and at a lower frequency in normal lung tissue (CHTF18 0.02%, MYADM 0.17%) and PBMCs (CHTF18 0.02%, MYADM 0.01%) (Fig 3A).

Further characterization of neo-antigen-reactive T cells in non-expanded samples was performed by flow cytometry. Although at low levels, CTLA-4 expression was confined to tumour-infiltrating CD8+ T cells for both L011 and L012, with highest levels identified on MTFR2, CHTF18 and MYADM-reactive T cells (Fig 3B, Fig 9A). High levels of PD-1 were expressed by >99% of MTFR2-, CHTF18- and MYADM-reactive tumour-infiltrating CD8+ T cells (Fig 3B, Fig 9A), whilst lower levels were observed on CD8+ MHC-multimer negative T cells in tumour, normal tissue and PBMCs. In L011, LAG-3 expression was higher on all tumour rinfiltrating CD8+ T cells, including MTFR2-reactive cells, relative to normal tissue and PBMCs (Fig 3B). LAG-3 expression was also observed in L012, although at lower levels (Fig 9A). IHC studies further supported these findings, identifying CD8+ T cells co-expressing LAG-3 in both L011 and L012 primary tumours (Fig 3D). Ki67 was expressed at higher levels on tumour infiltrating CD8+ T cells than in normal tissue or PBMCs (Fig 3B, Fig 9A), however the fraction of proliferating cells was low for both neo-antigen-reactive and MHC-multimer negative cells (<25%). In contrast, granzyme B (GzmB) was expressed at high levels on all studied CD8+ T cell subsets. Importantly, whereas a large proportion of neo-antigen reactive T cells in the tumours appeared highly activated expressing GzmB, the majority of these cells coexpressed PD- 1 (>60%) and appeared to be under proliferative control based on Ki67 levels (Fig 3C, Fig 9B).

Expression of LAG-3 and PD-1 on T cells reactive to clonal neo-antigens, together with tumour PD-L1 expression (Fig 3D), strongly supports the immune-signatures identified in high clonal lung tumours (Fig 1E). These data support a potential role for these specific checkpoints in restricting the activity of T cells recognizing clonal neo-antigens and future studies targeting these checkpoints in NSCLC with high clonal neo-antigen burden.

Next, it was explored whether the clonal status of putative neo-antigens might be associated with altered sensitivity to PD-1 blockade in NSCLC. Exome sequencing data from a recent study in which two independent NSCLC cohorts were treated with pembrolizumab was obtained (*2*)(Table S2), and the clonal architecture of each tumour was dissected by estimating the cancer cell fraction of each mutation (*13*) (Fig 10). As previously reported (*2*), neo-antigen burden was related to the clinical efficacy of pembrolizumab in the discovery and validation cohort, with a high neo-antigen repertoire associated with improved outcome (data not shown).

The relationship was also contingent upon the clonal architecture of each tumour (Fig. 4A-H). In the discovery cohort, every tumour exhibiting durable clinical benefit (DCB, defined as in (*2*) as partial response or stable disease lasting > 6 months) harbored a high clonal neo-antigen burden (defined as above or equal to the median number of clonal neo-antigens in the discovery cohort, 91) and a neo-antigen subclonal fraction lower than 5% (Fig. 4A-B). Conversely, every tumour exhibiting a non-durable benefit (NDB) harbored either a low clonal neo-antigen repertoire (<91) or high neo-antigen subclonal fraction (>5%). Thus, in the discovery cohort, combining both neo-antigen repertoire and neo-antigen heterogeneity (i.e. the ratio of clonal:sub-clonal neo-antigens or mutations) was able to predict sensitivity to pembrolizumab, better than either measure alone (Fig 4C).

Similarly, in the validation cohort, five of six tumours with a high clonal neo-antigen burden (defined as greater than or equal to the median of the validation cohort, 69) and low subclonal neo-antigen fraction (<5%) were associated with DCB (Fig 4D-F). Conversely, eight out of ten tumours with low clonal neo-antigen burdens or high neo-antigen heterogeneity were associated with NDB. For instance, despite a large neo-antigen burden, ZA6505 exhibited a nondurable clinical response, relapsing after 2 months. ZA6505 was one of the most heterogeneous tumours within the cohort, with over 80% of mutations classified as subclonal.

In summary, when the extent of neo-antigen heterogeneity and the clonal neo-antigen burden were considered together, outcome could be predicted in almost all cases (Fig 4G-H).

Moreover, in keeping with TCGA analysis (Fig 1E), we also observed greater PD-L1 expression in tumours harboring a large clonal neo-antigen burden and low neo-antigen heterogeneity compared to those with a low neo-antigen load or high neo-antigen heterogeneity (P= 0.0017, $\chi 2$- test, Fig 11). These results remained consistent when considering all mutations rather than class- I restricted putative neo-antigens (Fig 12), supporting the notion that unidentified MHC class II restricted neo-antigens may also play a significant role in immune reactivity (*6*) and the need for refinement of neo-antigen prediction algorithms (*16*). responding to anti-PD-1 therapy. Previous analysis of peripheral blood lymphocytes (PBLs) from CA9903, a tumour with exceptional response to pembrolizumab, identified a CD8+ T cell population in autologous PBLs recognizing a predicted neo-antigen resulting from a *HERC1*P3278S mutation (ASNA*S*SAAK) (*2*). Consistent with the relevance

of clonal neo-antigens, this mutation was likely present in 100% of cancer cells within the sequenced tumour (Fig 4I-J).

Supplementary Table S1: Differentially expressed immune genes between high and low clonal neo-antigen patient groups

	Mean	log2 Fold Change	p-value	adjusted p-value
GZMB	445.7851333	-1.78462652	1.18E-09	1.69E-07
TNFSF13	3532.313186	0.825249238	6.24E-08	4.13E-06
IL6	448.0627177	-1.816113115	1.13E-07	6.70E-06
TMEM173	3740.991274	0.924504705	7.26E-07	2.84E-05
IFNG	36.80074093	-1.756035642	8.98E-06	0.000202975
PD-L1	384.7156773	-1.42730081	1.44E-05	0.00029375
CXCL9	4559.513492	-1.511142807	1.50E-05	0.000302565
STAT1	17028.82171	-0.784356172	2.16E-05	0.000401426
LAG3	277.7770415	-1.129050218	2.51E-05	0.000454562
RORA	634.8938186	0.738052259	6.53E-05	0.000963038
PRDM1	1236.570377	-0.678822885	7.91E-05	0.001116763
TAP1	10724.07983	-0.763792013	0.000338393	0.003482612
GNLY	357.1056535	-1.047292568	0.000560104	0.005132755
CXCL10	2068.038219	-1.15715645	0.000568625	0.005180037
CD8A	788.7253219	-0.945122724	0.000589072	0.005306952
CSF3	49.20726456	-1.359216439	0.000907102	0.007361937
TBX21	76.56429476	-1.021741775	0.000939277	0.007541139
GZMH	186.2030449	-0.979136784	0.001007872	0.007952958
TAP2	4538.140019	-0.559264738	0.001188075	0.008964565
PD-L2	332.3493386	-0.771675574	0.002964397	0.017858103
PVR	2940.716051	-0.567512648	0.003336494	0.019565463
CD70	71.58250113	-1.050709857	0.003388254	0.019734624
PD1	147.129952	-0.886846385	0.003492638	0.020231348
VTCN1	822.1971729	1.238388678	0.005797085	0.029375393
CHUK	1499.467613	-0.303245758	0.006444708	0.031673895
SOCS2	795.7802019	0.663903969	0.008580641	0.039140625
TNFRSF14	2676.212113	0.359555349	0.009918008	0.043580894
CD8B	213.6837071	-0.734726572	0.012492819	0.051665805
IL1B	462.025209	-0.701631604	0.012892938	0.052917717
IL12A	29.20823765	-0.68364274	0.013729832	0.055446871
IL12B	14.43557004	0.783016768	0.01707779	0.065407095
IL2	2.444225976	0.986346011	0.018986691	0.070900521
CX3CL1	4506.608644	0.67426411	0.027478555	0.092915037
NOS2	41.22675253	-0.68521024	0.029626595	0.098142202
TNFRSF18	360.9297496	-0.729059895	0.029718057	0.098354315
KLRK1	182.5573646	-0.643726062	0.030255564	0.099453935
MADCAM1	7.273228749	-0.784565543	0.048684055	0.140368258
GZMA	578.3953638	-0.635858589	0.050652532	0.144348847

VEGFA	10928.73621	-0.451684004	0.056171355	0.155239718
PRF1	618.5783468	-0.473152277	0.058353066	0.159377477
LGALS9	5310.012377	0.422760767	0.05909457	0.160839147
IL7	207.9545877	0.496316279	0.061102723	0.164605503
PTGS2	6159.88191	-0.747090631	0.063658001	0.169174461
TNFRSF4	197.5157825	-0.379177736	0.07230099	0.184487923
CD160	25.1062924	-0.460251517	0.074003068	0.187576278
TNFRSF13B	38.64965649	0.578468093	0.077739763	0.194381934
TIGIT	239.3616733	-0.479398507	0.079715132	0.19772176
TNFRSF9	107.0383741	-0.538183729	0.088224384	0.211935421
IL8	4258.061194	-0.592100426	0.102069566	0.234512774
CD86	1011.868757	-0.311180799	0.112427315	0.250957613
IRF1	3898.872307	-0.322486714	0.116076728	0.256631346
CCL5	2614.171996	-0.472610458	0.122548084	0.266275852
CD28	215.0051414	0.33366846	0.124587781	0.26940173
CD200	390.5585743	-0.295026768	0.13116523	0.27888167
HAVCR2	1325.180665	-0.296647885	0.140180116	0.291003086
MS4A1	390.2558862	0.523928974	0.15233134	0.307579262
IL12RB1	198.3081926	-0.327226759	0.161228022	0.319922079
TGFB1	3705.083958	-0.203702547	0.164951696	0.325330473
STAT3	14213.7378	0.150804216	0.169624324	0.33151912
CXCR5	95.03997316	0.416011181	0.183763767	0.348650978
IDO1	1858.956376	-0.407500734	0.23727483	0.413799917
CD79A	1175.710552	-0.341052525	0.263424151	0.443401388
IL10RB	3285.337481	0.152074186	0.292309086	0.475665005
IRF5	980.613802	-0.193865677	0.294881352	0.478239401
CXCR3	271.2315304	-0.283719077	0.29632793	0.479863349
TNFSF9	294.2710962	-0.291633453	0.298080985	0.481806159
NR4A1	4233.840543	0.308559097	0.307663118	0.491861702
CD69	636.205301	0.310698903	0.313829669	0.498082357
TNFRSF13C	23.54714796	-0.27661096	0.320174619	0.504889662
CTLA4	144.1973513	-0.252213052	0.337165917	0.523370695
CD80	118.3959403	-0.219168442	0.344168801	0.531186145
VEGFB	3813.436412	0.111428746	0.348255092	0.535082065
CD276	4839.942708	-0.110880457	0.354884606	0.541381686
TNFSF4	285.0994787	-0.251740957	0.367394794	0.552571633
IL15	251.954005	-0.227790082	0.372118713	0.557431842
HLA-B	117419.6406	-0.189404462	0.392700995	0.577313935
TNFSF18	10.50871731	0.278307077	0.434127114	0.615308001
CSF2	82.00905002	0.258772851	0.456961522	0.635760218
IKBKB	3029.277618	0.123880242	0.462066231	0.639685204
HLA-E	28263.91252	0.112748395	0.463864656	0.641508215
CD3D	468.5251485	-0.215789423	0.464458271	0.642153767
EOMES	73.96153664	-0.207485464	0.471039076	0.647525273

LTA	47.75821054	0.202745276	0.48236162	0.656627117
CD244	84.89011319	-0.186732614	0.494820217	0.667103087
HLA-C	76083.94895	-0.133189426	0.505495645	0.676055645
TGFBR1	3202.195003	-0.08370058	0.506750828	0.677080529
CXCL5	944.0905877	-0.270235841	0.521955968	0.689704336
HLA-G	411.9342955	0.179225426	0.52944301	0.695460893
TGFB3	950.0375094	-0.121079367	0.553603166	0.71443979
B2M	172378.9968	0.110580042	0.561947299	0.72111769
ICAM1	22061.71601	-0.143421343	0.563916412	0.722858104
CD40	1772.030076	-0.119567048	0.600081755	0.750332383
IL10	48.9483278	-0.143330001	0.611619228	0.759687642
CD3E	926.6666625	-0.132795883	0.613950098	0.761354179
HLA-F	6351.352815	0.120347259	0.628199687	0.771810162
VCAM1	2159.418805	-0.130117222	0.642682858	0.782745807
CD79B	343.535879	0.127051943	0.65365394	0.790469331
CCL2	2809.121226	0.112856687	0.682702276	0.810562013
FOXP3	194.391431	0.111548571	0.689381473	0.815575495
BTLA	62.23164316	-0.102004115	0.732037222	0.845834192
SOCS1	433.4195773	0.082676919	0.734857322	0.84806292
CD2	948.4310392	-0.086505902	0.742632845	0.85329242
CD3G	131.2700215	-0.09041107	0.743838306	0.853911324
CXCL13	1506.896218	-0.118364398	0.755758076	0.861471947
ICOS	120.5538816	-0.086753989	0.760044117	0.864093608
CXCL1	960.6911493	0.085909113	0.800874166	0.889816067
CD4	5270.88003	-0.045153828	0.813688182	0.898429619
BCL6	2893.728602	-0.030670947	0.831857609	0.909937895
IL1A	52.50184149	-0.071209064	0.835686639	0.912278655
CD19	119.1478854	-0.060259488	0.8618787	0.926697933
HLA-A	92787.6356	-0.033095882	0.871822173	0.932461535
CD38	401.6927917	0.038510446	0.899895838	0.945971695
CD27	637.0929506	-0.02998534	0.908371198	0.951425293
STAT5A	1460.392543	0.003525758	0.977657023	0.99064235
IKBKG	1236.266327	0.002038592	0.989162825	0.995429737
ARG1	1.115682528	-0.318253465	0.491866502	NA
IL21	0.790890355	-1.092615876	0.040972459	NA
IL4	0.54010078	0.784036263	0.143480734	NA

