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Loss Of COP9-Signalosome Genes At 2q37 Is Associated With IMiD Agent Resistance In Multiple Myeloma

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Abstract:
The acquisition of a multi-drug refractory state is a major cause of mortality in myeloma. Myeloma drugs that target the Cereblon (CRBN) protein include widely-used immunomodulatory drugs (IMiDs), and newer CRBN E3 ligase modulator drugs (CELMoDs), in clinical trials. CRBN genetic disruption causes resistance and poor outcomes with IMiDs. Here we investigate alternative genomic associations of IMiD resistance, using large whole genome sequencing patient datasets (n=522 cases) at newly diagnosed, lenalidomide (LEN)-refractory and lenalidomide-then-pomalidomide (LEN-then-POM)-refractory timepoints.

Selecting gene targets reproducibly identified by published CRISPR/shRNA IMiD resistance screens, we found little evidence of genetic disruption by mutation associated with IMiD resistance. However, we identified a chromosome region, 2q37, containing COP9-signalosome members *COPS7b* and *COPS8*, copy loss of which significantly enriches between newly-diagnosed (incidence 5.5%), LEN-refractory (10.0%) and LEN-then-POM-refractory states (16.4%), and may adversely affect outcomes when clonal fraction is high. In a separate dataset (50 patients) with sequential samples taken throughout treatment, we identified acquisition of 2q37 loss in 16% cases with IMiD exposure, but none in cases without IMiD exposure. The COP9 signalosome is essential for maintenance of the CUL4-DDB1-CRBN E3 Ubiquitin Ligase. This region may represent a novel marker of IMiD resistance with clinical utility.

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Title

Loss Of COP9-Signalosome Genes At 2q37 Is Associated With IMiD Agent Resistance In Multiple Myeloma

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Key Points

1. 2q37 copy loss enriches significantly between newly-diagnosed (~5%), LEN-resistant (10%) and POM-resistant (16.4%) myeloma

2. 2q37 carries *COPS7b* and *COPS8*, required for CRBN stability. Their partial loss leads to CRBN partial loss, which may blunt LEN/POM efficacy.

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Abstract

The acquisition of a multi-drug refractory state is a major cause of mortality in myeloma. Myeloma drugs that target the Cereblon (CRBN) protein include widely-used immunomodulatory drugs (IMiDs), and newer CRBN E3 ligase modulator drugs (CELMoDs), in clinical trials. CRBN genetic disruption causes resistance and poor outcomes with IMiDs. Here we investigate alternative genomic associations of IMiD resistance, using large whole genome sequencing patient datasets (n=522 cases) at newly diagnosed, lenalidomide (LEN)-refractory and lenalidomide-then-pomalidomide (LEN-then-POM)-refractory timepoints.

Selecting gene targets reproducibly identified by published CRISPR/shRNA IMiD resistance screens, we found little evidence of genetic disruption by mutation associated with IMiD resistance. However, we identified a chromosome region, 2q37, containing COP9-signalosome members *COPS7b* and *COPS8*, copy loss of which significantly enriches between newly-diagnosed (incidence 5.5%), LEN-refractory (10.0%) and LEN-then-POM-refractory states (16.4%), and may adversely affect outcomes when clonal fraction is high. In a separate dataset (50 patients) with sequential samples taken throughout treatment, we identified acquisition of 2q37 loss in 16% cases with IMiD exposure, but none in cases without IMiD exposure. The COP9 signalosome is essential for maintenance of the CUL4-DDB1-CRBN E3 Ubiquitin Ligase. This region may represent a novel marker of IMiD resistance with clinical utility.

