Title: Genomic profiling of plasma circulating tumor DNA reveals genetics and residual disease in extranodal NK/T-cell lymphoma

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Abstract

Extranodal NK/T-cell lymphoma, nasal type (ENTKL), is an aggressive hematological malignancy with poor prognosis. Early detection of tumors at initial diagnosis or during routine surveillance is important for improving survival outcomes. Molecular profiling of circulating tumor DNA (ctDNA) is a promising noninvasive tool for monitoring disease status. Here, we assessed the gene mutation spectrum of plasma ctDNA in ENKTL by cancer personalized profiling sequencing (CAPP-Seq). We found that the most frequently mutated genes were *MGA* (33.3%), *TP53* (30%), *ASXL3* (28.3%), *DDX3X* (25%), *BCORL1* (25%), *TRAF3* (21.7%), *NOTCH3* (21.7%), *STAT5B* (21.7%), *EP300* (21.7%), *APC* (20%), *ATM* (18.3%), *TNFRSF14* (16.7%), *KMT2D* (16.7%), *EZH2* (16.7%) and *SETD2* (16.7%). The mutation frequencies of *MGA* and *TP53* were significantly higher in stage III-IV, and mutations in *MGA*, *TP53*, and *NOTCH3*, *et al* were significantly correlated with the metabolic tumor burden of the patients. Compared with tumor tissue DNA, ctDNA profiling showed good concordance. Serial ctDNA analysis showed that treatment with chemotherapy could decrease the number and mutation allele frequency of genes. Compared with PET/CT, ctDNA has more advantages for tracking residual disease in patients. In addition, we also found that mutated *MGA*, *TP53*, *SETD2*, and *APC* predicted poor prognosis in patients. Collectively, our results provide evidence that ctDNA may serve as a novel precision medicine biomarker in ENKTL.

Keywords: ENKTL, circulating tumor DNA, mutation allele frequency, minimal residual disease, prognosis.

Introduction

Extranodal NK/T-cell lymphoma, nasal type (ENTKL), is an aggressive extranodal lymphoma of NK-cell or T-cell lineage, which is highly aggressive and has heterogenicity, with predominance in males(Suzuki, 2014; Tse & Kwong, 2013; Xiong & Zhao, 2019). Current treatment strategies (such as combination chemotherapy and radiotherapy and targeted therapy) can improve the complete remission of patients, but most of them will ultimately relapse and progress(Au et al, 2009; Kwong et al, 2012). For these patients, standard tests, including computed tomography (CT) and serum protein markers at initial diagnosis and during routine surveillance, are pivotal for therapeutic evaluation; however, these tests have limitations and low sensitivity and specificity or false positivity. Therefore, there is an urgent need for a highly sensitive, standardized and noninvasive assay that can be used for the accurate detection of early relapse and/or progression of disease.

Current treatment response criteria for non-Hodgkin lymphoma (non-HL) rely on CT scans or positron emission tomography (PET) scans. Imaging scans at initial diagnosis or recurrence can provide macro overviews of tumor volume and location, but CT scans have some limitations due to cost and radiation exposure, and PET lacks the specificity(Adams et al, 2015; Kim et al, 2015; Trotman et al, 2014). Moreover, imaging scans cannot monitor dynamic tumor response and the clonal evolution process. Therefore, clinically validated technology is needed to overcome the current monitoring treatment response. One potential biomarker is the detection of circulating cell-free DNA (cfDNA), which comes from dying cells that release DNA fragments into the circulation, and in tumor patients, some of the cfDNA primarily originates from apoptotic and necrotic cancer cells, which carry tumor-specific alterations (named circulating tumor DNA, ctDNA)(Bettegowda et al, 2014; Crowley et al, 2013; Forshew et al, 2012; Siravegna et al, 2017). ctDNA sequencing in plasma is a promising tool for real-time monitoring of tumor progression, and its application has been explored in multiple solid tumors. ctDNA can reflect the temporal evolution of

tumors, so current studies have shown that ctDNA detection can be used to monitor minimal residual disease and track recurrence after treatment(Esposito et al, 2016; Zhou et al, 2016). ctDNA sequencing is a noninvasive and tumor-specific assay, and ctDNA in circulation has a half-life of less than two hours, so ctDNA is more sensitive than protein biomarkers and more specific than routine surveillance imaging with CT scans or PET scans(Chen et al, 2018). Thus, ctDNA might become an important marker in the molecular diagnosis of tumors.

Clinical application of ctDNA has been performed in multiple types of lymphoma, such as diffuse large B-cell lymphoma (DLBCL), classical HL, follicular lymphoma and peripheral T-cell lymphoma(Delfau-Larue et al, 2018; Roschewski et al, 2015; Scherer et al, 2016; Spina et al, 2018; Suehara et al, 2018) by using immunoglobulin next-generation sequencing (Ig-NGS), T-cell receptor gene next-generation sequencing (TCR-NGS) and cancer personalized profiling sequencing (CAPP-Seq). ctDNA assessment is a prognostic tool used prior to treatment that acts as a dynamic marker of burden during treatment. Although most studies evaluating ctDNA assessment in lymphoma focused on B-cell lymphomas and HL, recent evidence of ctDNA assessment in peripheral T-cell lymphoma by TCR-NGS suggested that ctDNA detection in T-lineage lymphoma is feasible(Melani et al, 2017). In the present study, we thoroughly determined the molecular spectrum of ctDNA mutations in ENTKL and characterized the relationship between mutations in ctDNA and clinical factors. 6 paired tumor tissues and peripheral blood samples were collected to compare the concordance of tumor DNA and ctDNA. Moreover, dynamic ctDNA mutation alterations during the treatment were also observed.

Results

Patient characteristics

To examine the feasibility of ctDNA detection in plasma using CAPP-Seq, from February 2017 to September 2019, 60 newly diagnosed ENKTL patients were

recruited, and initial and longitudinal plasma specimens were obtained from patients undergoing chemotherapy (Fig. 1). The median age at first blood draw was 46 years (range, 40 to 55 years), and 41 patients were men. At disease onset, 24 patients (40%) exhibited B symptoms. According to the Ann Arbor staging criteria, 13 patients were identified as stage I, 17 patients were identified as stage II, 13 patients were identified as stage III, and 17 patients were identified as stage IV (Table 1). ctDNA was successfully extracted from all patients and healthy individuals, and the average concentration of cfDNA was 0.87 ng/μl (range, 0.49 to 1.31 ng/μl).

ctDNA mutation spectrum of newly diagnosed ENKTL

Of the 60 patients recruited in this study, three or more somatic mutations were detected in the plasma of 54 (90%) patients with a median of 7 per sample (range, 2 to 27) (Fig. 2, upper panel), and no mutations were found in the healthy control blood. The range of mutant allele frequencies in each gene of the sample is shown in Table S1. The most frequently mutated genes were *MGA* (33.3%), *TP53* (30%), *ASXL3* (28.3%), *DDX3X* (25%), *BCORL1* (25%), *TRAF3* (21.7%), *NOTCH3* (21.7%), *STAT5B* (21.7%), *EP300* (21.7%), *APC* (20%), *ATM* (18.3%), *TNFRSF14* (16.7%), *KMT2D* (16.7%), *EZH2* (16.7%) and *SETD2* (16.7%). Other genes, such as *KDM6A*, *ZNF608*, and *ARID1A*, were mutated less frequently (<10% of the patients) than the above genes (Fig. 2, middle panel). Consistent with the somatic SNV spectrum in other tumors, we found that C>T/G>A was a preferred alteration (Fig. 2, lower panel).

