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Longitudinal analysis of 25 sequential sample-pairs using a custom multiple myeloma mutation sequencing panel (M³P)

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Conflict of interests KMK, CL, JM, LBr, YXZ, CXS, PJ, JBE, JO, LBu, MK, GA, LR, SK, HE, AKS, and EB declare that they have no conflict of interest.

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Abstract

Recent advances in genomic sequencing technologies now allow results from deep next-generation sequencing to be obtained within clinically meaningful timeframes, making this an attractive approach to better guide personalized treatment strategies. No multiple myeloma-specific gene panel has been established so far; we therefore designed a 47-gene-targeting gene panel, containing 39 genes known to be mutated in ≥3%of multiple myeloma cases and eight genes in pathways therapeutically targeted in multiple myeloma (MM). We performed targeted sequencing on tumor/germline DNA of 25 MM patients in which we also had a sequential sample post treatment. Mutation analysis revealed *KRAS* as the most commonly mutated gene (36 % in each time point), followed by *NRAS* (20 and 16 %), *TP53* (16 and 16 %), *DIS3* (16 and 16 %), *FAM46C* (12 and 16 %), and *SP140* (12 and 12 %). We successfully tracked clonal evolution and identified mutation acquisition and/or loss in *FAM46C, FAT1, KRAS, NRAS, SPEN, PRDM1, NEB*, and *TP53* as well as two mutations in *XBP1*, a gene associated with bortezomib resistance. Thus, we present the first longitudinal analysis of a MM-specific targeted sequencing gene panel that can be used for individual tumor characterization and for tracking clonal evolution over time.

Keywords

Multiple myeloma; Targeted sequencing

Introduction

Initial therapy in multiple myeloma (MM) consistently induces high-quality remission, including CR, in the majority of patients. However, relapses occur in almost all patients over time, best explained by the existence of tumor clone heterogeneity already existent at initial diagnosis with different drug susceptibilities, leading to clonal selection and evolution over time [1–3]. Successful treatment of the disease therefore needs a broad target that includes minor subclones, from which a final, ultimately resistant clone could arise [4]. Consequently, awareness of the individual genetic profile of the tumor cell population and the surveillance of changes over time under therapeutic selective pressure is needed to assess treatment efficacy. Today, whole genome/exome sequencing data of more than 300 MM patients are publically available from recent large sequencing studies [5–7, 3] describing

genomic complexity of the disease including baseline clonal heterogeneity [4], linear and branching evolution [3], and therapeutic selection of clones and subclones resulting in clonal tides [2]. However, whereas genetic diagnostics in MM as cytogenetics, fluorescence-in-situ-hybridization and gene expression profiling are well established [8], individual mutation profiling has not yet been adopted to the routine risk assessment. In this work, we report on an innovative gene panel investigating 25 MM patients at sequential time points before and after therapy. We employed semiconductor sequencing technology that provides rapid mutation analysis at reasonable costs, needing low sample input (10 ng) and a sample turnaround time in clinically meaningful timeframes (hours).

Material and methods

We obtained DNA of 22 newly diagnosed and 3 pretreated MM patients, including a later time point sample and corresponding germline from peripheral blood mononuclear cells from the German MM trial group (DSMM). All samples were collected with informed consent according to the Declaration of Helsinki. Plasma cells were enriched using CD138+ beads (median purity 95 %). The purity of the samples was assessed cytologically. DNA was subsequently extracted using the Qiagen AllPrep DNA/RNA Mini Kit according to the manufacturer's recommendations. The time between the sample biopsies ranged from 63 to 2054 days, with an average of 374 days. Treatment information and response data were not available. Baseline FISH was available in 23 of 25 pairs and revealed a increased risk cohort defined by 70 % gain of 1q21 (16/23), 48 % of del13q (11/23), 35 % of t(4;14) (8/23), 9 % of t(11;14) (2/23) and t(14;16) (2/23), and 26 % (6/23) of del17p.