Main Text

Introduction

Identification of causes and biomarkers of drug resistance in myeloma guide understanding of treatment failures, and development of targeted therapeutics. Genetic changes driving myeloma development are well described, including structural variants, copy number aberrations (CNAs) and gene mutations. Tumour genetic/epigenetic changes conferring survival advantage during drug exposure, and their contribution to drug resistant tumour clones, are less understood[1,2]. We previously reported genetic aberrations in Cereblon (CRBN), the target of immunomodulatory (IMiD) and CRBN E3 ligase modulator (CELMoD) drugs, associated with IMiD resistance. These include mutations, high levels of a splice variant skipping exon 10, CRBN structural variants, and heterozygous loss of the CRBN-containing 3p region; they exhibit strong therapeutic selection on lenalidomide (LEN) and/or pomalidomide (POM) treatment[3]. We hypothesized that homozygous or heterozygous genetic alterations in additional genes required for CRBN-targeting drug activity may be clinically relevant. Pharmacogenetic screens identifying genes essential for IMiD agent sensitivity *in vitro* have recurrently identifed COP9 signalosome (CSN) complex genes[4-9] (**Supplementary Tables 1&2**), required for maintenance of the CUL4-DDB1-CRBN E3 Ubiquitin Ligase. Although pharmacological CSN inhibiton is toxic to many cancer cell lines[10], in myeloma decrease in even one CSN subunit results in decreased CRBN protein levels and reduced LEN efficacy, because the CSN acts as a deneddylating 'off switch' for the CUL4-DDB1-CRBN E3 Ubiquitin Ligase. If deneddylating activity falls, CRBN protein is auto-ubiquitinated and degraded[4,5]. All CSN subunits 1-9 are required for deneddylating activity, although only one of COP7A or COP7B[11].

We asked whether disruption of any genes identified by *in vitro* IMiD agent-essential genetic screens bear relevance to IMiD agent resistance in the clinic. Due to the dominance of CNAs in myeloma[12], we interrogated association of screen-identified gene deletion in the IMiD-response pathway, with selection during IMiD agent exposure. These results assign novel significance to genomic regions in

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RRMM patients, specifically regarding therapy acquired-resistance to IMiDs and potentially CELMoDs.

Methods

Illumina whole-genome sequencing (WGS, coverage 60/30x tumour/germline) from 522 cases and RNASeq data from 189 cases was analysed as reported[3], although sample size was increased (**Supplementary Figure 1, Supplementary Table 3**). DNA was extracted from germline peripheral blood and baseline/relapse timepoint bead-enriched CD138+ myeloma cells, from patients in CC4074-MM010 (STRATUS; NCT01712789[13]), CC-4047-MM-007 (OPTIMISMM; NCT01734928[14]), CC-4047-MM-014-B (NCT01946477), CC220-MM001 (NCT02773030[15]) and CC122-ST-001-MM2 (NCT01421524) trials. Newly diagnosed (ND) patient data from IFM/DFCI-2009 (NCT01191060[16]) were also used.

Separately, clinically-annotated sequential myeloma patient samples from two UK biobanks (**Supplementary Table 4**) were analysed. Patients consented to research use of bone marrow aspirates from sequential disease timepoints. WGS was performed as above, although in certain instances the CD138- bone marrow fraction was used for germline DNA.

Results and Discussion

We adopted a hypothesis-driven approach identifying candidate genes whose loss may favour IMiD drug resistance from published pharmacogenetic screens (n=5 screens, **Supplementary Table 1**). We shortlisted 23 genes essential for LEN/POM activity in ≥2 screens (**Figure 1A, Supplementary Table 2**). In each WGS cohort (ND n=198, LEN-refractory n=269, LEN-then-POM-refractory n=55, defined in **Supplementary Methods**), incidence of LEN/POM-essential gene mutation in drug-refractory cohorts was rare, as previously found with *CRBN*[3]. Copy loss was more frequent (**Figure 1B**), although not uniformly increased across all genes and timepoints. We identified regions containing any of these 23 genes with a trend in enrichment of copy loss between ND, LEN, and LEN-then-POM-