Correlation of detectable ctDNA with clinical characteristics of ENKTL patients

To assess the concordance of the ctDNA test with the tissue NGS test, 6 paired tumor biopsies were genotyped. Compared with the results of the plasma ctDNA spectrum, most of the genes overlapped (Fig. 3A). In 3 patients, biopsy-confirmed tumor mutations were all detectable in ctDNA samples (Fig. 3B), but in patient #23, *TP53* variation was not detected in tumor DNA, and *KMT2D* variation could not be detected

in plasma ctDNA. In patient #8, *TRAF3* could not be detected in plasma ctDNA (Fig. 3C). These differences might be caused by tumor anatomical heterogeneity or tumor-associated stromal tissue infiltration.

To validate the potential clinical utility of mutations detected in plasma ctDNA, we investigated the association between clinical factors and ctDNA levels. The ctDNA concentration of patients with stage III-IV (0.96±0.24 ng/ul) was significantly higher than that of patients with stage I-II (0.79±0.24 ng/ul) (P=0.01), and no significant difference was obtained in patients with relapse and without relapse (Fig. 4A). As MTV measured from PET/CT could quantitatively reflect disease burden, we also found that the plasma ctDNA concentration was significantly correlated with MTV (P<0.001) (Fig. 4B), suggesting that ctDNA could be identified as a promising biomarker for tumor stage and burden. In patients with stage III-IV disease, the mutation frequencies of *MGA* (14/30) and *TP53* (13/30) were significantly higher than that of patients with stage I-II disease. Interestingly, the mutation frequency of *DDX3X* (3/30) in patients with stage III-IV disease was significantly lower than that of patients with stage I-II disease (Fig. 4C). Regarding the remaining most frequently mutated genes, no significant differences were found between patients with stage I-II and III-IV disease. Additionally, the mutation allele frequencies of *MGA*, *TP53* and *DDX3X* were not correlated with patient stage (Fig. 4D). In patients with relapse and no relapse, the mutation frequency of *MGA, TP53, DDX3X* and *SETD2* showed significant differences (Fig. 4E). Furthermore, the correlation of the mutation status of each gene and metabolic tumor burden was investigated. We found that patients with mutations in *MGA, TP53, NOTCH3, DDX3X*, *APC*, *ATM*, *EZH2*, and *TNFRSF14* showed significantly higher metabolic tumor burden (Fig. 4F), suggesting that these genes might be positively correlated with disease malignancies.

serial ctDNA detection during therapy could complement the response assessment of the patients, and patients with mutated MGA, TP53, SETD2, and APC predicted

poor prognosis

We further investigated the potential role of plasma ctDNA in the therapeutic monitoring of patients with chemotherapy. Ten patients were recruited, and in one patient (#22), the number and mutation allele frequency of ctDNA detection decreased with chemotherapy. When patients finished 4 cycles of therapy, no tumor was found by PET/CT scan, but plasma ctDNA could detect a low frequency of *MGA* and *SETD2* mutations. With more cycles, patients could achieve complete molecular remission. Patient #23 achieved complete remission assessed by PET/CT, but the ctDNA status was still positive; therefore, auto-hematopoietic cell transplantation (HCT) was conducted. After these interventions were administered, the patient also achieved complete molecular remission (Fig. 5A). Fortunately, we have not yet observed a relapse among these recruited patients, so a long follow-up term should be performed to investigate the relapse/progression ctDNA spectrum. Next, the prognosis of ctDNA mutation status was also investigated. Patients with mutated *MGA, TP53, SETD2*, and *APC* had a shorter OS (Fig. 5B-E). No significant difference was observed in patients with other mutated genes (Fig. 5F-K), which might be due to the small sample recruited in this clinical trial. The results of univariate and multivariate analysis for risk factor of OS in patients were summarized in Tables 2. univariate analysis of factors revealed that stage, IPI scores, recurrence status, MTV, *MGA* mutation, *TP53* mutation and *SETD2* mutation were independent prognostic indicators of the overall survival of patients, and multivariate analysis showed that *MGA* mutation and *TP53* mutation was solely prognostic factor. All these results suggested that dynamic detection of ctDNA during therapy could better reflect disease status compared with PET/CT scan, and patients with mutated *MGA, TP53, SETD2*, and *APC* predicted poor prognosis.

Discussion

This study was the first to determine the plasma ctDNA mutation gene spectrum of ENKTL using CAPP-Seq and demonstrated that ctDNA sequencing could be

considered a promising biomarker for monitoring the minimal residual disease (MRD) of patients. The most frequently mutated genes in plasma ctDNA were *MGA* (33.3%), *TP53* (30%), *ASXL3* (28.3%), *DDX3X* (25%), *BCORL1* (25%), *TRAF3* (21.7%), *NOTCH3* (21.7%), *STAT5B* (21.7%), *EP300* (21.7%), *APC* (20%), *ATM* (18.3%), *TNFRSF14* (16.7%), *KMT2D* (16.7%), *EZH2* (16.7%) and *SETD2* (16.7%). ctDNA concentration was positively correlated with tumor stage and MTV. Consistent with tumor tissue, almost all of the gene mutations detected in plasma ctDNA could be found in tumor DNA, and further analysis demonstrated that a decreased number and MAF of certain genes in plasma ctDNA could complement the therapeutic response of the patients, and patients with mutated *MGA, TP53, SETD2* and *APC* had poor prognosis, suggesting that the gene mutations in plasma could serve as a promising biomarker for the diagnosis or monitoring of ENKTL disease courses.

Tumor biopsies can only offer information about lymphoma at one specific time in a specific area and can be accompanied with complications, and PET/CT may lead to false negatives due to technology limitations or false positive caused by tumor flares or pseudoprogression(Cheson et al, 2016). ctDNA detection is an optimal noninvasive and specific technique to overcome these traditional monitoring methods. A rapidly growing body of evidence has established that ctDNA detection during curative therapies can identify patients for whom there remains evidence of residual, radiographically occult cancer. In DLBCL, ctDNA can be used to predict tumor load and treatment outcome of newly diagnosed patients, and patients with a pretreatment ctDNA level above the median had a significantly poorer prognosis than those below the median(Kurtz et al, 2015). Later, Kurtz et al. reported that serial ctDNA measurements of 125 patients were an effective tool for prognostication, patients with 2-log or greater decreases in ctDNA had a better event-free survival (EFS) after one cycle of therapy, and patients with 2.5-log or greater reduction in ctDNA had a significantly better EFS after two cycle treatments. These studies suggested that serial ctDNA detection could be used to dynamically assess the therapy response and guide personalized target therapy. In another study, Daigle et al. found that mutation of

CREBBP in R/R DLBCL had the greatest association with tazemetostat treatment (an EZH2 inhibitor), and patients with mutations in *PIM1*, *BCL6*, *HIST1H1E* and *TP53* lacked response to treatment(Daigle et al, 2017). In HL, Valeria et al. determined the molecular mutation spectrum using ctDNA measurement. The most commonly mutated genes were *STAT*, *TNFAIP3* and *ITPKB*, and detection sensitivity was 87.5%, suggesting that ctDNA could reflect the spectrum of tumor tissue mutation well. Compared with newly diagnosed and refractory HL mutation profiles, most mutations overlapped, and mutations of *STAT*, *TNFAIP3*, *GNA13* and *ITPKB* might be an ancestral clone persisted throughout disease course(Spina et al, 2018). Although only a few studies have focused on assessing ctDNA in T-cell lymphoma, preliminary evidence has also demonstrated that ctDNA measurement in TCL is feasible(Melani et al, 2017; Sakata-Yanagimoto et al, 2017). Our study is the first to determine the mutation spectrum of ctDNA in ENKTL. The most common mutation genes are *MGA*, *TP53*, *ASXL3*, *DDX3X*, *BCORL1*, and *TRAF3*. Our results revealed that ctDNA could reflect MTV, and by longitudinally profiling patients treated with chemotherapy, we provide evidence that serial ctDNA assessment could monitor the disease status, and the results could partially reflect which mutated gene was sensitive to chemotherapy drugs. However, none of our 10 patients recruited for serial ctDNA analysis relapsed; for relapsed patients, whether primary mutation clone recurrence or new mutation occupies the main role needs to be further investigated. In addition, future trials should include more patients and long-term follow-up to explore the plasma gene clone evolution process.