Supplementary Table S2: Detailed clinical characteristics of individual patients

#	Study ID	Cohort (Discovery. Validatio	Histolog	Age (yea	Se	Smok	Pack-yea	PD-L1*	Priors ^^	Dose (mg/k	Sched. **	PFS (m	Event ^^	Resp ^^^^	Durable Clinical Bene
1	SA9755	Valid	NSCLC NOS	63	F	Former	36	Weak	1	10	3	18.8	0	PR	DCB
2	HE3202	Disc	Adeno	63	F	Former	58	Strong	3	10	3	14.7	0	PR	DCB
3	TU0428	Disc	Adeno	66	M	Current	48	Negative	0	10	3	2.1	1	POD	NDB
4	Y2087	Valid	Adeno	68	F	Never	0	Weak	5	10	3	8.3	1	SD	DCB
5	M4945	Valid	Adeno	66	M	Former	40	Unknown	3	10	2	21.1	1	PR	DCB
6	RI1933	Disc	Adeno	60	F	Former	21	Strong	1	10	3	25.2	0	PR	DCB
7	ZA6505	Valid	Adeno	76	F	Never	0	Negative	6	10	3	1.9	1	POD	NDB
8	CU9061	Valid	Squam	57	M	Former	39	Weak	1	2	3	6.2	1	SD	NDB
9	CA9903	Disc	Adeno	57	M	Former	80	Strong	3	10	3	14.5	1	PR	DCB
10	SC0899	Disc	Adeno	64	F	Current	25	Weak	0	10	3	14.8	1	PR	DCB
11	FR9547	Valid	Adeno	65	F	Current	25	Strong	1	2	3	12.4	1	PR	DCB
12	KA3947	Disc	Adeno	64	F	Former	52.5	Strong	0	10	3	8.1	1	SD	DCB
13	MA7027	Disc	Adeno	56	M	Former	37.5	Weak	1	10	2	1.8	1	POD	NDB
14	ZA6965	Valid	Adeno	57	F	Former	25	Strong	1	2	3	14.5	0	PR	DCB
15	AL4602	Valid	Adeno	59	M	Former	34	Strong	0	10	3	16.8	0	SD	DCB
16	JB112852	Disc	Adeno	60	M	Never	0	Negative	5	10	2	3.3	1	POD	NDB
17	SR070761	Valid	Squam	51	F	Former	2.5	Negative	4	10	2	3.4	1	POD	NDB
18	DI6359	Disc	Adeno	61	F	Current	60	Strong	6	10	3	9.8	0	PR	DCB
19	SB010944	Valid	Squam	68	M	Never	0	Unknown	2	10	3	35.7	0	PR	DCB
20	RH090935	Valid	Adeno	78	F	Former	60	Strong	0	10	3	20.9	0	PR	DCB
21	SC6470	Disc	Adeno	59	M	Current	15	Weak	0	10	2	8.3	1	SD	DCB
22	BL3403	Disc	Adeno	73	F	Former	43.75	Weak	1	10	2	6.5	1	SD	NDB
23	GR4788	Disc	Squam	59	M	Current	45	Negative	0	10	2	1.9	1	POD	NDB
24	DM123062	Valid	Adeno	50	M	Never	0	Weak	6	10	2	1.9	1	POD	NDB
25	R7495	Valid	Adeno	63	M	Former	73.5	Weak	2	2	3	1.4	1	POD	NDB
26	WA7899	Valid	Adeno	49	M	Never	0	Strong	2	10	3	1.9	1	POD	NDB
27	RO3338	Disc	Adeno	71	M	Former	20	Weak	1	10	3	2.1	1	POD	NDB
28	LO3793	Valid	Adeno	62	F	Former	6	Weak	2	2	3	3.5	1	SD	NDB
29	LO5004	Valid	Adeno	56	F	Former	8	Weak	0	10	2	6.3	1	SD	NDB
30	GR0134	Valid	Adeno	80	M	Former	56	Negative	0	10	3	8.3	1	PR	DCB
31	VA1330	Disc	Adeno	71	F	Former	0.5	Unknown	1	10	3	4.1	1	SD	NDB
32	NI9507	Valid	Adeno	41	F	Current	2.25	Weak	1	10	3	1.9	1	POD	NDB
33	AU5884	Disc	Adeno	64	M	Former	10	Weak	2	10	2	1.8	1	POD	NDB
34	VA7859	Disc	Adeno	57	F	Former	3.15	Unknown	1	10	3	6.3	1	SD	NDB

#,patient number. Adeno, adenocarcinoma. Squam, squamous cell carcinoma. NSCLC NOS, non small-cell lung cancer, not otherwise specified. P

**Pembrolizumab dosed every 2 or 3 weeks as indicated.

*PDL-1 expression. Strong, >/=50% membraneous staining; Weak, 1-49% membraneous staining; Negative, <1% membraneous staining; Unknown

^^^^Resp. denotes best overall response to pembrolizumab.

^^^Event (1) or censure (0) for progression-free survival

^^Prior courses of cytotoxic chemotherapy. Combination chemotherapy counted as a single course. No patient had received prior immunotherap

^Self-reported smoking status.

DCB, durable clinical benefit beyond 6 months. NDB, no durable benefit. NR, not reached 6 months follow-up.

F, Female. M, Male. P, positive. No, negative. U, unknown. Smok., Smoking status. Pack-years, product of number of packs per day and number of

Sched., Schedule of administration in weeks. Mos, months. Resp., best overall response.

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All documents referred to herein are hereby incorporated by reference in their entirety, with special attention to the subject matter for which they are referred Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology, cellular immunology or related fields are intended to be within the scope of the following claims.

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Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

The reference in this specification to any prior publication (or information derived from it), or to any matter which is known, is not, and should not be taken as an acknowledgment or admission or any form of suggestion that that prior publication (or information derived from it) or known matter forms part of the common general knowledge in the field of endeavour to which this specification relates.

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THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method for identifying a subject with cancer who is suitable for treatment with an immune checkpoint modulator, said method comprising:

(i) determining the number of clonal neo-antigens in one or more cancer cells from said subject; and/or

(ii) determining the ratio of clonal: sub-clonal neo-antigens and/or sub-clonal neo-antigen fraction in more than one cancer cell from said subject;

wherein a higher number of clonal neo-antigens, and/or a higher ratio of clonal:sub-clonal neo-antigens, or lower (or low) sub-clonal neo-antigen fraction in comparison to a reference sample is indicative of response to an immune checkpoint modulator.

2. The method according to claim 1, further comprising determining the expression profile of immune checkpoint molecules in cancer cells and/or tumour infiltrating immune cells from said subject, or tumour type, wherein differential immune checkpoint molecule expression in comparison to a reference sample is indicative of response to an immune checkpoint modulator.

3. The method according to claim 2, wherein determining the expression profile of immune checkpoint molecules is performed by a transcriptome-wide differential gene expression analysis to identify differentially expression immune checkpoint-related genes.

4. A method for predicting or determining the prognosis of a subject with cancer, the method comprising:

(i) determining the number of clonal neo-antigens in one or more cancer cells from said subject; and/or

(ii) determining the ratio of clonal: sub-clonal neo-antigens and/or sub-clonal neo-antigen fraction in more than one cancer cell from said subject,

wherein a higher number of clonal neo-antigens and/or a higher ratio of clonal:sub-clonal neo-antigens, or lower (or low) sub-clonal neo-antigen fraction, is indicative of improved prognosis.

5. A method of treating or preventing cancer in a subject, wherein said method comprises the following steps:

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i) identifying a subject with cancer who is suitable for treatment with an immune checkpoint modulator according to the method of any one of claims 1 to 3; and

ii) treating said subject with an immune checkpoint modulator.

6. A method of treating or preventing cancer in a subject which comprises treating a subject with cancer with an immune checkpoint modulator, wherein the subject has been determined to have:

(i) a higher number of clonal neo-antigens; and/or

(ii) a higher ratio of clonal:sub-clonal neo-antigens, or lower (or low) sub-clonal neo-antigen fraction;

in comparison to a reference sample.

7. An immune checkpoint modulator when used in a method of treatment or prevention of cancer in a subject, the method comprising:

i) identifying a subject with cancer who is suitable for treatment with an immune checkpoint modulator according to the method of any one of claims 1 to 3; and

ii) treating said subject with an immune checkpoint modulator.

8. An immune checkpoint modulator when used in the treatment or prevention of cancer in a subject, wherein the subject has:

(i) a higher number of clonal neo-antigens; and/or

(ii) a higher ratio of clonal:sub-clonal neo-antigens, or lower (or low) sub-clonal neo-antigen fraction;

in comparison to a reference sample.

9. Use of an immune checkpoint modulator in preparation of a medicament for treatment or prevention of cancer in a subject, wherein the subject has:

(i) a higher number of clonal neo-antigens; and/or

(ii) a higher ratio of clonal:sub-clonal neo-antigens, or lower (or low) sub-clonal neo-antigen fraction;

in comparison to a reference sample.

10. The method, immune checkpoint modulator or use according to any one of claims 6, 8 or 9, wherein the subject further has a differential immune checkpoint molecule expression in comparison to a reference sample.

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11. The method, immune checkpoint modulator or use according to any one of the preceding claims, wherein the immune checkpoint modulator interacts with CTLA4, PD-1, PD-L1, Lag-3, Tim-3, TIGIT or BTLA.

12. The method, immune checkpoint modulator or use according to claim 11 wherein the immune checkpoint modulator is pembrolizumab, nivolumab, atezolizumab or ipilimumab.

13. The method, immune checkpoint modulator or use according to any one of the preceding claims, wherein the cancer is selected from bladder cancer, gastric cancer, oesophageal cancer, breast cancer, colorectal cancer, cervical cancer, ovarian cancer, endometrial cancer, kidney cancer (renal cell), lung cancer (small cell, non-small cell and mesothelioma), brain cancer (gliomas, astrocytomas, glioblastomas), melanoma, lymphoma, small bowel cancers (duodenal and jejunal), leukemia, pancreatic cancer, hepatobiliary tumours, germ cell cancers, prostate cancer, head and neck cancers, thyroid cancer and sarcomas.

14. The method, immune checkpoint modulator or use according to claim 13 wherein the cancer is lung cancer or melanoma.

15. The method, immune checkpoint modulator or use according to claim 14 wherein the cancer is non-small cell lung cancer (NSCLC).

16. The method, immune checkpoint modulator or use according to any one of the preceding claims, wherein the subject is a mammal, preferably a human, cat, dog, horse, donkey, sheep, pig, goat, cow, mouse, rat, rabbit or guinea pig.

17. The method, immune checkpoint modulator or use according to claim 16 wherein the subject is a human.

FIGURE 1A

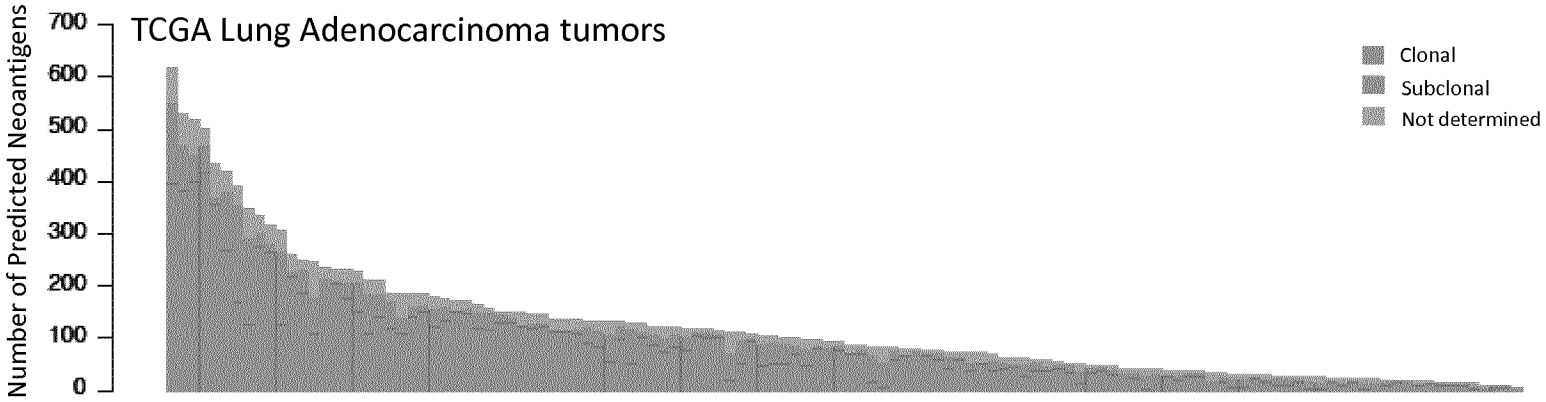

TCGA Lung Adenocarcinoma tumors
Number of Predicted Neoantigens
700
600
500
400
300
200
100
0
Clonal
Subclonal
Not determined