The 47 gene multiple myeloma mutation panel (M^3P)

We established a MM-specific 47 gene mutation panel [9] including a selection of 39 genes expressed in MM (by analyzing gene expression profiling public datasets) with nonsynonymous mutations found in ≥3 % of published MM genomes [6, 10]. To this, we added eight genes targeted by the most commonly used MM therapies, associated with resistance to IMiDs (*CRBN*, *CUL4A*, *CUL4B*, *DDB1*, and *IRF4*), proteasome inhibitors (*PSMG2*, *PSMB5*) and glucocorticoid therapies (*NR3C1*) (Table 1). We employed the Ion Torrent semiconductor sequencing platform (PGM, Life Technologies, Carlsbad, CA, USA), using 20 ng of starting DNA for each sample (10 ng per primer pool). The coding regions of the 47 genes were amplified in 200-bp libraries using customized oligos (Ion AmpliSeq Designer, Life Technologies). Overall, 2875 amplicons, covering 96 % of the M^3P exons, were analyzed per sample, multiplexed in two library preparations (Ion AmpliSeq Library Kit 2.0, Life Technologies). Template preparation and enrichment of DNA libraries was done on the Ion OneTouch2 and Ion OneTouch ES (Life Technologies) automated system, respectively. Batches of four samples were barcoded (Ion Xpress Barcode Adapters, Life Technologies), pooled, and sequenced using Ion 318 and 318v2 chips and the Ion Sequencing 200 Kit v2 (Life Technologies). Sequencing data were analyzed using the Ion Reporter Software v1.6 (protocols applied: "TumorNormalTemplate 1.6.2" and "Ion QC protocol", Life Technologies, Carlsbad, CA, USA), visualized, and manually reviewed using the Integrative Genomics Viewer (IGV, Broad Institute, Cambridge, MA, USA). Variants were analyzed using SIFT, Provean (J. Craig Venter Institute) [11, 12], PolyPhen-2

(Harvard University) [13], and the Catalogue of Somatic Mutations in Cancer (COSMIC, Welcome Trust Sanger Institute, UK) [14]. Mutation calls were considered positive when called by ≥10 % variant reads and >20 times sequencing coverage depth in the tumor sample. In already characterized cancer-related mutations (COSMIC database), the threshold was reduced to 3 %. We additionally considered mutations called below threshold if a matching variant above the threshold was found in the corresponding tumor sample.

Results

First we validated the targeted sequencing technology by investigating a MM tumor sample previously analyzed by whole exome sequencing (WES) and thus known to be harboring 12 mutations included in the M^3P panel [15]. To determine the sequencing accuracy, especially in low allele frequency mutations, additional targeted sequencing was performed at 1:3 and 1:10 dilutions of the tumor sample. The sample was diluted using a commercially available lymphoproliferative cell line (GM19240, Coriell Cell Repositories, Camden, NJ, USA) for which sequencing results were publicly available from the HapMap project.

We obtained a mean coverage of × 278 read depth for the M^3P panel. All 12 mutations initially found by WES were also reported using targeted sequencing. The mutation allelic frequencies, ranging between 15 and 60 % by WES, correlated very well with the results obtained by targeted sequencing (R=0.98, p<0.0001). A good correlation was also found in the 1:3 (p=0.0002, R=0.84) and 1:10 (p=0.006, R=0.75) dilution (Fig. 1), and all mutations were found in both dilutions, including a mutation with a lowest non reference allele read frequency of 1.32 % in the 1:10 dilution.