Plasma cfDNA includes tumor cell-derived plasma ctDNA and normal cell apoptosis-derived cfDNA, which are DNA fragments. ctDNA could be a more comprehensive reflection of gene mutations in all tumors than conventional biopsy(Herrera & Armand, 2017; Scherer et al, 2017). Therefore, ctDNA-based detection methods are critical for identifying which mutation is tumor-specific. As sensitivity increases with the development of new methods and because age-related

somatic mutations could also lead to hematopoietic clonal expansions, the possibility of false-positive results might occur(Genovese et al, 2014; Jaiswal et al, 2014). In the present study, to decrease the bias caused by age, the age range of the included patients was narrow, and the sequencing panel was conducted according to a previous gene-expression profiling study on ENKTL. Zhao et al. confirmed that the most frequently mutated genes in NKTCL were the RNA helicase gene *DDX3X*, tumor suppressors (*TP53* and *MGA*), JAK-STAT pathway molecules (*STAT3* and *STAT5b*) and epigenetic modifiers (*MLL2, ARID1A, EP300* and *ASXL3*)(Jiang et al, 2015). In addition to those recurrent mutations, *JAK3, KMT2D, EZH2, NOTCH1* and *TET2* were also identified by different groups. Combined with the most frequent gene mutation spectrum in Chinese individuals, we selected 41 mutated genes for our study. Interestingly, we found that *MGA* alterations possessed a higher frequency (36.7%, 22 of 60 cases) and which were all non-synonymous mutations (exon5: c. A2146T: p.T716S, exon14:c.C4567G: p.P1523A). MGA mutation was correlated with tumor load and stage, and patients with MGA mutation showed poor prognosis. MGA is a dual-specificity transcription factor that contains a T-domain DNA-binding motif, and it can inhibit myc-dependent cell transformation via binding Max(Hurlin et al, 1999; Jo et al, 2016; Romero et al, 2014). Recently, MGA mutations were also reported to occur in high-risk chronic lymphocytic leukemia, lung cancer and colorectal cancer(De Paoli et al, 2013; Jo et al, 2016; Romero et al, 2014). However, the underlying significance in NKTCL has not been explored to date, and further studies regarding MGA mutation in NKTCL lymphomagenesis are urgently recommended. To our knowledge, this is the first study to prospectively evaluate the potential utility of ctDNA analysis in NKTCL patients. We have demonstrated that ctDNA assessment could predict the therapy response in NKTCL, and the highest mutation frequencies were in *MGA* and *TP53,* and MGA mutation was significantly correlated with ctDNA and PET-CT. Our results could define the optimal strategy for patient follow-up.

Materials and methods

1. Study design and patient selection

In this prospective cohort study, 60 patients recently diagnosed with ENKTL at the hematology medical center of Xinqiao Hospital from February 2017 to September 2019 were enrolled (ClinicalTrials identifier: ChiCTR1800014813). All consecutive patients who were deemed appropriate for this study during the study period were included without selection. Histological diagnoses were established independently by at least two experienced senior pathologists according to the WHO classification of Tumors of Hematopoietic and Lymphoid tissue criteria. All patients underwent baseline staging using laboratory, radiographic, and bone marrow examinations. Eastern Cooperative Oncology Group (ECOG) performance status was assessed at diagnosis. Stage was evaluated in accordance with the Ann Arbor staging system. The International Prognostic Index (IPI) was calculated based on serum lactate dehydrogenase, stage, extranodal status and performance status. Patient characteristics and treatment regimens of each therapy cycle were collected from each patient. Three healthy individuals were also recruited as control participants to test the accuracy of our sequencing platform for ctDNA profiling. All participants provided informed written consent before undergoing any study-related procedures in accordance with the Declaration of Helsinki. This study was approved by the China Ethics Committee of Registering Clinical Trials (ChiECRCT-20180005).

After the initial stage assessment, all patients were given front-line therapy according to National Comprehensive Cancer Network (NCCN) guidelines. Patients were reviewed routinely with a combination of clinical assessment and CT or fluorodeoxyglucose-PET (FDG-PET). FDG-PET was often used as an interim scan, and metabolic tumor volume (MTV) was determined from the initial and interim PET images using PET Edge software (MIMSoftware Inc., Cleveland, OH, USA). ctDNA profiling was conducted according to the therapy response during the patient's therapy course.

2. Sample collection and DNA extraction

Before treatment, peripheral blood samples were collected using 10 ml EDTA vacutainer tubes and processed within 4 hours. cfDNA was extracted from plasma using the QIAamp Circulating Nucleic Acid Kit (Qiagen, Valencia, California) following the manufacturer's instructions. Genomic DNA was extracted from formalin fixed paraffin-embedded (FFPE) tissue using the QIAamp DNA FFPE Tissue Kit (Qiagen). DNA concentration and quality were estimated using a Qubit fluorometer (Invitrogen). cfDNA quality was assessed using an Agilent 2100 Bioanalyzer and DNA HS Kit (Agilent Technologies, Palo Alto, CA, USA).

3. Multiregional targeted NGS of patients' plasma and tumor sample

CAPP-Seq was performed on FFPE tumor DNA and cfDNA, and targeted sequencing gene panels including coding exons and splice sites of 41 genes (Yuanqi Biopharmaceutical Co., Shanghai, China) that are recurrently mutated in NK/T-cell lymphoma were specifically designed for this study, including ADAM3A, *APC, ARID1A, ARID1B, ARID2, ASXL3, ATM, BCOR, BCORL1, CD28, CHD8, CREBBP, DDX3X, DNMT3A, EP300, EZH2, FYN, IDH2, IL2RG, JAK1, JAK3, KDM6A, KMT2A, KMT2D, MGA, NF1, NOTCH1, PRDM1, PTPN1, RHOA, SETD2, SOCS1, STAT3, STAT5B, STAT6, TET1, TET2, TNFRSF14, TP53, TRAF3* and *ZAP608*. Tumor DNA was sheared through sonication before library construction to obtain an almost 200-bp fragment for cfDNA, which possesses a fragmented DNA nature. No additional fragmentation was performed before library construction. The NGS libraries were constructed using the SureSelect Library Prep Kit (Agilent Technologies, Palo Alto, CA, USA). Quantification of the library was performed using the Agilent DNA 1000 Kit (Agilent Technologies). Sequencing was performed following the manufacturer's protocol on the Illumina MiSeq system (Illumina, San Diego, CA). For tumor DNA, the mean depth of each sample was 2500×. The length of cfDNA fragments primarily ranged from 100 to 200 bp, and the average coverage depths for sequencing were 813.42× (range, 462×-6513×), with an average of 5% of

the target sequence being covered with sufficient depth for variant calling. Bioinformatics analysis was performed to verify the sample sequence and mutation site and calculate the mutated allele frequency (MAF) compared with the human genome sequence (hg19) using Burrows-Wheel Aligner (BWA) sequence alignment software. Samtools version 1.3 was used for single nucleotide variant (SNV)/indel calling and filter workflow.