FIGURE 1

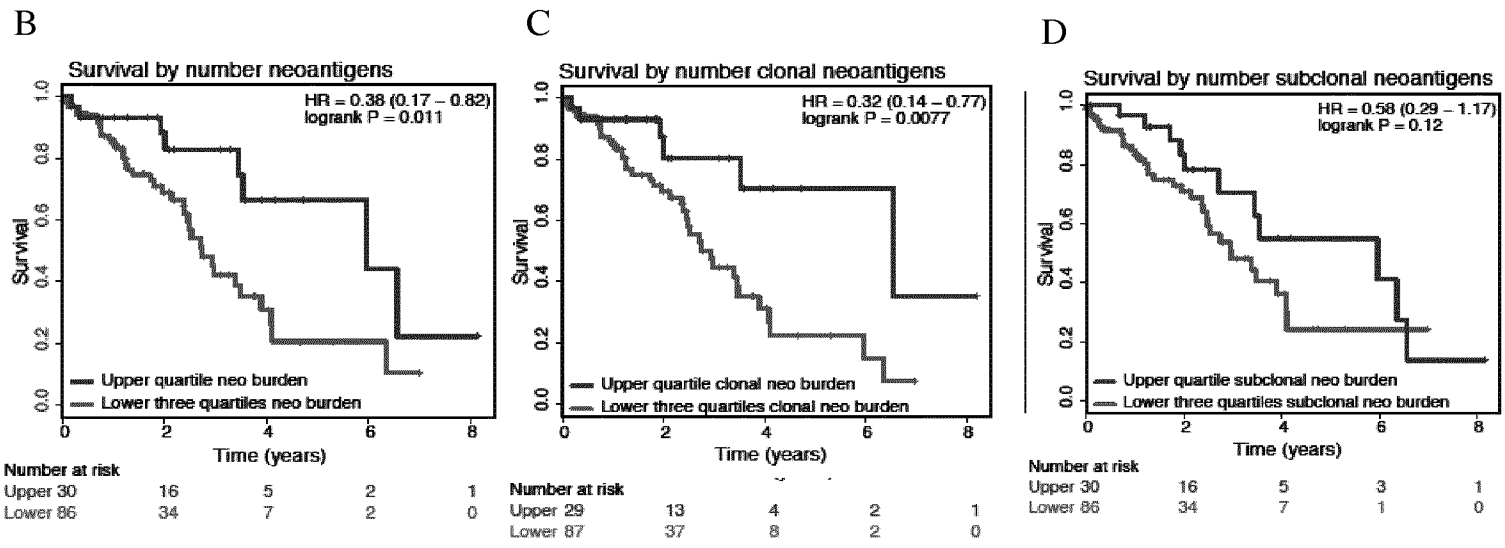

B
Survival by number neoantigens
HR = 0.38 (0.17 – 0.82)
logrank P = 0.011
Survival
Upper quartile neo burden
Lower three quartiles neo burden
Time (years)
Number at risk
Upper 30 16 5 2 1
Lower 86 34 7 2 0
C
Survival by number clonal neoantigens
HR = 0.32 (0.14 – 0.77)
logrank P = 0.0077
Survival
Upper quartile clonal neo burden
Lower three quartiles clonal neo burden
Time (years)
Number at risk
Upper 29 13 4 2 1
Lower 87 37 8 2 0
D
Survival by number subclonal neoantigens
HR = 0.58 (0.29 – 1.17)
logrank P = 0.12
Survival
Upper quartile subclonal neo burden
Lower three quartiles subclonal neo burden
Time (years)
Number at risk
Upper 30 16 5 3 1
Lower 86 34 7 1 0

FIGURE 1E

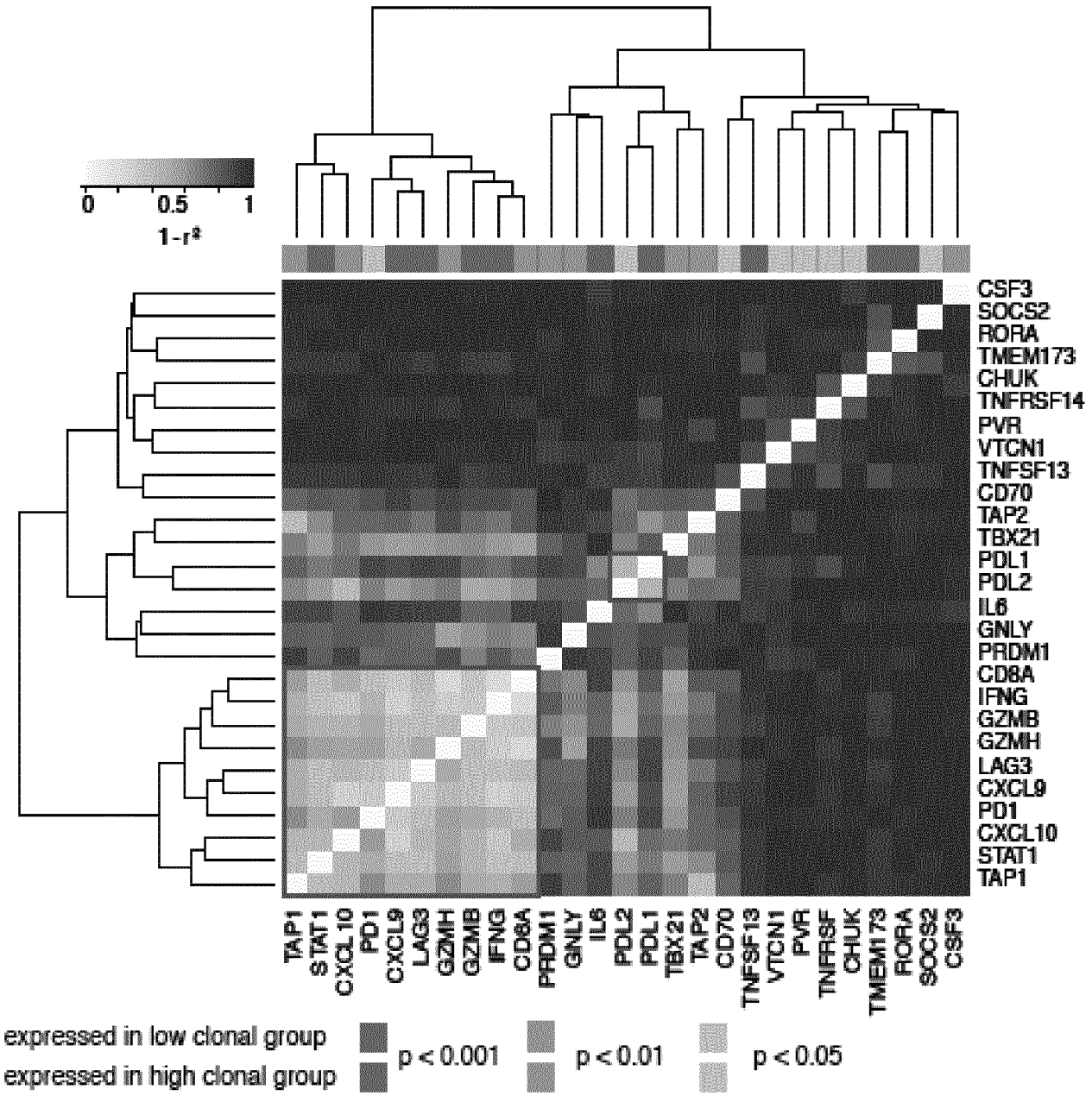
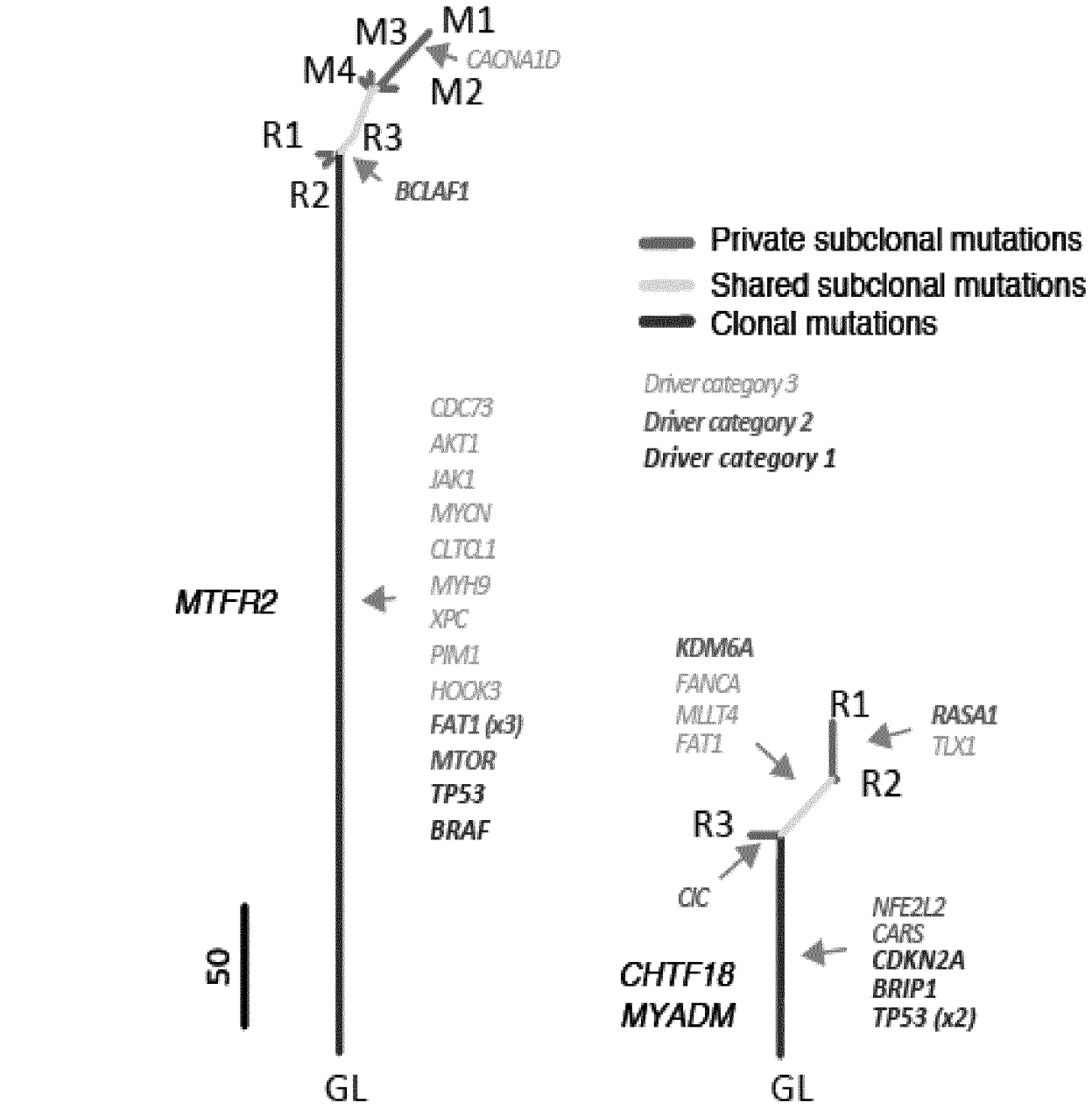

0
0.5
1
1-r²
CSF3
SOCS2
RORA
TMEM173
CHUK
TNFRSF14
PVR
VTCN1
TNFSF13
CD70
TAP2
TBX21
PDL1
PDL2
IL6
GNLY
PRDM1
CD8A
IFNG
GZMB
GZMH
LAG3
CXCL9
PD1
CXCL10
STAT1
TAP1
TAP1
STAT1
CXCL10
PD1
CXCL9
LAG3
GZMH
GZMB
IFNG
CD8A
PRDM1
GNLY
IL6
PDL2
PDL1
TBX21
TAP2
CD70
TNFSF13
VTCN1
PVR
TNFRSF
CHUK
TMEM173
RORA
SOCS2
CSF3
expressed in low clonal group
expressed in high clonal group
p < 0.001
p < 0.01
p < 0.05

FIGURE 2A



Patient	L011	L012
Age	49	69
Smoking status (pack-years)	Smoker (45)	Smoker (40)
Subtype	LUAD	LUSC
Subclonal fraction (primary tumor)	4%	44%