An average read sequencing depth of × 280 (median × 255, amplicons with at least 20 reads in >90 %) was achieved across the 25 paired samples. In 20 patients (80 %) and for 23 of the 47 M^3P genes (49 %), mutations were identified at the cutoffs employed. The number of mutations per patient varied from 0 to 8, with a total of 101 mutations identified of which 82 (81.2 %) were predicted damaging/deleterious by Polyphene-2, Provean, or SIFT. In the earlier time point, an average of 1.92 variants was found per patient. In time point 2, the mean mutation prevalence increased slightly to 2.12 mutations per patient. Changes in mutation abundance exceeding 20 % were seen in 52 % of the patients (measured by purity corrected variant reads). Six genes were mutated in more than 10 % of the patients at both time points, with *KRAS* as the most prevalent (36 % in each time point), followed by NRAS (20 %, 16 %), *TP53* (20 %, 24 %), *DIS3* (16 %, 16 %), *FAM46C* (12 %, 16 %), and *SP140* (12 %, 12 %) (Fig. 2). Acquisition of mutations over time was seen in several genes, including *FAM46C* (p.Ile276Thr), *FAT1* (p.His3512Asn), *KRAS* (p.Gln61His, known activating), *SPEN* (p.Thr2747Ala), *PRDM1* (p.Lys620Arg), *NEB* (p.Thr3677Ser), and *TP53* (p.Tyr163Cys). Disappearance of mutations was found in *KRAS* (p.Gly12Val, known activating) and *NRAS* (p.Pro54Leu). Probable parallel evolution [3] in the RAS/MAPK pathway was observed in two patients with one patient presenting two mutations in one gene (*KRAS*, p.Tyr64Asn, p.Ala146Thr) and one patient harboring mutations in different genes of the pathway (*KRAS* and *BRAF*). Furthermore, multiple mutations on one gene were found in *ANK2* (p.Glu2857Lys, p.His923Tyr), *ATM* (truncating p.Glu431*, p.Leu2945Met), and *TP53* (p.Tyr163Cys, p.Arg181His, p.Met246Ile). Of interest, only the mutations in ATM

showed concordant changes of abundance over time, whereas the mutations in *ANK2, KRAS*, and *TP53* had opposing changes, indicating that these mutations are present in different subclones of the tumor cell population. Accordingly, in 8 of 12 patients with more than one mutation, the existence of coexisting tumor (sub)clones could be determined by opposing changes in variant read abundance over time. Most strikingly, in one relapsed del(17p) patient, we detected a *TP53, KRAS*, and *FAM46C* mutations in a pleural effusion of a pretreated patient at time point A while 5 months later, this patient developed a plasma cell leukemia in which loss of this *TP53*-mutated subclone and emergence of a second subclone with a different *TP53* mutation was seen. Notably, a third clonal *TP53* mutation was found to be shared by both subclones (Table 2).

Damaging mutations in drug resistance related genes were rare, even when we checked below the chosen threshold of significance. However, variants in *XBP1*, a gene related to proteasome inhibitor resistance [16], were found in two patients. While one of the mutations did not change significantly between the time points (p.Glu99Lys), one was reduced in abundance by therapy by 74% of the reads (p.Arg94Gln) over time.

Discussion

The investigation of tumor samples on multiple time points allows insights in the pathomechanisms that lead to tumor progression and the development of drug resistance in MM patients. Whole genome/whole exome sequencing data as well as single cell analyses on sequential MM samples have been performed, confirming baseline clonal heterogeneity, linear and branching evolution, and the selection of clones and subclones under selective pressure of drug therapy resulting in clonal tides [3, 7, 17, 2, 4]. We established a MM-specific next-generation targeted sequencing panel (M^3P) to investigate the most commonly mutated genes in MM as well as genes for which a targetable drug is available or that are related to drug resistance [9]. This approach allows to obtain results faster (within clinically meaningful timeframes), cheaper, and with far less sample demand than established WES/WGS sequencing technologies. We investigated 22 untreated and 3 pretreated tumor normal pairs and a subsequent time point sample by M^3P. We observed clonal evolution in the majority of patients including clonal expansion or contraction, as well as complete extinction of subclones (*KRAS, TP53*) and the emergence of new subclones (*FAM46C, FAT1, SPEN, TP53*). Of interest, we found the majority of mutations in our cohort to be present in both patient time points and true extinction of clones or subclones by therapy was uncommon. Baseline FISH data indicate increased incidence of high-risk markers in our cohort, including del17p or t(4;14), explaining the increased incidence of TP53 mutations in our cohort(16 %). DIS3 incidences matched t(4;14)/t(11;14) restricted cohorts [18] with four patients harboring a mutations in this gene. *DIS3*, located on chromosome 13, is one of the most commonly mutated genes in MM, but its role in the pathophysiology of MM remains undiscovered. *DIS3* is component of the RNA exosome complex and may be involved in Ig class switch recombination and Ig variable region somatic hypermutation in human B lymphocytes [19]. Mutations of *DIS3* have been described in other malignant diseases such as medulloblastoma [20], acute myeloid leukemia [21], and nodular melanoma [22]. In MM, *DIS3* mutations have been associated to t(4;14), t(11;14) and a dependency on del(13q) was reported [18]. In our cohort, four *DIS3* mutations were identified prior to treatment of which