4. **Statistical analysis**

Statistical analysis was conducted using SPSS software (Version 18.0, LEAD Corp). Descriptive statistics were used to analyze clinical, demographic and genetic test result characteristics. Correlation of gene mutation and clinicopathologic features of patients were conducted using the Pearson χ^2 test. Overall survival (OS) measured the proportion of patients who were alive at a specific time after diagnosis. Survival estimates were obtained using the Kaplan–Meier method, and comparisons were made using a log-rank test. Unpaired Student's t-test for two groups and one-way ANOVA for multiple group data were used in this study. A COX proportional regression model was used to calculate the survival hazard ratio (HR). Statistical differences were considered significant if the *P* value was less than 0.05.

Declaration of interests

All authors declare no competing interests.

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Author contributions

JR, YL, and XZ conceived and designed the study. JR, WZ and QL developed the methodology and analyzed and interpreted the data. JR and WZ wrote the manuscript.

All authors reviewed and revised the manuscript.

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The paper explained

Problem

Extranodal NK/T-cell lymphoma, nasal type (ENTKL), is an aggressive hematological malignancy with poor prognosis, early detection of tumor at initial diagnosis or during routine surveillance is important for precision medicine. PET/CT or tissue biopsies sequencing had some limitations; therefore, more sensitive and real-time monitoring method was urged to explored.

Results

In this study, we assessed the gene mutation spectrum of plasma ctDNA by cancer personalized profiling sequencing (CAPP-Seq), and found that *MGA, TP53, ASXL3* et al were the most frequently mutated genes, mutation frequency of MGA and TP53 were significantly higher in stage III-IV, and mutation of MGA, TP53, NOTCH3 were significantly correlated with metabolic tumor burden of the patients. Compared with tumor tissue DNA, ctDNA profiling showed good concordance. We also aimed at tracking the genetics modifications upon treatment, serial ctDNA analysis showed that treatment with chemotherapy could decrease the number and mutation allele frequency of genes, compared with PET/CT, ctDNA has much more sensitivity for track residual disease of the patients. Survival analysis demonstrated that patients with mutated *MGA, TP53, SETD2,* and *APC* showed a shorter survival time.

Impact

These results demonstrated that ctDNA assessment was feasible in NKTCL, and gene mutation spectrum of plasma ctDNA was figured out, serial ctDNA monitoring had more advantage over PET/CT, which could define the optimal strategy for patient during follow-up.

References

Adams HJ, Nievelstein RA, Kwee TC (2015) Prognostic value of complete remission status at

end-of-treatment FDG-PET in R-CHOP-treated diffuse large B-cell lymphoma: systematic review and meta-analysis. Br J Haematol 170: 185-191

Au WY, Weisenburger DD, Intragumtornchai T, Nakamura S, Kim WS, Sng I, Vose J, Armitage JO, Liang R (2009) Clinical differences between nasal and extranasal natural killer/T-cell lymphoma: a study of 136 cases from the International Peripheral T-Cell Lymphoma Project. Blood 113: 3931-3937

Bettegowda C, Sausen M, Leary RJ, Kinde I, Wang Y, Agrawal N, Bartlett BR, Wang H, Luber B, Alani RM et al (2014) Detection of circulating tumor DNA in early- and late-stage human malignancies. Sci Transl Med 6: 224ra224

Chen Y, George AM, Olsson E, Saal LH (2018) Identification and Use of Personalized Genomic Markers for Monitoring Circulating Tumor DNA. Methods Mol Biol 1768: 303-322

Cheson BD, Ansell S, Schwartz L, Gordon LI, Advani R, Jacene HA, Hoos A, Barrington SF, Armand P (2016) Refinement of the Lugano Classification lymphoma response criteria in the era of immunomodulatory therapy. Blood 128: 2489-2496

Crowley E, Di Nicolantonio F, Loupakis F, Bardelli A (2013) Liquid biopsy: monitoring cancer-genetics in the blood. Nat Rev Clin Oncol 10: 472-484

Daigle S, McDonald AA, Morschhauser F, Salles G, Ribrag V, McKay P, Tilly H, Schmitt A, Gerecitano J, Fruchart C et al (2017) Discovery of Candidate Predictors of Response to Tazemetostat in Diffuse Large B-Cell Lymphoma and Follicular Lymphoma Using NGS Technology on ctDNA Samples Collected Pre-Treatment. Blood 130: 4013-4013

De Paoli L, Cerri M, Monti S, Rasi S, Spina V, Bruscaggin A, Greco M, Ciardullo C, Fama R, Cresta S et al (2013) MGA, a suppressor of MYC, is recurrently inactivated in high risk chronic lymphocytic leukemia. Leuk Lymphoma 54: 1087-1090

Delfau-Larue MH, van der Gucht A, Dupuis J, Jais JP, Nel I, Beldi-Ferchiou A, Hamdane S, Benmaad I, Laboure G, Verret B et al (2018) Total metabolic tumor volume, circulating tumor cells, cell-free DNA: distinct prognostic value in follicular lymphoma. Blood Adv 2: 807-816

Esposito A, Criscitiello C, Locatelli M, Milano M, Curigliano G (2016) Liquid biopsies for solid tumors: Understanding tumor heterogeneity and real time monitoring of early resistance to targeted therapies. Pharmacol Ther 157: 120-124

Forshew T, Murtaza M, Parkinson C, Gale D, Tsui DW, Kaper F, Dawson SJ, Piskorz AM, Jimenez-Linan M, Bentley D et al (2012) Noninvasive identification and monitoring of cancer mutations by targeted deep sequencing of plasma DNA. Sci Transl Med 4: 136ra168

Genovese G, Kahler AK, Handsaker RE, Lindberg J, Rose SA, Bakhoum SF, Chambert K,

Mick E, Neale BM, Fromer M et al (2014) Clonal hematopoiesis and blood-cancer risk inferred from blood DNA sequence. N Engl J Med 371: 2477-2487

Herrera AF, Armand P (2017) Minimal Residual Disease Assessment in Lymphoma: Methods and Applications. J Clin Oncol 35: 3877-3887

Hurlin PJ, Steingrimsson E, Copeland NG, Jenkins NA, Eisenman RN (1999) Mga, a dual-specificity transcription factor that interacts with Max and contains a T-domain DNA-binding motif. EMBO J 18: 7019-7028

Jaiswal S, Fontanillas P, Flannick J, Manning A, Grauman PV, Mar BG, Lindsley RC, Mermel CH, Burtt N, Chavez A et al (2014) Age-related clonal hematopoiesis associated with adverse outcomes. N Engl J Med 371: 2488-2498

Jiang L, Gu ZH, Yan ZX, Zhao X, Xie YY, Zhang ZG, Pan CM, Hu Y, Cai CP, Dong Y et al (2015) Exome sequencing identifies somatic mutations of DDX3X in natural killer/T-cell lymphoma. Nat Genet 47: 1061-1066