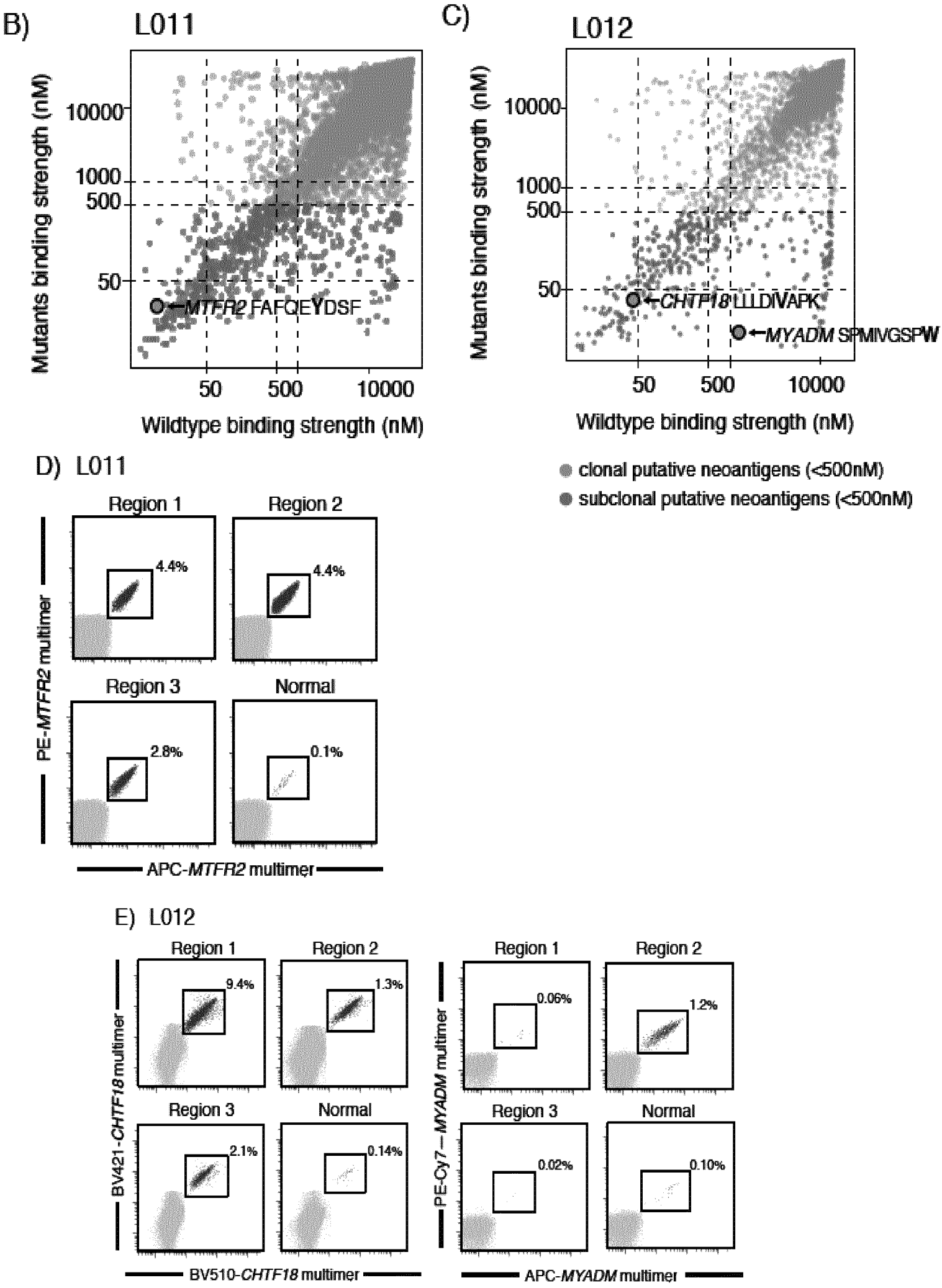

B)
L011
Mutants binding strength (nM)
10000
1000
500
50
MTFR2 FAFQEYDSF
50
500
10000
Wildtype binding strength (nM)
C)
L012
Mutants binding strength (nM)
10000
1000
500
50
CHTF18 LLLDIVAPK
MYADM SPMIVGSPW
50
500
10000
Wildtype binding strength (nM)
clonal putative neoantigens (<500nM)
subclonal putative neoantigens (<500nM)
D) L011
Region 1
4.4%
Region 2
4.4%
Region 3
2.8%
Normal
0.1%
PE-MTFR2 multimer
APC-MTFR2 multimer
E) L012
Region 1
9.4%
Region 2
1.3%
Region 3
2.1%
Normal
0.14%
BV421-CHTF18 multimer
BV510-CHTF18 multimer
Region 1
0.06%
Region 2
1.2%
Region 3
0.02%
Normal
0.10%
PE-Cy7—MYADM multimer
APC-MYADM multimer

FIGURE 3A

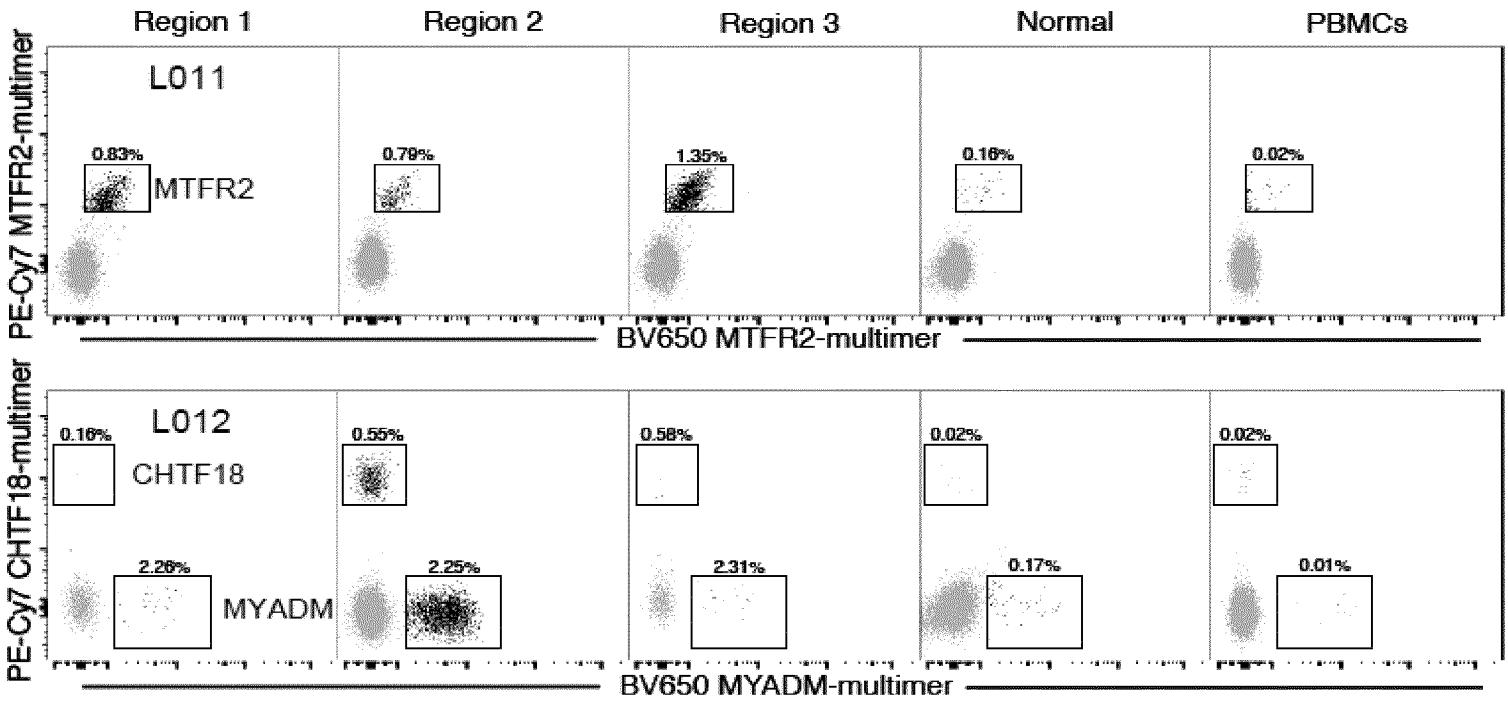

Region 1
Region 2
Region 3
Normal
PBMCs
L011
0.83%
MTFR2
0.78%
1.35%
0.16%
0.02%
PE-Cy7 MTFR2-multimer
BV650 MTFR2-multimer
L012
0.16%
CHTF18
0.55%
0.58%
0.02%
0.02%
2.26%
MYADM
2.25%
2.31%
0.17%
0.01%
PE-Cy7 CHTF18-multimer
BV650 MYADM-multimer

FIGURE 3B

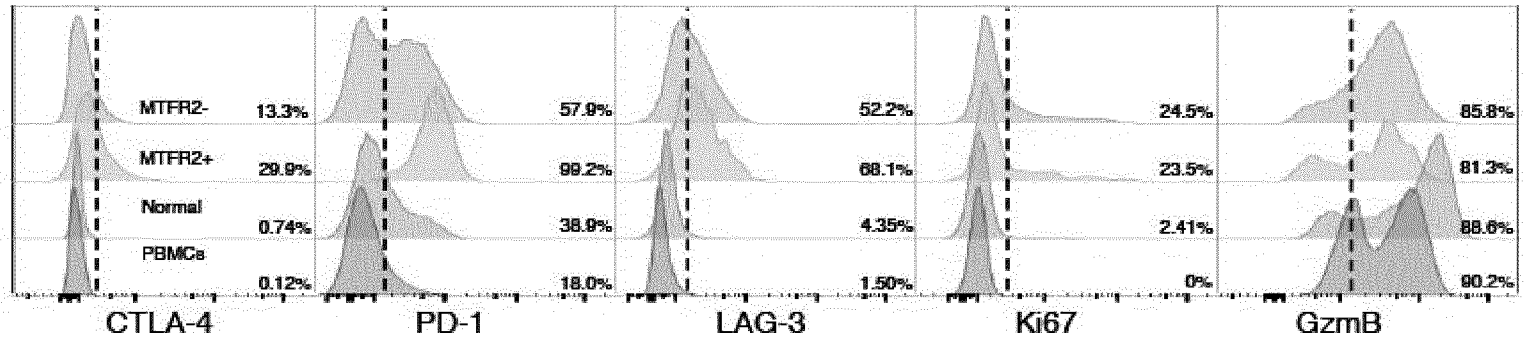
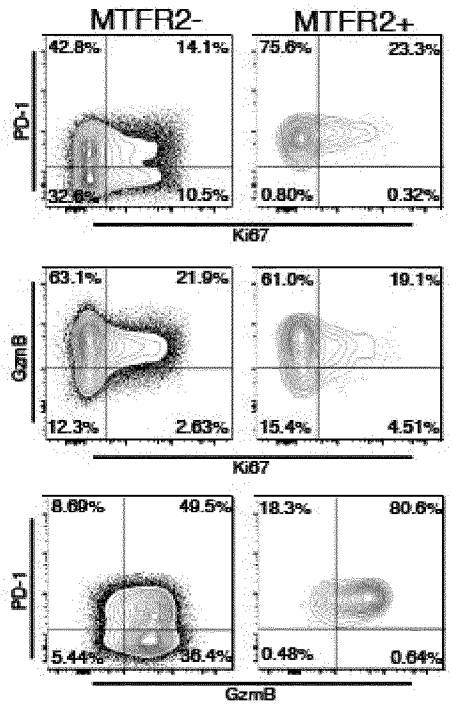

MTFR2-
MTFR2+
Normal
PBMCs
13.3%
29.9%
0.74%
0.12%
57.9%
99.2%
38.9%
18.0%
52.2%
68.1%
4.35%
1.50%
24.5%
23.5%
2.41%
0%
85.8%
81.3%
88.6%
90.2%
CTLA-4
PD-1
LAG-3
Ki67
GzmB

FIGURE 3

C


MTFR2-
MTFR2+
42.8%
14.1%
75.6%
23.3%
PD-1
32.6%
10.5%
0.80%
0.32%
Ki67
63.1%
21.9%
61.0%
19.1%
GzmB
12.3%
2.63%
15.4%
4.51%
Ki67
8.69%
49.5%
18.3%
80.6%
PD-1
5.44%
36.4%
0.48%
0.64%
GzmB