two were clonal (p.Pro412Leu and p.Asp784His) and two subclonal (p.Arg780Lys and p.Arg780Thr). Of interest, mutations in *DIS3* in MM at amino acid position 780 have been described to alter gene function by causing significant aberrations of hDIS3 exoribonucleolytic activity [23]. We could see clonal evolution caused by therapy-induced selective pressure in all *DIS3* mutations in our cohort: In three of them, we observed a significant decrease of variant read (VR) abundance over time (p.Asp784His −73 %, p.Pro412Leu −34 %, and p.Arg780Lys −29 %), whereas in one patient an increase was seen (p.Arg780Thr +17 %).

Another gene, frequently mutated in our cohort, was *SP140*. This gene is expressed in mature B cells and on plasma cell lines. It is the lymphoid-restricted homolog of SP100 which contributes to EBV-mediated B cell immortalization [24]. It is involved in the pathogenesis of chronic lymphocytic leukemia [25] and squamous cell carcinoma [26]. However, the clinical impact of mutations in *SP140* in MM is not yet determined. Truncating mutations of *SP140* have been recently described in MM [7], and indeed, we also saw two truncating *SP140* mutations (p.Arg576* and p.Glu75*) and one missense mutation (p.Glu856Lys) in our cohort. We observed a 72 % decrease in abundance of the missense mutation after treatment; however, the truncating mutations remained stable over time.

Of interest, mutations in genes related to drug resistance were rare. We identified mutations in *XBP1* in two patients, a gene reported to be associated with resistance against proteasome inhibition. Mutation taster predicts splice site changes by both mutations and it has been shown that only spliced *XBP1s* is transcriptionally active and other mutations affecting the *XBP1* splicesite have been demonstrated to cause bortezomib resistance [16]. Furthermore, both mutations occurred in highly conserved regions, affecting the bzip domain and the Leucine zipper region of the gene and both were predicted as damaging or possibly damaging by Polyphene-2 or SIFT. Thus, both mutations might alter sensitivity to proteasome inhibition; however, no data on treatment are available; therefore, the effect of these mutations remains speculative.