Jo YS, Kim MS, Yoo NJ, Lee SH (2016) Somatic mutation of a candidate tumour suppressor MGA gene and its mutational heterogeneity in colorectal cancers. Pathology 48: 525-527

Kim SJ, Choi JY, Hyun SH, Ki CS, Oh D, Ahn YC, Ko YH, Choi S, Jung SH, Khong PL et al

(2015) Risk stratification on the basis of Deauville score on PET-CT and the presence of Epstein-Barr virus DNA after completion of primary treatment for extranodal natural killer/T-cell lymphoma, nasal type: a multicentre, retrospective analysis. Lancet Haematol 2: e66-74

Kurtz DM, Green MR, Bratman SV, Scherer F, Liu CL, Kunder CA, Takahashi K, Glover C, Keane C, Kihira S et al (2015) Noninvasive monitoring of diffuse large B-cell lymphoma by immunoglobulin high-throughput sequencing. Blood 125: 3679-3687

Kwong YL, Kim WS, Lim ST, Kim SJ, Tang T, Tse E, Leung AY, Chim CS (2012) SMILE for natural killer/T-cell lymphoma: analysis of safety and efficacy from the Asia Lymphoma Study Group. Blood 120: 2973-2980

Melani C, Pittaluga S, Yee L, Lucas A, Shovlin M, Jacob A, Chan K, Vignali M, Jaffe ES, Wilson WH et al (2017) Next-Generation Sequencing Based Monitoring of Circulating-Tumor DNA in Untreated Peripheral T-Cell Lymphoma. Blood 130: 2728-2728

Romero OA, Torres-Diz M, Pros E, Savola S, Gomez A, Moran S, Saez C, Iwakawa R, Villanueva A, Montuenga LM et al (2014) MAX inactivation in small cell lung cancer disrupts MYC-SWI/SNF programs and is synthetic lethal with BRG1. Cancer Discov 4: 292-303

Roschewski M, Dunleavy K, Pittaluga S, Moorhead M, Pepin F, Kong K, Shovlin M, Jaffe ES, Staudt LM, Lai C et al (2015) Circulating tumour DNA and CT monitoring in patients with

untreated diffuse large B-cell lymphoma: a correlative biomarker study. Lancet Oncol 16: 541-549

Sakata-Yanagimoto M, Nakamoto-Matsubara R, Komori D, Nguyen TB, Hattori K, Nanmoku T, Kato T, Kurita N, Yokoyama Y, Obara N et al (2017) Detection of the circulating tumor DNAs in angioimmunoblastic T- cell lymphoma. Ann Hematol 96: 1471-1475

Scherer F, Kurtz DM, Diehn M, Alizadeh AA (2017) High-throughput sequencing for noninvasive disease detection in hematologic malignancies. Blood 130: 440-452

Scherer F, Kurtz DM, Newman AM, Stehr H, Craig AF, Esfahani MS, Lovejoy AF, Chabon JJ, Klass DM, Liu CL et al (2016) Distinct biological subtypes and patterns of genome evolution in lymphoma revealed by circulating tumor DNA. Sci Transl Med 8: 364ra155

Siravegna G, Marsoni S, Siena S, Bardelli A (2017) Integrating liquid biopsies into the management of cancer. Nat Rev Clin Oncol 14: 531-548

Spina V, Bruscaggin A, Cuccaro A, Martini M, Di Trani M, Forestieri G, Manzoni M, Condoluci A, Arribas A, Terzi-Di-Bergamo L et al (2018) Circulating tumor DNA reveals genetics, clonal evolution, and residual disease in classical Hodgkin lymphoma. Blood 131: 2413-2425

Suehara Y, Sakata-Yanagimoto M, Hattori K, Nanmoku T, Itoh T, Kaji D, Yamamoto G, Abe Y,

Narita K, Takeuchi M et al (2018) Liquid biopsy for the identification of intravascular large B-cell lymphoma. Haematologica 103: e241-e244

Suzuki R (2014) Pathogenesis and treatment of extranodal natural killer/T-cell lymphoma. Semin Hematol 51: 42-51

Trotman J, Luminari S, Boussetta S, Versari A, Dupuis J, Tychyj C, Marcheselli L, Berriolo-Riedinger A, Franceschetto A, Julian A et al (2014) Prognostic value of PET-CT after first-line therapy in patients with follicular lymphoma: a pooled analysis of central scan review in three multicentre studies. Lancet Haematol 1: e17-27

Tse E, Kwong YL (2013) How I treat NK/T-cell lymphomas. Blood 121: 4997-5005

Xiong J, Zhao W (2019) What we should know about natural killer/T-cell lymphomas. Hematol Oncol 37 Suppl 1: 75-81

Zhou J, Huang A, Yang XR (2016) Liquid Biopsy and its Potential for Management of Hepatocellular Carcinoma. J Gastrointest Cancer 47: 157-167

Figure legends

Figure 1. consort diagram of patient enrolment, specimen collection.

Figure 2. The mutational profile of newly diagnosed ENKTL. The heatmap shows individual nonsynonymous somatic mutations detected in ctDNA of newly diagnosed patients (n=60). Each row represents a gene, and each column represents a primary tumor. Mutations are color coded in red. The upper bar graph shows the number and type of mutated genes, the horizontal bar graph shows the gene mutation frequency, the middle bar graph shows the percentage of nonsynonymous somatic mutations, and the lower graph shows the clinical characteristics of each sample.

Figure 3. A. The consistent gene mutation spectrum and mutation allele frequency detected in plasma ctDNA and tumor tissue of the patients. B. Venn diagram summarizing the detailed mutations discovered in both plasma ctDNA (gray) and tumor DNA (blue). C. For each patient, the fraction of tumor biopsy-confirmed mutations that were detected in plasma ctDNA is shown. The gray portion of the bar marks the part of tumor biopsy-confirmed mutation that was not found in plasma ctDNA.

Figure 4. Correlation of ctDNA assessment with patient clinical characteristics. A. The general plasma ctDNA concentration in each specific patient with different tumor stage and relapse status; B. Linear regression of plasma ctDNA concentration with metabolic tumor volume. C. The mutation frequency of *MGA*, *TP53*, and *DDX3X* in patients with different tumor stages. D. The mutation allele frequency of *MGA*, *TP53*, and *DDX3X* in patients with different tumor stages. E. The mutation frequency of *MGA*, *TP53*, and *DDX3X* in patients with different relapse statuses. F. The general metabolic tumor volume in patients with different mutation statuses of *MGA*, *TP53*, *DDX3X*, *NOTCH3*, *APC*, *ATM*, *EZH2* and *TNFRSF14*.

Figure 5. Serial ctDNA detection during therapy could complement the response assessment of the patients, and patients with mutated *MGA*, *TP53*, *SETD2*, and *APC* had poor prognosis. A. Dynamic plasma ctDNA gene mutation change in a representative patient sample (#22, 23) during the therapy courses; Kaplan-Meier estimated the OS of plasma *MGA* (B), *TP53* (C), *SETD2* (D), *APC* (E), *DDX3X* (F), *ASCL3* (G), *ATM* (H), *BCORL1* (I), *TRAF3* (J), *KMT2D* and (K) mutation status in patients with ENKTL.