D

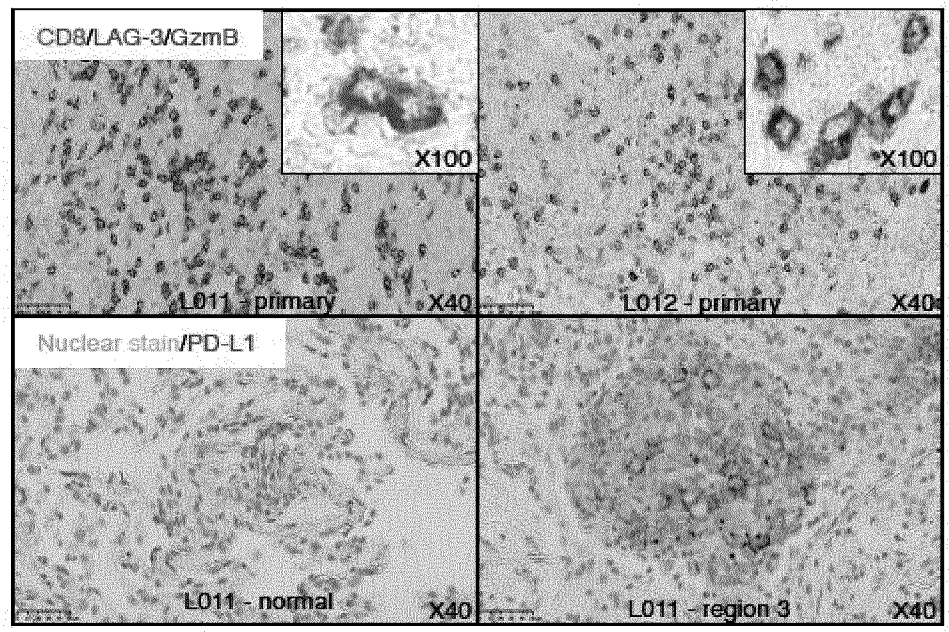
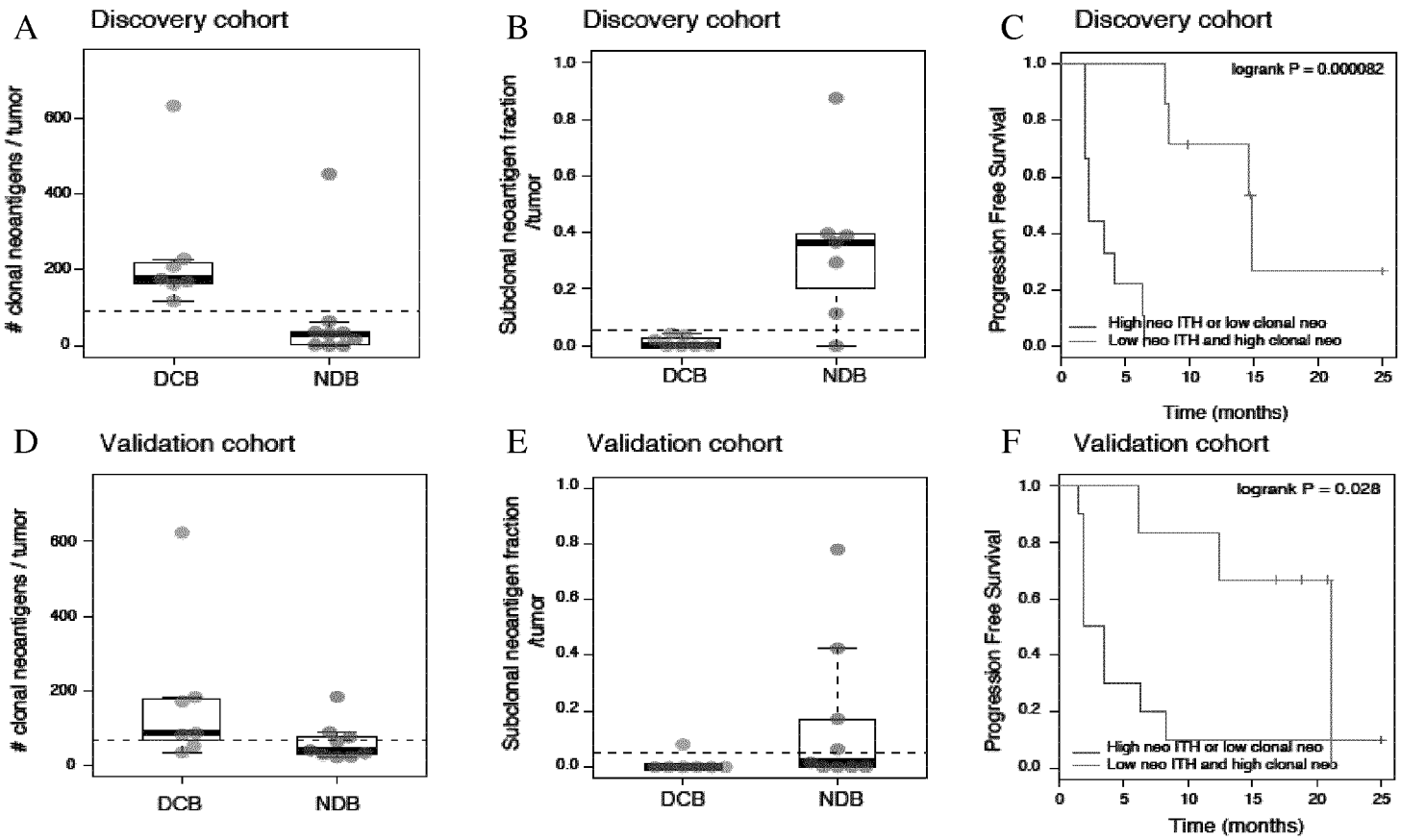

CD8/LAG-3/GzmB
X100
X100
L011 - primary
X40
L012 - primary
X40
Nuclear stain/PD-L1
L011 - normal
X40
L011 - region 3
X40

FIGURE 4


A
Discovery cohort
clonal neoantigens / tumor
600
400
200
0
DCB
NDB
B
Discovery cohort
Subclonal neoantigen fraction /tumor
1.0
0.8
0.6
0.4
0.2
0.0
DCB
NDB
C
Discovery cohort
logrank P = 0.000082
Progression Free Survival
1.0
0.8
0.6
0.4
0.2
0.0
High neo ITH or low clonal neo
Low neo ITH and high clonal neo
0
5
10
15
20
25
Time (months)
D
Validation cohort
clonal neoantigens / tumor
600
400
200
0
DCB
NDB
E
Validation cohort
Subclonal neoantigen fraction /tumor
1.0
0.8
0.6
0.4
0.2
0.0
DCB
NDB
F
Validation cohort
logrank P = 0.028
Progression Free Survival
1.0
0.8
0.6
0.4
0.2
0.0
High neo ITH or low clonal neo
Low neo ITH and high clonal neo
0
5
10
15
20
25
Time (months)

10/25

FIGURE 4

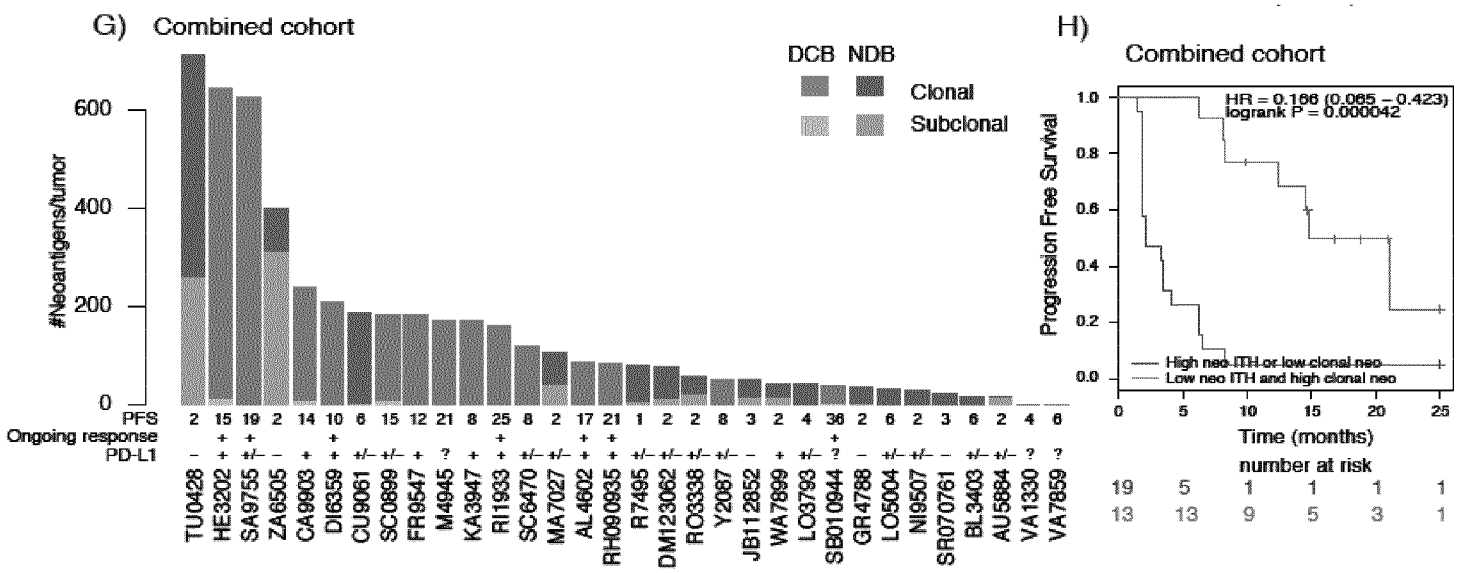
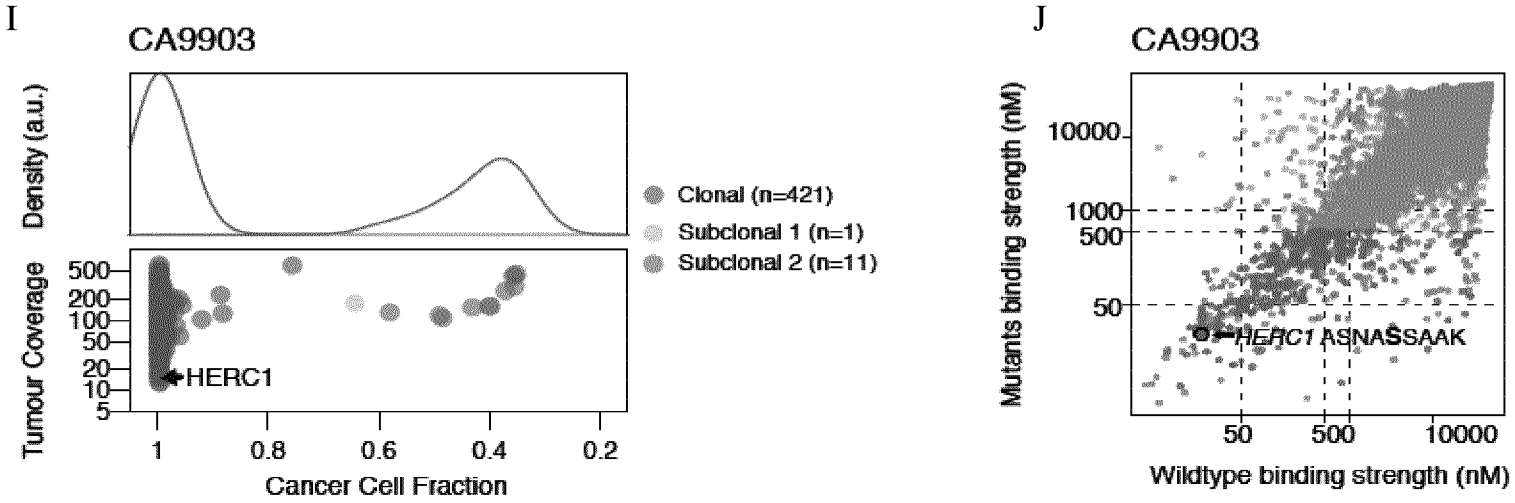
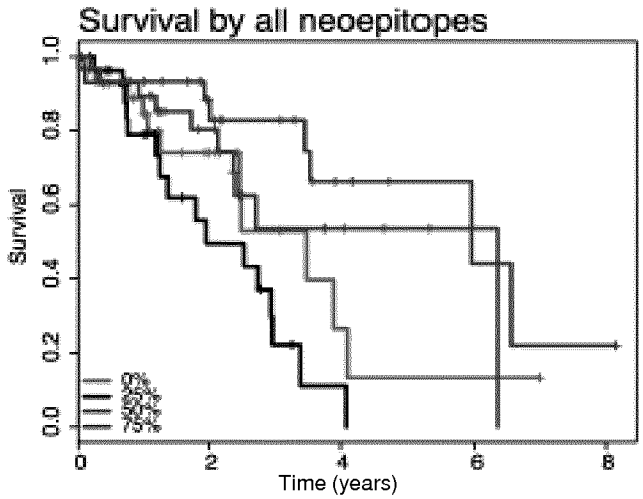


FIGURE 4


I
CA9903
Density (a.u.)
Tumour Coverage
500
200
100
50
20
10
5
HERC1
1
0.8
0.6
0.4
0.2
Cancer Cell Fraction
Clonal (n=421)
Subclonal 1 (n=1)
Subclonal 2 (n=11)
J
CA9903
Mutants binding strength (nM)
10000
1000
500
50
HERC1 ASNASSAAK
50
500
10000
Wildtype binding strength (nM)

12/25

FIGURE 5A



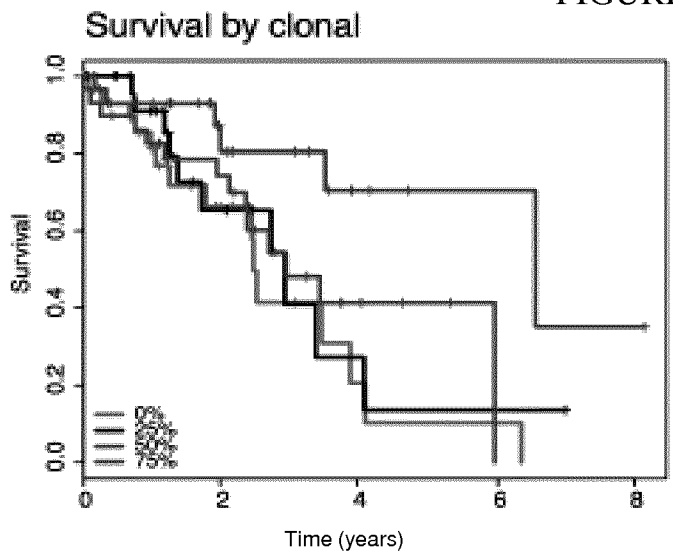
75%-50%	log-rank P = 0.22
75%-25%	log-rank P = 7.4e-05
75%-0%	log-rank P = 0.064
50%-25%	log-rank P = 0.026
50%-0%	log-rank P = 0.45
25%-0%	log-rank P = 0.1

Number at risk

0%	29	11	2	1	0
25%	**28**	**8**	**1**	**0**	**0**
50%	29	15	4	1	0
75%	30	16	5	2	1