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refractory states, using criteria of an overall incidence of >10% copy loss at the LEN-then-POM-refractory state, and incidence of copy loss that increased from ND to LEN-then-POM-refractory states by ≥1.5-fold (**Supplementary Figure 2**). These criteria delivered 3 regions demonstrating enrichment: a) 3p (*CRBN* locus as reported[3]) b) 17p (reported to be selected during myeloma progression[17] as site of *TP53,* in addition to three of the shortlisted IMiD response-essential genes: *UBE2G1*, *NCOR1* and *COPS3*; enrichment of loss may be driven by shared loss of *TP53*) and c) 2q37, previously unidentified as relevant in myeloma, but which contains two CSN members (*COPS7B, COPS8*) (**Figure 1c, Supplementary Figures 3/4**). The proportion of cases with *COPS8* loss significantly increased from 11/198 (5.5%, ND), to 27/269 (10.0%, LEN-refractory) and 9/55 (16.4%, LEN-then-POM-refractory), p=0.028. COPS7b loss increased from 8/198 (4.0%, ND), to 21/269 (7.8%, LEN-refractory) and 7/55 (12.7%, LEN-then-POM-refractory), p=0.034 (FDR-corrected chi-squared proportion trend tests). The proportion of cases where the copy loss was clonal also increased, between ND (36.4% *COPS8*, 50.0% *COPS7B*) and LEN-then-POM-refractory states (77.8% *COPS8*, 85.7% *COPS7B*) (**Figure 1C**). Patients who lost a copy of *COPS7B/COPS8* also demonstrated variable but significant reduction in their gene expression (p<0.01 both genes, 2-tailed T-test) (**Figure 1D**).

Outcome data with subsequent POM treatment was available in the LEN-refractory STRATUS cohort (n=188)[13], and with RVD induction in the ND cohort[16]. Although underpowered, when all 2q37 clonal fraction sizes were considered there was no difference in either cohort PFS (**Supplementary Figure 5a/c**) or ORR (31.5% vs 32.6%, STRATUS only) between cases with 2q37 loss and those without. In STRATUS, median PFS was 4.6 months and median POM-based treatment duration 4.9 months[13], likely inadequate time for small clones to drive a PFS different from the background rate. We therefore next confined analysis to 2q37-loss cases with cancer clonal fraction (CCF)>0.75 (following an approximated bimodal split of CCF distribution (**Figure 1C, arrow**). Small cohort size (7/188) hindered power, but in CCF>0.75 cases a trend to lower PFS was noted (p=0.09) (**Supplementary Figure 5b**), and ORR was 25% (2/8 responded). This association, including CCF>0.75 cut-off validation, will need confirming with larger cohorts.

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In a separate cohort of myeloma patients (n=50) with sequential WGS analysis before and after treatment (total n=127 tumours; ND n=32, non-LEN/POM exposure n=42, LEN-exposed/refractory n=41, LEN-then-POM-refractory n=12, **Figure 2A**), we traced acquisition and/or expansion of CNA-defined subclones post LEN/POM therapy vs timepoints post non-IMiD therapies (**Figure 2A-C**). 5/31 (16%) patients who had a LEN/POM-exposed timepoint acquired either clonal or subclonal loss of the 2q37 region containing *COPS7B* and *COPS8* at their LEN/POM-exposed timepoint. In four cases, this CNA had been either absent or below limit of detection pre-LEN/POM exposure. In one case, it was present at low level before LEN/POM exposure, but the patient had previous thalidomide (THAL)-based therapy. In this case, when LEN-based treatment ceased, the clone disappeared again (**Figure 2Cv**). In contrast to the LEN/POM-exposed timepoints, in the 42 timepoints without prior LEN/POM exposure, and the 32 ND timepoints, the only incidence of *COPS7B*/*COPS8*-containing regional 2q37 loss was the case who had had THAL-based prior therapy. In 2 cases, sequential histological material was available; plasma cell CRBN protein levels fell after 2q37 loss emergence, although variably on a per-cell basis (**Figure 2D**). We performed correlatory *in vitro* modelling of partial COPS7B or COPS8 loss, resulting in CRBN protein loss and reduced LEN-induced growth arrest (**Supplementary Figure 6**).

Although not previously shown to confer therapy-specific clonal advantages in clinical myeloma, CNAs may represent biomarkers of drug resistance. We demonstrated the contribution of CRBN copy loss in LEN/POM-refractory patients, and now identify a second therapy-related CNA, 2q37 loss, whose incidence increases through LEN- and POM-refractory states, emerging as a marker of dominant clones in IMiD-resistant disease. CRBN is critical to IMiD function, but whether these CNAs will mark resistance to novel CELMoD agents[18], or the kinetics of CELMoD-CRBN binding are as sensitive to relative CRBN protein loss or mutation, remains unaddressed. Both CRBN and CSN-

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member CNAs may be cost-effectively detected by additions to targeted sequencing approaches[19], which may prove useful in future therapeutic decision-making.