Table 1 Patients' demographic and clinical characteristics

Prognostic variables	No.	Proportion (%)
Gender		
Male	41	68.33
Female	19	31.67
clinical stage		
I	13	21.67
II	17	28.33
III	13	21.67
IV	17	28.33
B symptoms		
with	24	40.00
without	36	60.00
IPI Scores		
1	19	31.67
2	23	38.33
3	13	21.67
4	5	8.33
Recurrence status		
with	8	13.33
without	52	86.67
Ki67 index		
Low	33	55.00
High	27	45.00

Table 2 Univariate and multivariate analyses of the overall survival of patients

Prognostic variables	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*p value*	HR (95% CI)	*p value*
Gender (female vs. male)	0.868(0.332-2.266)	0.772		
Stage (I-II vs. III-IV)	3.444(1.223-9.696)	0.019	2.688(0.506-14.058)	0.247
B symptons (with vs. without)	1.165(0.460-2.951)	0.748		
IPI	1.593(1.040-2.441)	0.033	1.068(0.500-2.282)	0.866
Recurrence status	3.197(1.236-8.268)	0.017	3.729(0.931-14.933)	0.063
MTV	3.123(1.099-8.875)	0.033	2.908(0.882-9.583)	0.079
ctDNA concentraion	1.754(0.653-4.715)	0.265		
MGA mutation (without vs. with)	0.293(0.113-0.762)	0.012	1.706(0.474-0.644)	0.014
TP53 mutation (without vs. with)	0.367(0.144-0.993)	0.035	0.175(0.047-0.652)	0.009
ASXL3 mutation (without vs. with)	0.638(0.245-1.659)	0.356		
BCORL1 mutation (without vs. with)	0.821(0.307-2.196)	0.695		
DDX3X mutation (without vs. with)	1.502(0.426-5.299)	0.527		
TRAF3 mutation (without vs. with)	1.126(0.370-3.429)	0.835		
STAT5B mutation (without vs. with)	1.306(0.378-4.515)	0.674		
EP300 mutation (without vs. with)	1.407(0.406-4.880)	0.59		
NOTCH3 mutation (without vs. with)	1.037(0.339-3.170)	0.95		
APC mutation (without vs. with)	0.396(0.153-1.027)	0.057		
ATM mutation (without vs. with)	0.605(0.215-1.702)	0.341		
KMT2D mutation (without vs. with)	0.540(0.151-1.927)	0.342		
EZH2 mutation (without vs. with)	0.761(0.248-2.332)	0.632		
STED2 mutation (without vs. with)	0.294(0.103-0.843)	0.023	0.661(0.195-2.233)	0.505
TNFRSF14 mutation (without vs. with)	1.438(0.41-5.044)	0.57		

Supplementary Materials:

Table S1: The range of mutant allele frequencies in each gene of each sample.