13/25

FIGURE 5B



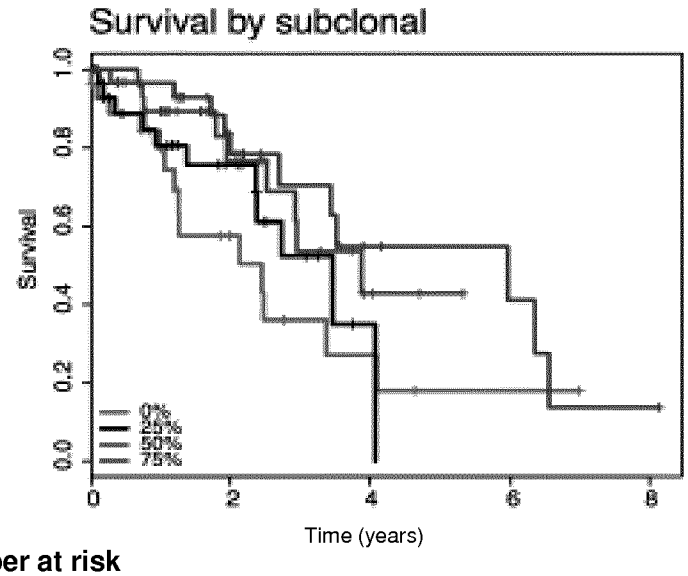
75%-50%	log-rank P = 0.041
75%-25%	log-rank P = 0.036
75%-0%	log-rank P = 0.0027
50%-25%	log-rank P = 0.79
50%-0%	log-rank P = 0.47
25%-0%	log-rank P = 0.54

Number at risk

0%	30	11	2	1	0
25%	**28**	**9**	**2**	**1**	**0**
50%	29	17	4	0	0
75%	29	13	4	2	1

14/25

FIGURE 5C



75%-50%	log-rank P = 0.52
75%-25%	log-rank P = 0.1
75%-0%	log-rank P = 0.031
50%-25%	log-rank P = 0.28
50%-0%	log-rank P = 0.056
25%-0%	log-rank P = 0.47

Number at risk

0%	27	9	3	1	0
25%	**29**	**13**	**1**	**0**	**0**
50%	30	12	3	0	0
75%	30	16	5	3	1

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FIGURE 6

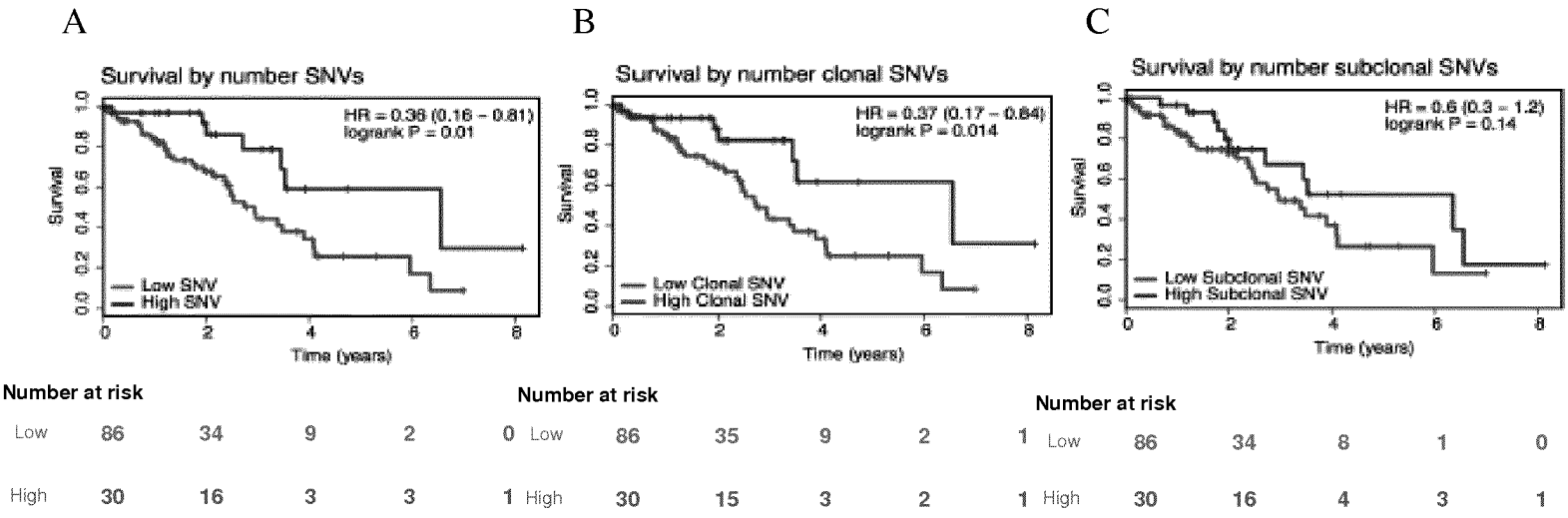

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FIGURE 7A

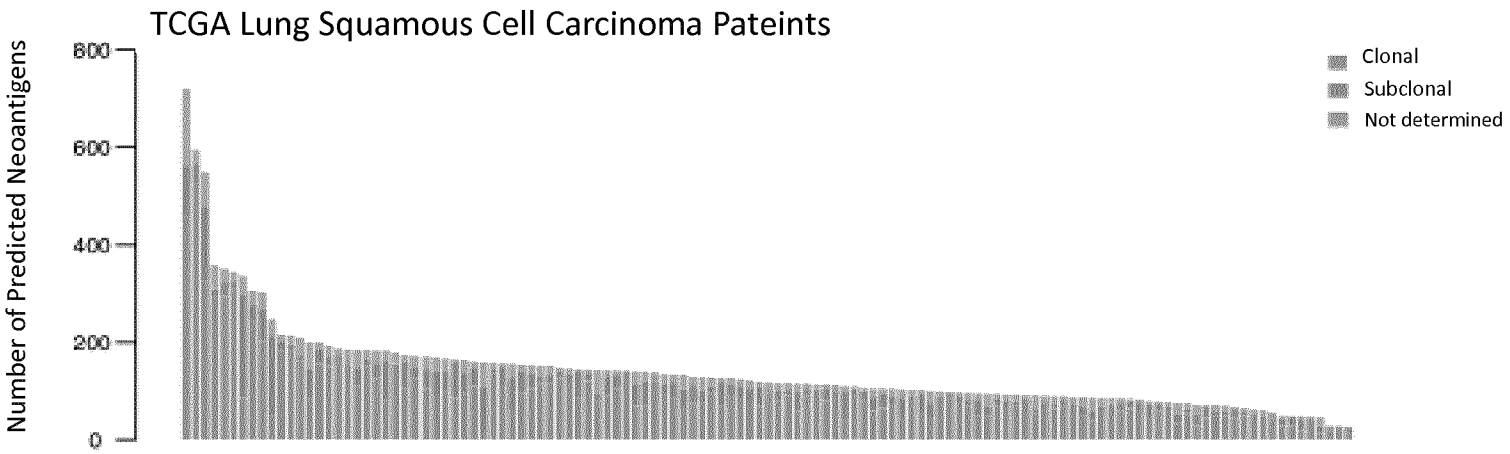

FIGURE 7

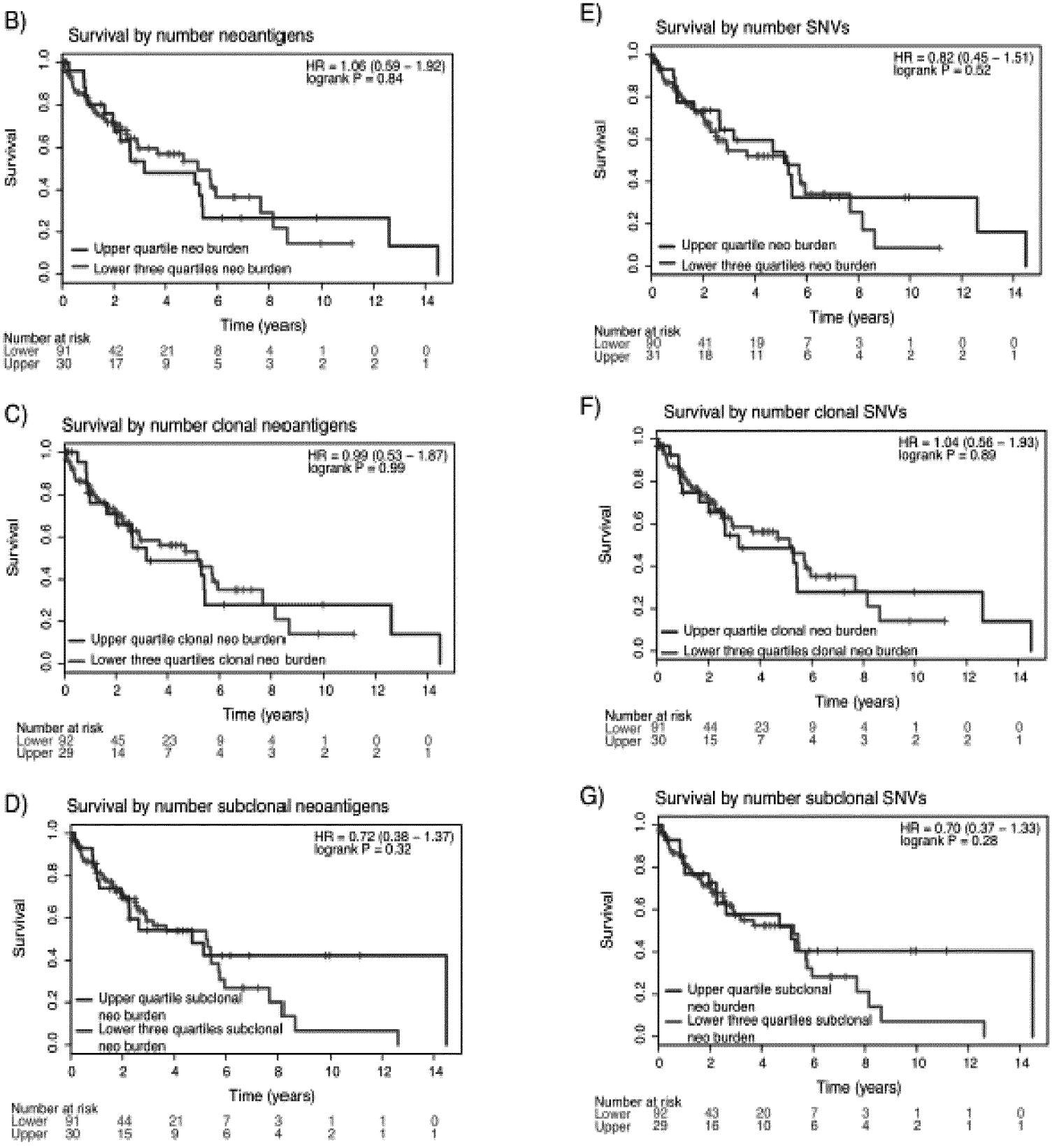
B)
Survival by number neoantigens
HR = 1.06 (0.59 – 1.92)
logrank P = 0.84
Survival
Upper quartile neo burden
Lower three quartiles neo burden
Time (years)
Number at risk
Lower 91 42 21 8 4 1 0 0
Upper 30 17 9 5 3 2 2 1
C)
Survival by number clonal neoantigens
HR = 0.99 (0.53 – 1.87)
logrank P = 0.99
Survival
Upper quartile clonal neo burden
Lower three quartiles clonal neo burden
Time (years)
Number at risk
Lower 92 45 23 9 4 1 0 0
Upper 29 14 7 4 3 2 2 1
D)
Survival by number subclonal neoantigens
HR = 0.72 (0.38 – 1.37)
logrank P = 0.32
Survival
Upper quartile subclonal neo burden
Lower three quartiles subclonal neo burden
Time (years)
Number at risk
Lower 91 44 21 7 3 1 1 0
Upper 30 15 9 6 4 2 1 1
E)
Survival by number SNVs
HR = 0.82 (0.45 – 1.51)
logrank P = 0.52
Survival
Upper quartile neo burden
Lower three quartiles neo burden
Time (years)
Number at risk
Lower 90 41 19 7 3 1 0 0
Upper 31 18 11 6 4 2 2 1
F)
Survival by number clonal SNVs
HR = 1.04 (0.56 – 1.93)
logrank P = 0.89
Survival
Upper quartile clonal neo burden
Lower three quartiles clonal neo burden
Time (years)
Number at risk
Lower 91 44 23 9 4 1 0 0
Upper 30 15 7 4 3 2 2 1
G)
Survival by number subclonal SNVs
HR = 0.70 (0.37 – 1.33)
logrank P = 0.28
Survival
Upper quartile subclonal neo burden
Lower three quartiles subclonal neo burden
Time (years)
Number at risk
Lower 92 43 20 7 3 1 1 0
Upper 29 16 10 6 4 2 1 1