We believe that targeted mutation profiling will likely become part of the clinical workup in MM in the near future. M^3P is the first MM-specific targeted sequencing panel so far and using this targeted sequencing panel, we were not only able to characterize the individual mutational tumor profiles of the 25 investigated patients, but also identified clonal heterogeneity and tracked successfully clonal evolution over time. Investigation in larger cohorts of clinically well annotated patients are needed; however, our study could already illustrate how M^3P may serve as a practical tool to provide information needed to more precisely and more efficiently conduct individualized therapy concepts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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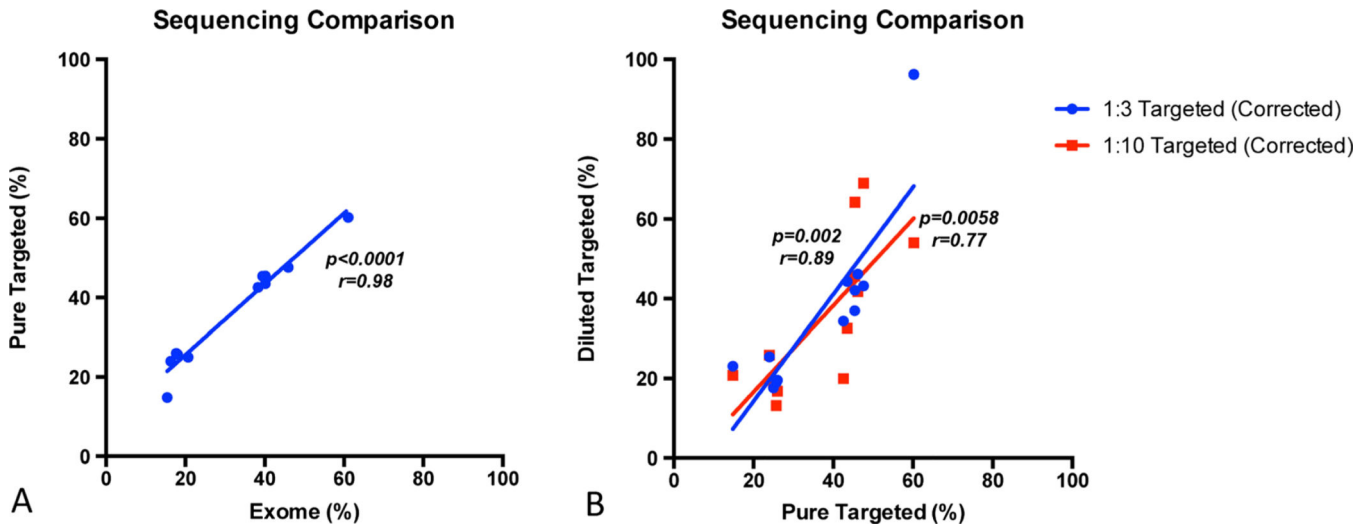

Fig. 1.

Correlation between Illumina exome and Torrent targeted semiconductor sequencing technology. **a** Correlation between techniques on the frequency of non reference allele reads of the undiluted tumor sample. **b** Correlation between WGS and the 1:3 and the 1:10 dilutions (dilution corrected)