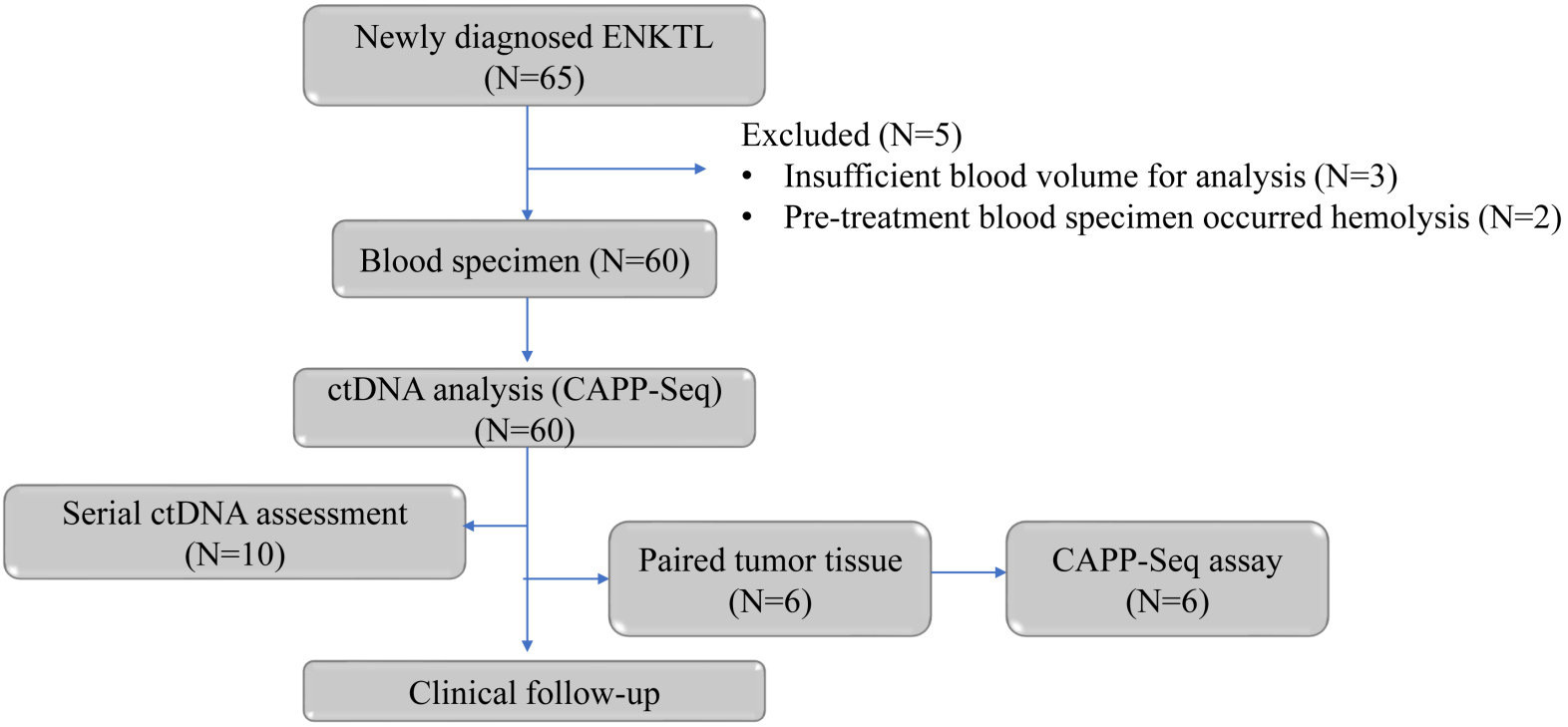
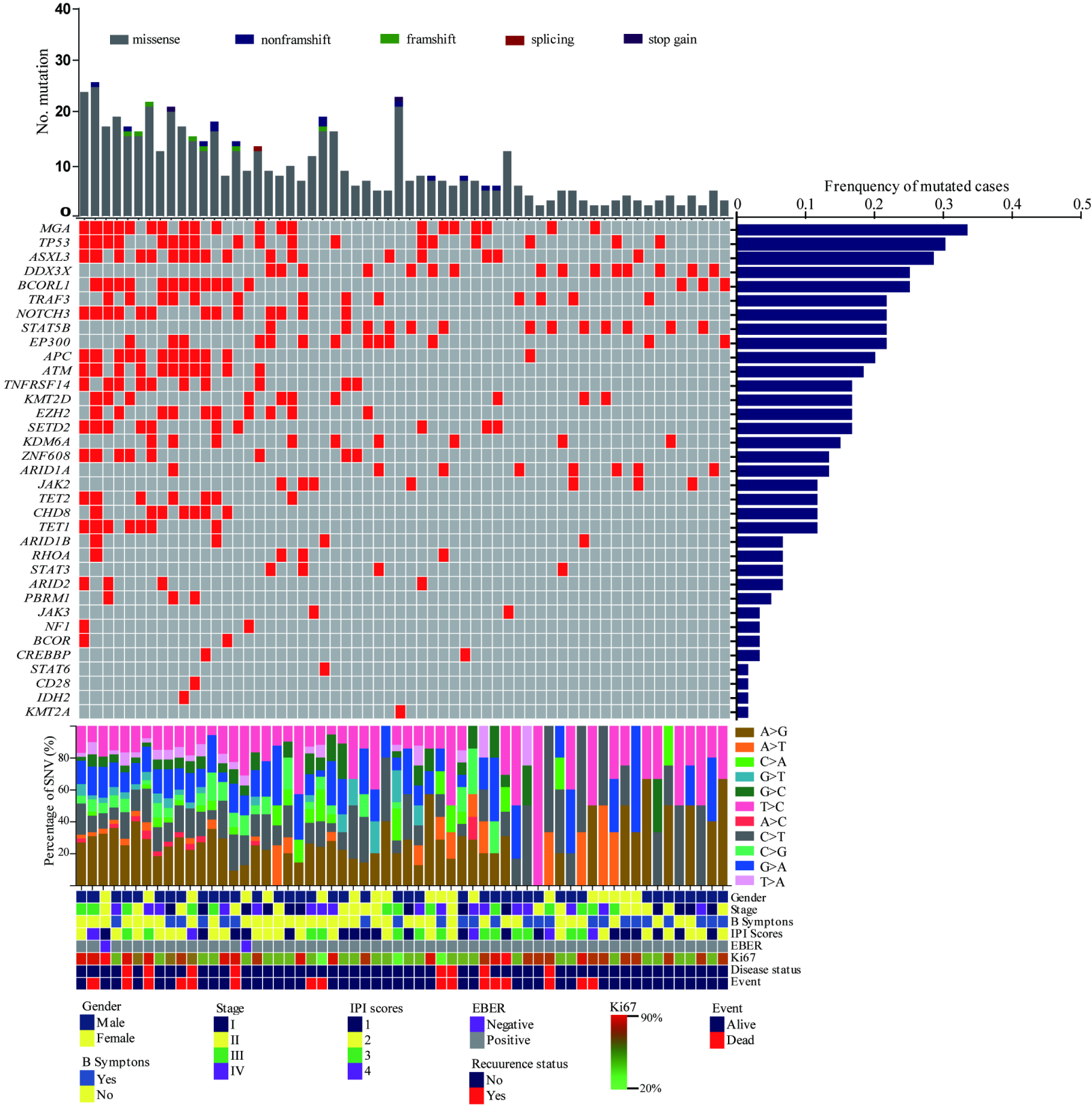
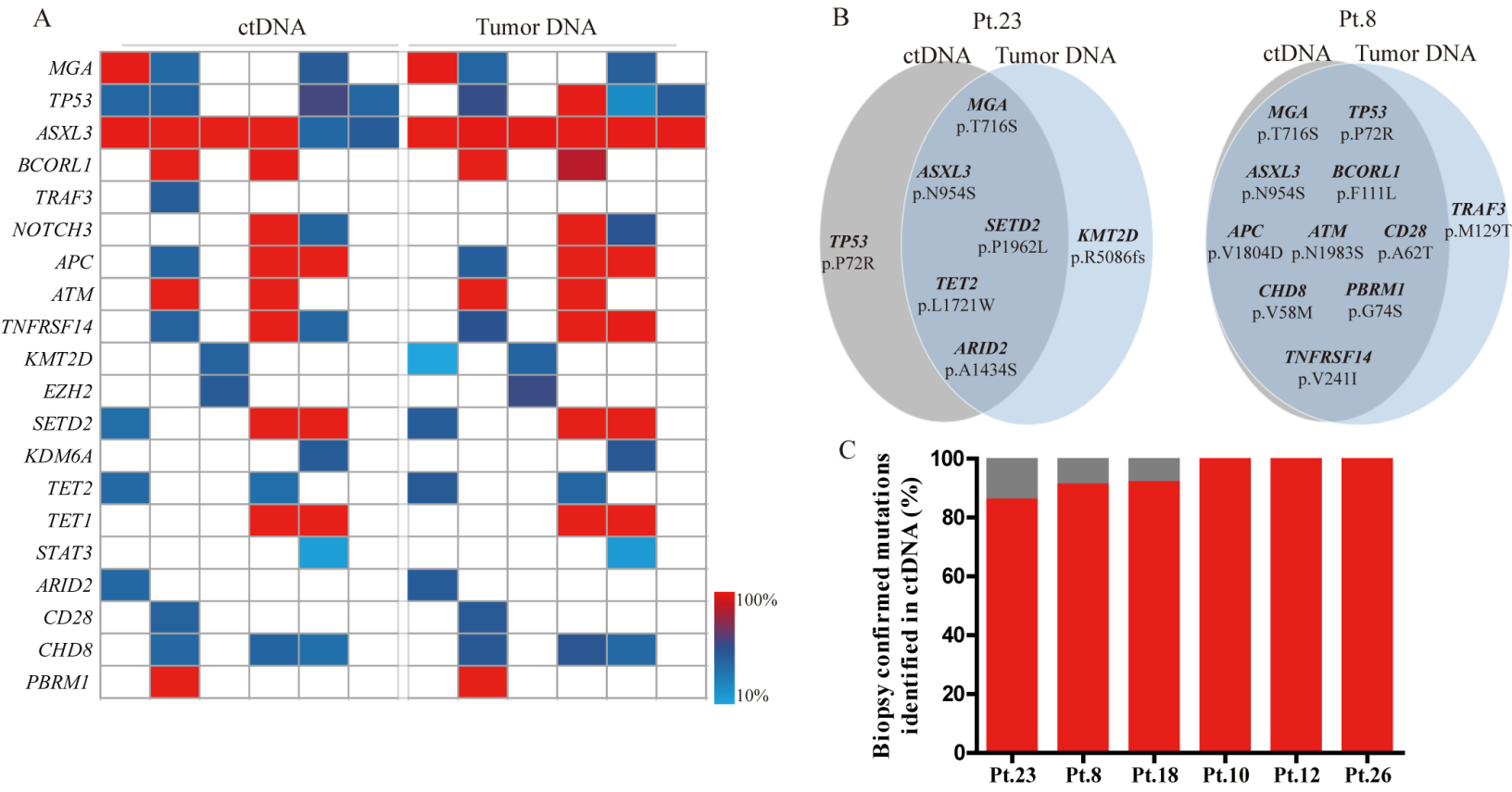

Newly diagnosed ENKTL
(N=65)
Excluded (N=5)
• Insufficient blood volume for analysis (N=3)
• Pre-treatment blood specimen occurred hemolysis (N=2)
Blood specimen (N=60)
ctDNA analysis (CAPP-Seq)
(N=60)
Serial ctDNA assessment
(N=10)
Paired tumor tissue
(N=6)
CAPP-Seq assay
(N=6)
Clinical follow-up