FIGURE 8

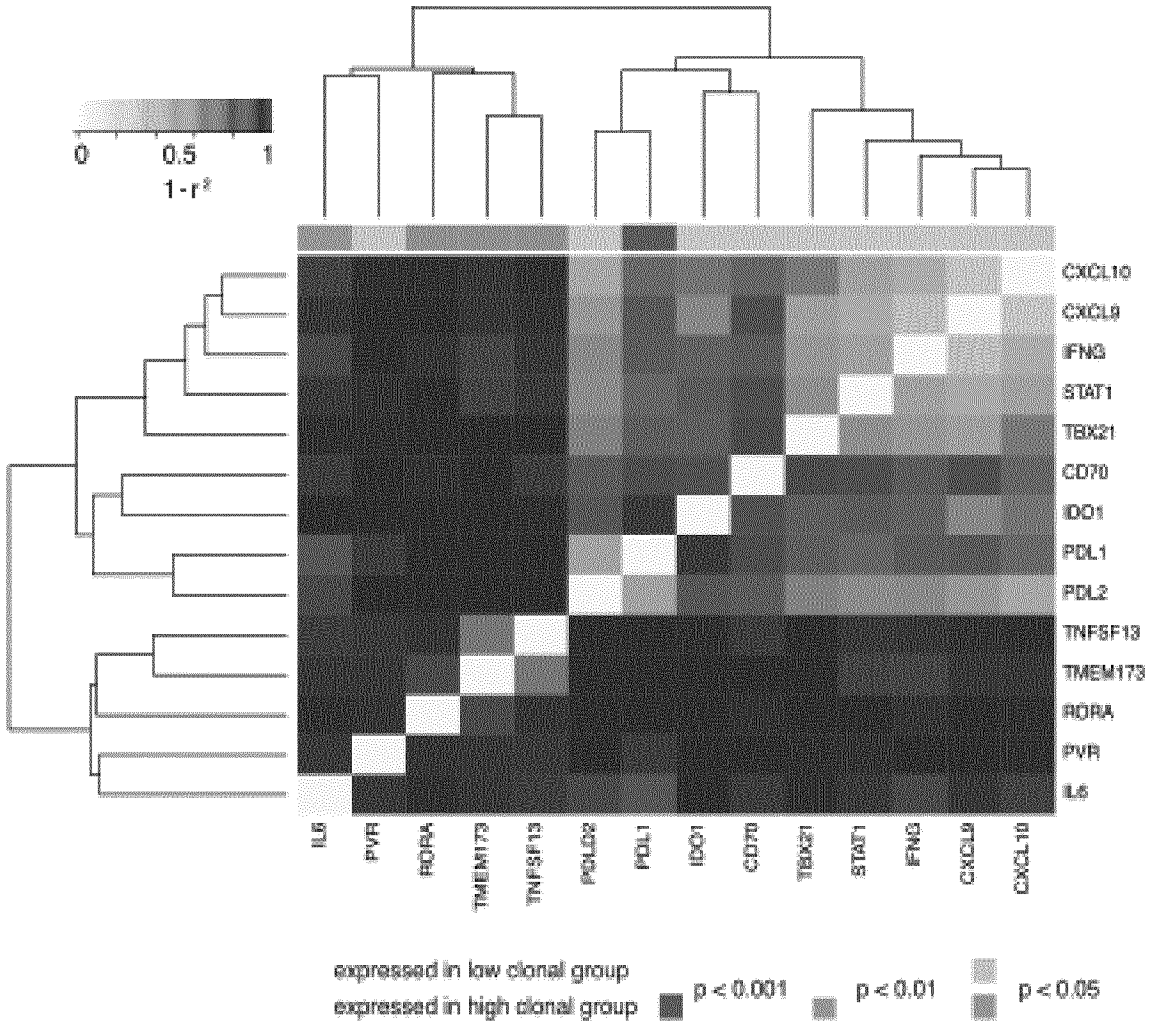
0
0.5
1
1-r²
CXCL10
CXCL9
IFNG
STAT1
TBX21
CD70
IDO1
PDL1
PDL2
TNFSF13
TMEM173
RORA
PVR
IL6
IL6
PVR
RORA
TMEM173
TNFSF13
PDL2
PDL1
IDO1
CD70
TBX21
STAT1
IFNG
CXCL9
CXCL10
expressed in low clonal group
expressed in high clonal group
p < 0.001
p < 0.01
p < 0.05

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FIGURE 9A

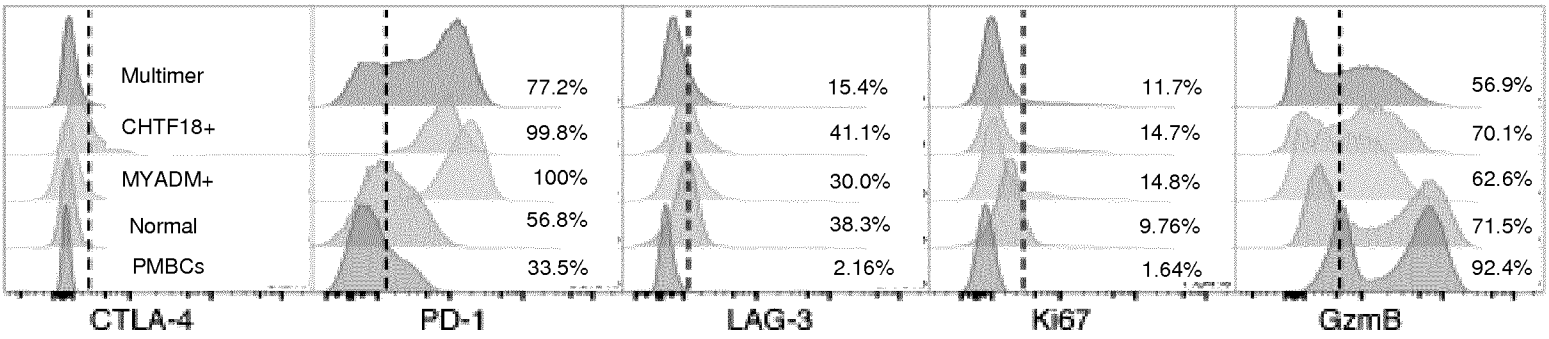

FIGURE 9B

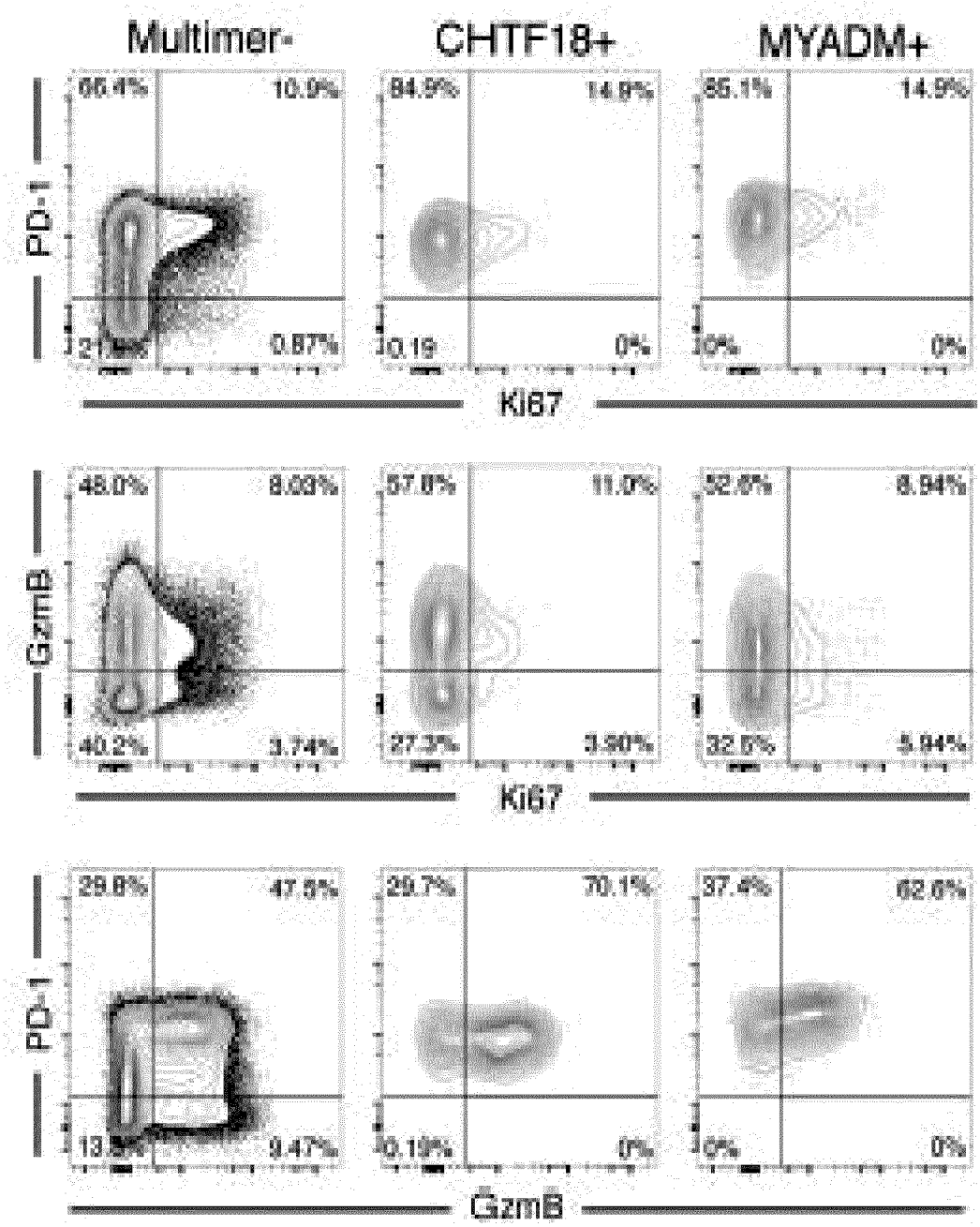

FIGURE 9

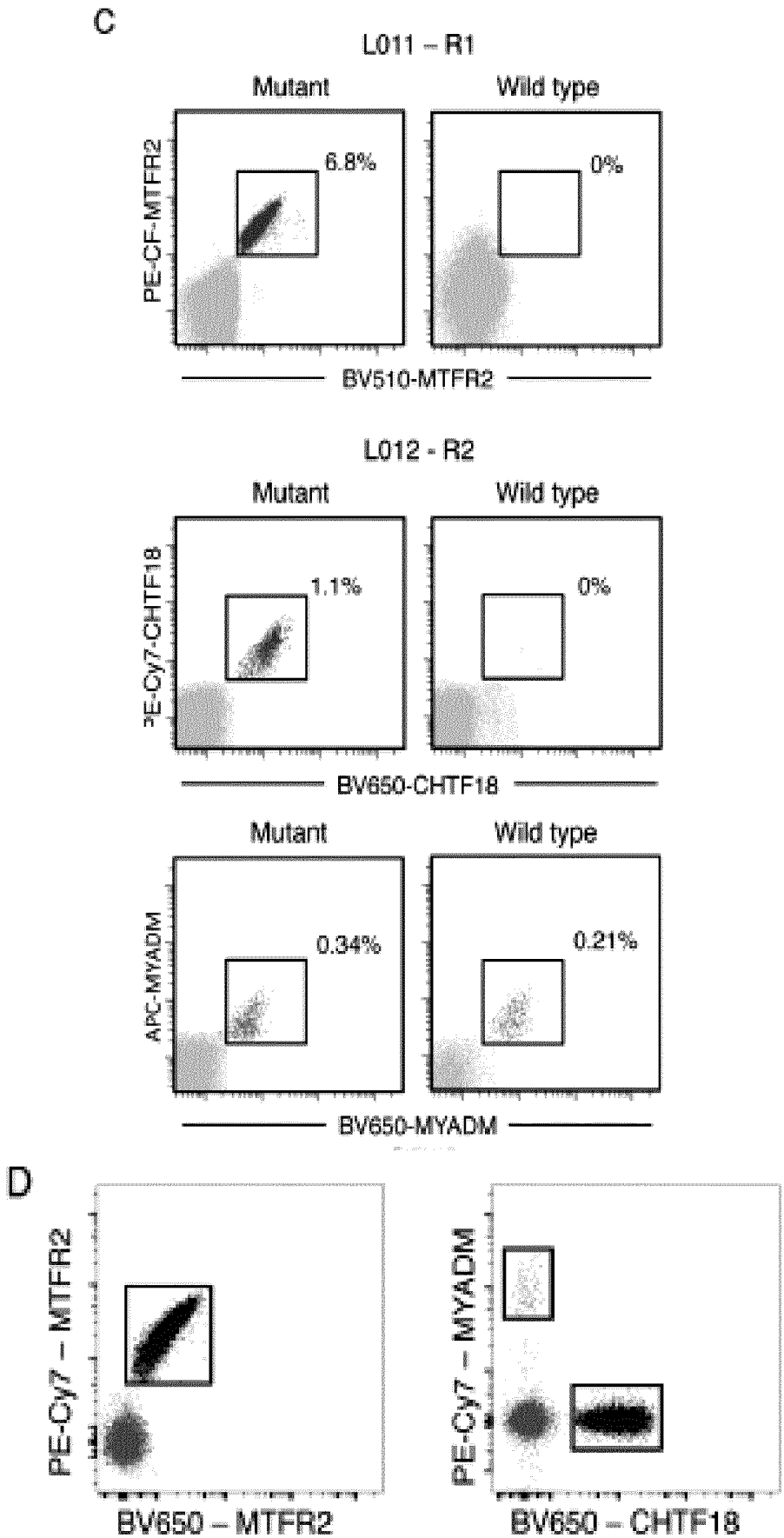
C
L011 – R1
Mutant
Wild type
6.8%
0%
PE-CF-MTFR2
BV510-MTFR2
L012 - R2
Mutant
Wild type
1.1%
0%
PE-Cy7-CHTF18
BV650-CHTF18
Mutant
Wild type
0.34%
0.21%
APC-MYADM
BV650-MYADM
D
PE-Cy7 – MTFR2
BV650 – MTFR2
PE-Cy7 – MYADM
BV650 – CHTF18