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Authorship Contributions

S.G. designed research questions, performed experiments, analyzed and interpreted the data, and wrote the manuscript; N.A.-P. led the computational and statistical analysis, interpreted the data and assisted with study design; M. K., K. K., F.T., and M.O.E. performed data analysis, statistical design, and data interpretation; M.S., Ev.F. and S.C. processed patient samples and collated clinical data; D.R. and K. S. performed IHC/ISH imaging and quantification; A.P. performed western blots; K.Y. and K.R. contributed clinical samples; E.F., K.R., K. Y. and P.V. assisted with study design and the

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manuscript; E.F. and F.T. performed oversight and management of MGP resources (data generation, infrastructure, and processing); and A.T. designed research questions, oversaw scientific direction, and assisted with the manuscript.

Conflict-of-interest disclosure:

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Figure Legends

Figure 1: Loss of *COPS7b* and *COPS8* genes on chromosome 2q37 increases in incidence at LEN and LEN-then-POM refractory states

(A) Genes (n = 23) and their chromosome location, identified from ≥2 published pharmacogenetic screens (n=5 screens, **Supplementary Tables 1 & 2**).

(B) Incidence of mutation or deletion (excluding cases with copy neutral LOH) in the 23 genes listed in (A) in 3 patient datasets: NDMM, LEN-refractory and POM-refractory. Incidence of 1q21 gain/amp (*CKS1B*), 1p loss (*CDKN2C*) and 17p loss (*TP53*) across the 3 patient datasets are provided for context.

(C) Proportion of samples with *COPS7b* and *COPS8* copy loss (excluding cases with copy neutral LOH) (LH y-axis) and their CCFs (RH y-axis) at NDMM, LEN-, and POM-refractory states. Significance detected by chi-squared test for trend in proportions with FDR correction (when compared with all 23 genes, for other genes see **Supplementary Figure 2**) Note: no instances of homozygous COPS7B or COPS8 loss were identified. Arrow in (C)ii. marks narrow point in CCF distribution taken as cut-off to divide high CCF (>0.75) from low CCF (<0.75) cases (used in **Supplementary Figure 5**)

(D) Difference in *COPS7b* and *COPS8* gene expression (mRNA expression by TPM) with presence or absence of gene copy loss. Significance detected by unpaired 2-sided T-test.

NDMM = Newly diagnosed multiple myeloma; LEN = Lenalidomide; POM = Pomalidomide; LOH = loss of heterozygosity; CCF = cancer clonal fraction; TPM = transcripts per million reads mapped.

Figure 2: Clonal fraction of 2q37 *COPS7b/COPS8* loss increases only during IMiD-based therapies

(A) Schematic showing sequential sample numbers analysed at each state of LEN or POM exposure/refractoriness

(B) Summary table of incidence of patients acquiring 2q37 loss (filtered to include loss of *COPS7b* and/or *COPS8* containing-regions only) during IMiD-based therapy vs no IMiD exposure

(C) 'Fishplot' diagrams demonstrating behavior of 2q37 loss-containing subclones (green-colored) over sequential samples, in relation to other CNA-defined subclones and drug exposure. Total tumour burden over time (grey area) is derived from serial serum M-protein/restricted free light chain measurements (taken at white vertical line timepoints). Only IMiD-containing drug regimes are marked. Subclone emergence points have been inferred by linear growth assumptions. Note behavior of subclones during intervening months/years between WGS sampling points (taken at black vertical line timepoints) is inferred, for example in plot (v), where precise max CCF of 2q37-containing subclone reached, and timing of its expiration is unknown. This plot shows one likely scenario.

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(D) For 2 of the cases in (C), CRBN immunohistochemistry (IHC) of bone marrow biopsies from same timepoints as the WGS data is shown, plus Kappa (κ) and Lambda (λ) light chain in situ hybridisation (ISH) to indicate tumour burden. Graphs show corresponding CRBN protein quantification (percentage of cell surface stained) across disease stage, each point representing one cell. Significance as shown determined by Mann Whitney (case 2, 2 timepoints) and Kruskal Wallis (case 3, 3 timepoints) tests for non-parametric data.