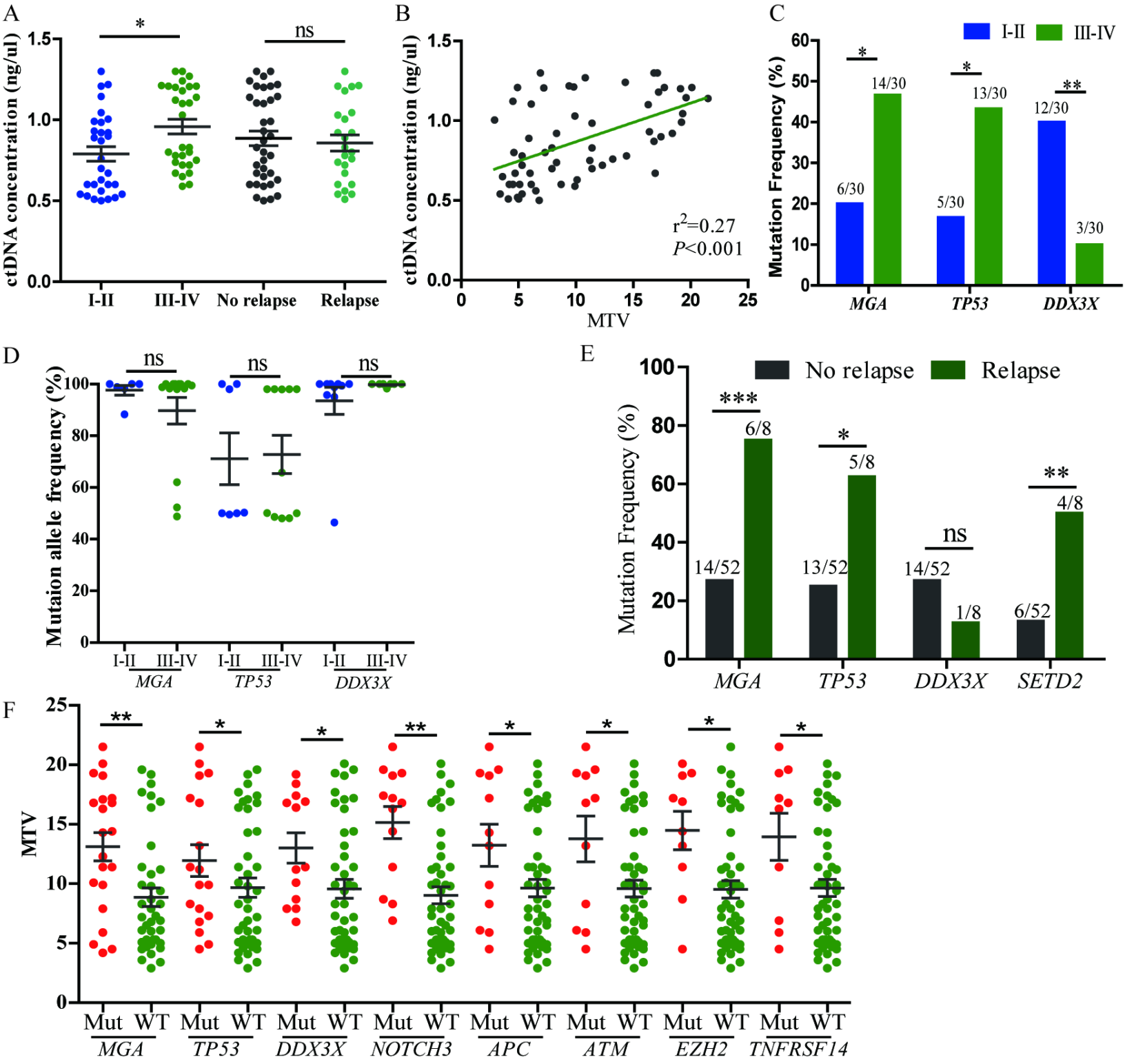
Figure. 1



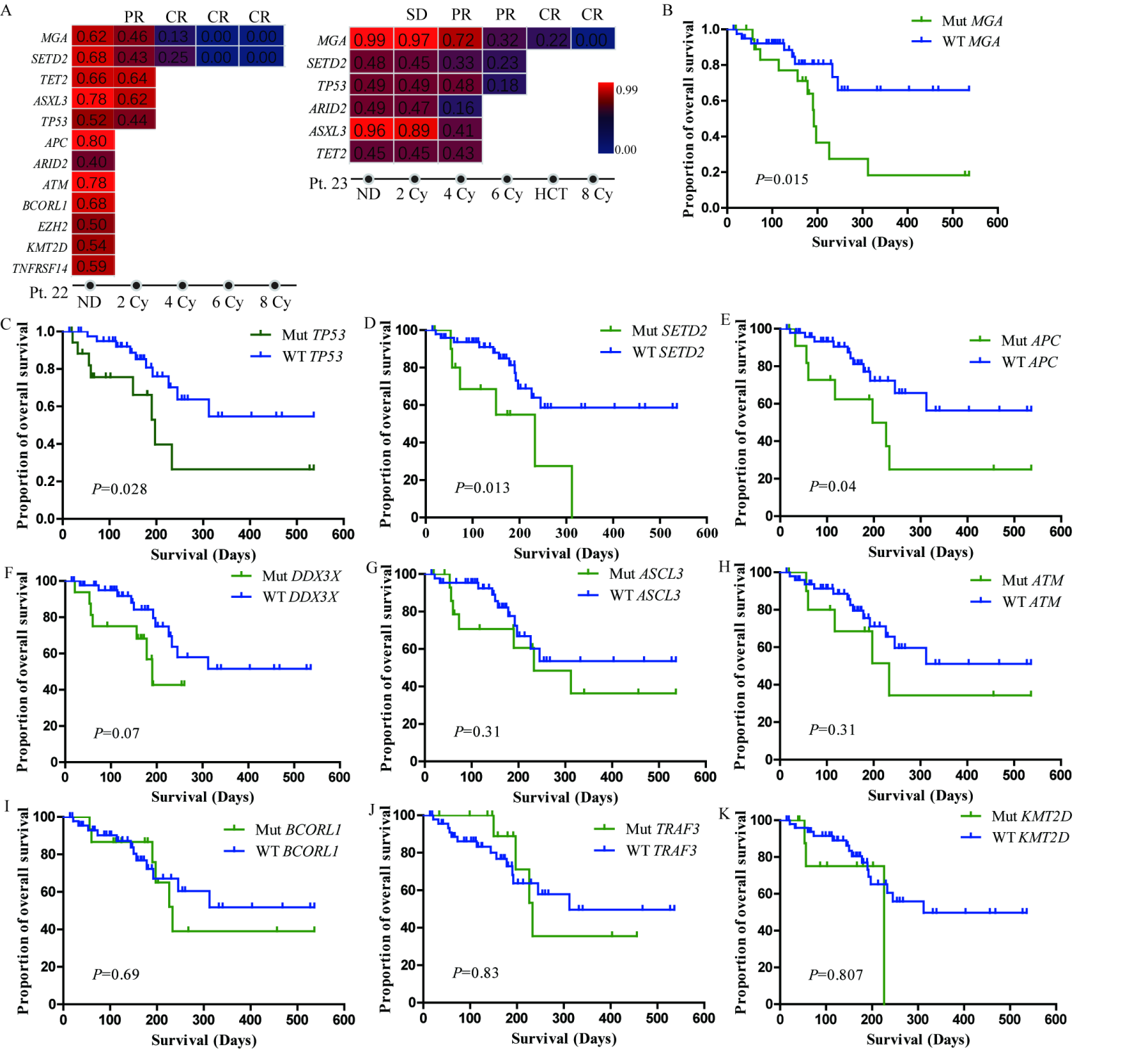
Rao Jun et al._Figure 2



Rao Jun et al._Figure 3



Rao Jun et al._Figure 4



Rao Jun, et al. Figure 5