FIGURE 10

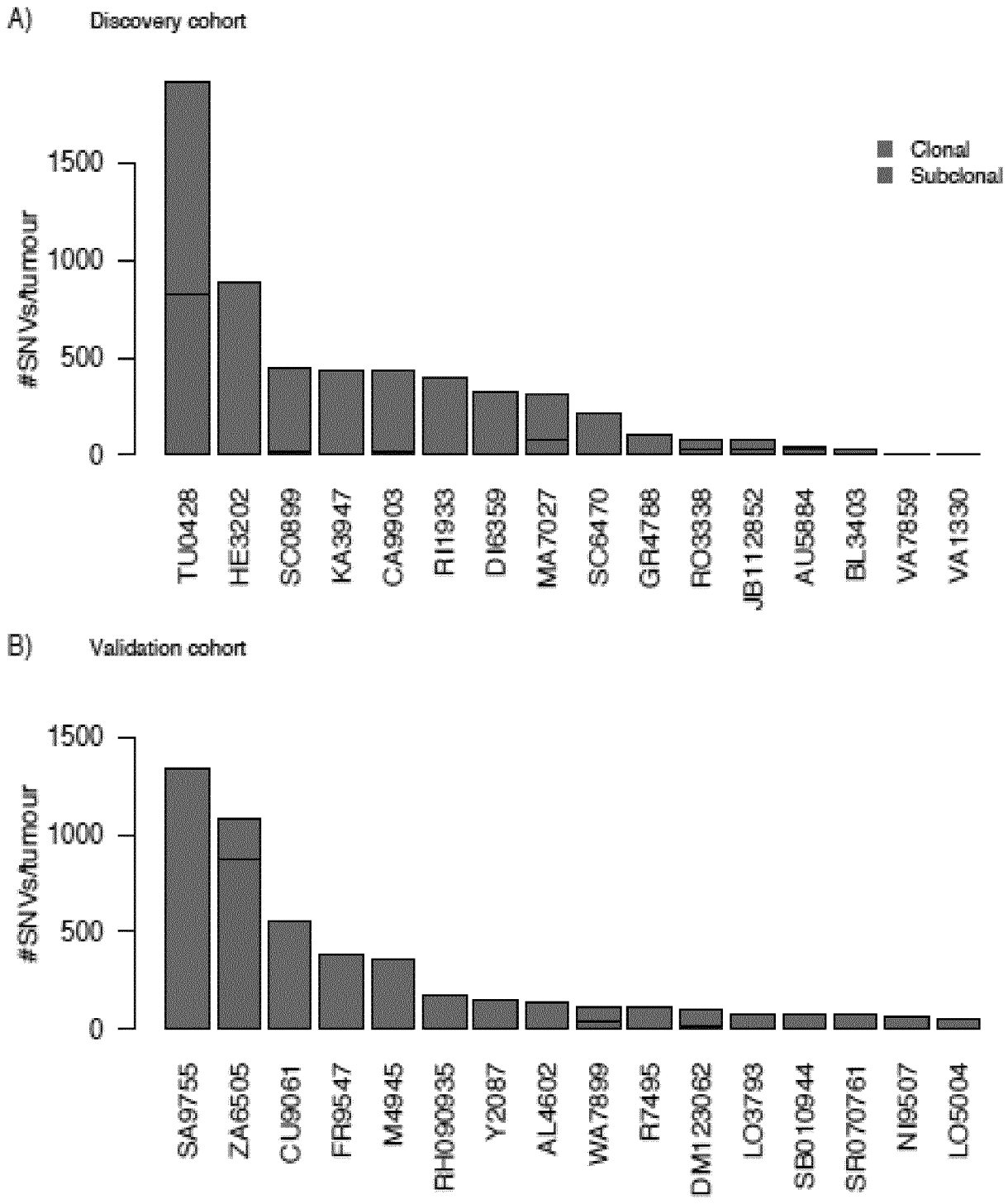
A)
Discovery cohort
#SNVs/tumour
1500
1000
500
0
Clonal
Subclonal
TU0428
HE3202
SC0899
KA3947
CA9903
RI1933
DI6359
MA7027
SC6470
GR4788
RO3338
JB112852
AU5884
BL3403
VA7859
VA1330
B)
Validation cohort
#SNVs/tumour
1500
1000
500
0
SA9755
ZA6505
CU9061
FR9547
M4945
RH090935
Y2087
AL4602
WA7899
R7495
DM123062
LO3793
SB010944
SR070761
NI9507
LO5004

FIGURE 11

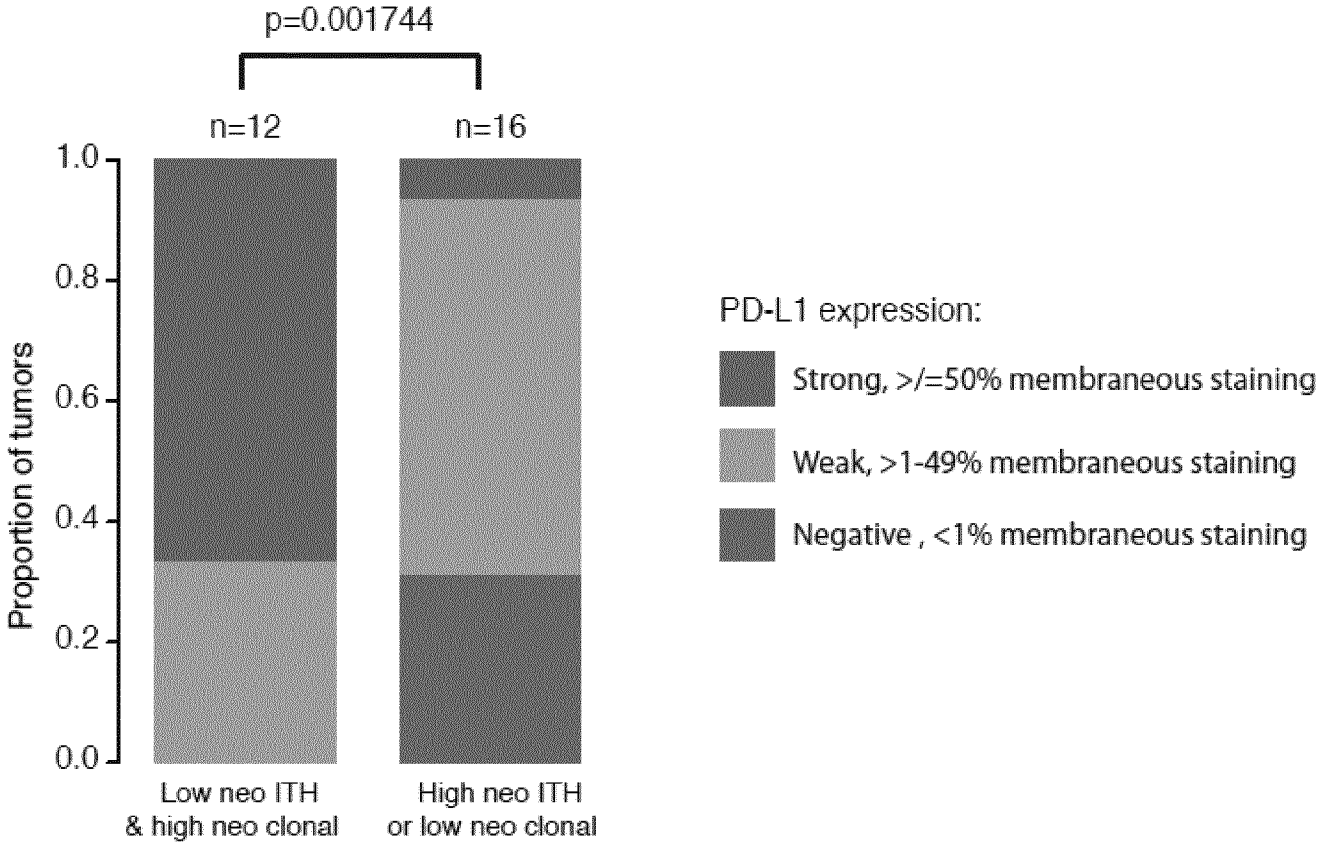
p=0.001744
n=12
n=16
1.0
0.8
0.6
0.4
0.2
0.0
Proportion of tumors
Low neo ITH
& high neo clonal
High neo ITH
or low neo clonal
PD-L1 expression:
Strong, >/=50% membraneous staining
Weak, >1-49% membraneous staining
Negative , <1% membraneous staining

FIGURE 12

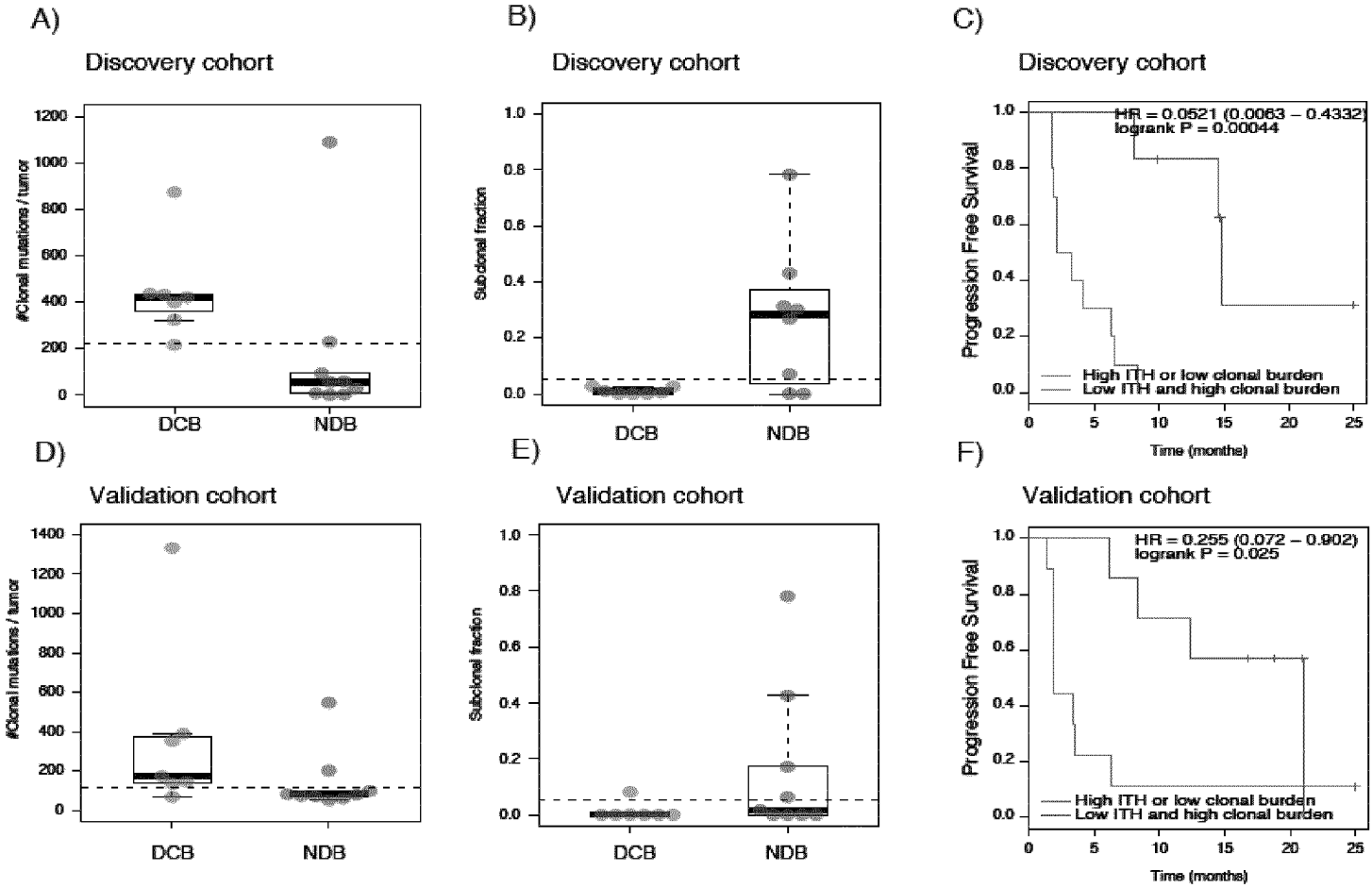
A)
Discovery cohort
#Clonal mutations / tumor
1200
1000
800
600
400
200
0
DCB
NDB
B)
Discovery cohort
Subclonal fraction
1.0
0.8
0.6
0.4
0.2
0.0
DCB
NDB
C)
Discovery cohort
HR = 0.0521 (0.0063 – 0.4332)
logrank P = 0.00044
Progression Free Survival
1.0
0.8
0.6
0.4
0.2
0.0
High ITH or low clonal burden
Low ITH and high clonal burden
0
5
10
15
20
25
Time (months)
D)
Validation cohort
#Clonal mutations / tumor
1400
1200
1000
800
600
400
200
0
DCB
NDB
E)
Validation cohort
Subclonal fraction
1.0
0.8
0.6
0.4
0.2
0.0
DCB
NDB
F)
Validation cohort
HR = 0.255 (0.072 – 0.902)
logrank P = 0.025
Progression Free Survival
1.0
0.8
0.6
0.4
0.2
0.0
High ITH or low clonal burden
Low ITH and high clonal burden
0
5
10
15
20
25
Time (months)

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FIGURE 12