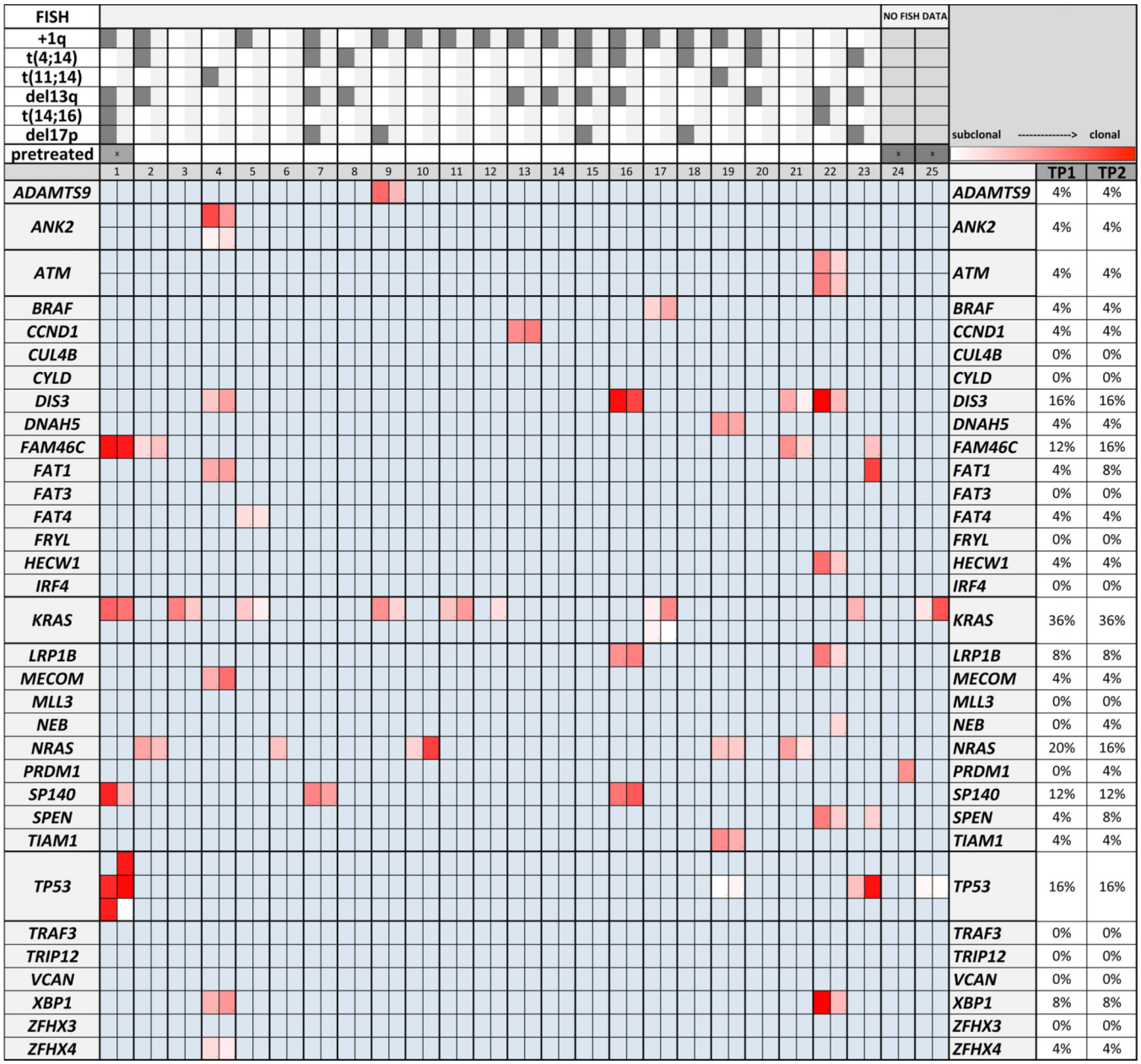

Fig. 2.

Track of clonal evolution in the longitudinal analysis and mutation prevalence in 22 untreated and 3 pretreated MM patients. Clonal evolution is evident in the majority of patients, with changes in clonal size (indicated by the heatmap), acquisition of a *TP53*, *FAM46C*, *FAT1*, *SPEN*, *PRDM1*, and *NEB* and a gain and loss of a *NRAS* and *KRAS* mutation over time, representing individual change of mutational profile under therapeutic selection pressure. Multiple mutations in one gene at the same time point were identified in *ANK2*, *ATM*, *KRAS*, and *TP53*; each mutation is represented by an individual line. FISH: Analysis was performed on time point 1 in patients 1–23

Table 1

The multiple myeloma mutation panel (M^3P) v1.0

ADAMTS9	DIS3	IRF4	NRAS	TP53
ANK2	DNAH5	KRAS	PRDM1	TRAF3
ATM	EGFR	LRP1B	PSMB5	TRIP12
BRAF	EGR1	LTN1	PSMG2	VCAN
CCND1	FAM46C	LYST	PTPRD	XBP1
CRBN	FAT1	MECOM	RASA2	ZFHX3
CUL4A	FAT3	MLL3	RB1	ZFHX4
CUL4B	FAT4	NBPF1	SP140	
CYLD	FRYL	NEB	SPEN	
DDB1	HECW1	NR3C1	TIAM1	

Table 2

This table illustrates the individual change of the mutational profile over time tracked by M^3P

	TP 1	TP 2
FAM46C	87 %	83 %
KRAS	57 %	51 %
SP140	81 %	22 %
TP53	78 %	90 %
	82 %	0 %
	0 %	84 %

Percentage in box represents purity corrected variant reads. In this patient one *TP53* mutation gets eradicated over time, whereas a second *TP53* mutation expands between the time points, suggesting the presence of at least two different clones. Additionally, a *SP140* mutation is decreased but not eradicated, suggesting the existence of a third clone. Furthermore, the mutation frequency of a *FAM46C*, a *KRAS* and a third *TP53* mutation stay unchanged over time and are not affected by the clonal evolution tracked by M^3 P, providing evidence that these mutations are clonal