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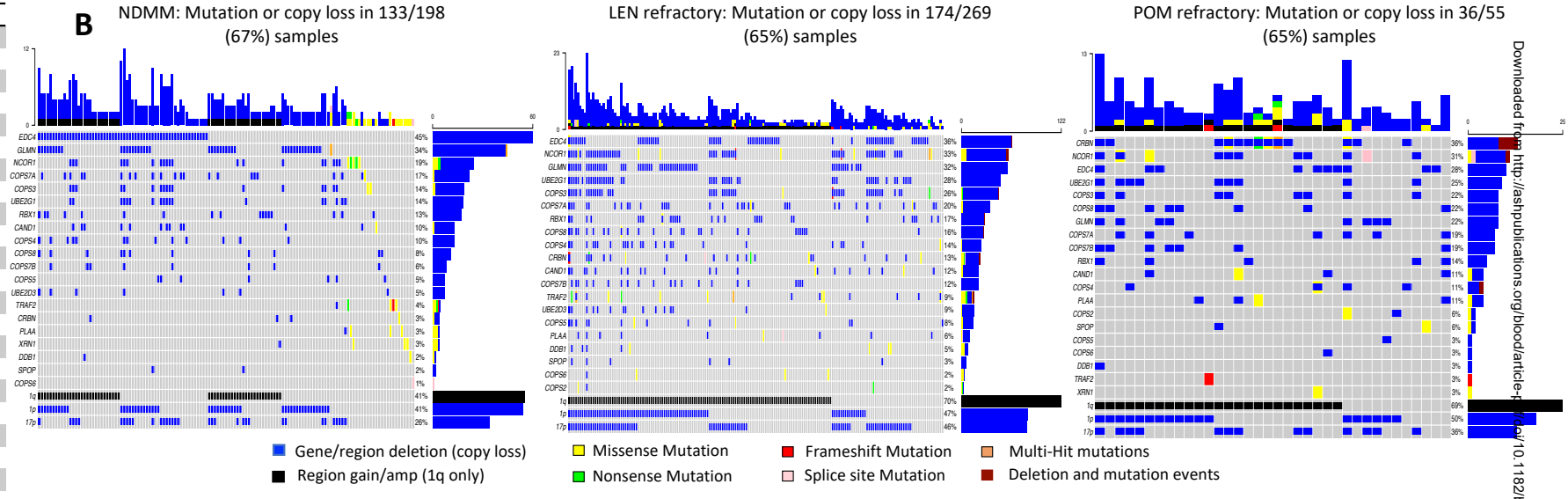
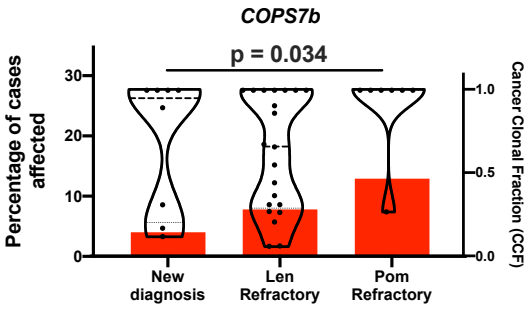
A

Gene	Chromosome
CAND1	chr12
COPS2	chr15
COPS3	chr17
COPS4	chr4
COPS5	chr8
COPS6	chr7
COPS7A	chr12
COPS7B	chr2
COPS8	chr2
CRBN	chr3
DDB1	chr11
EDC4	chr16
GPS1	chr17
PLAA	chr9
RBX1	chr22
TRAF2	chr9
UBE2D3	chr4
UBE2G1	chr17
UBE2M	chr19
SPOP	NA
XRN1	NA
NCOR1	chr17
GLMN	chr1

B



C i.



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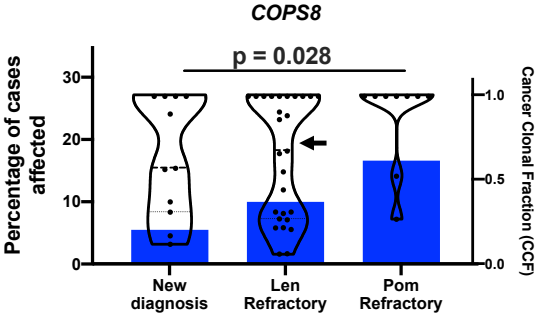

D

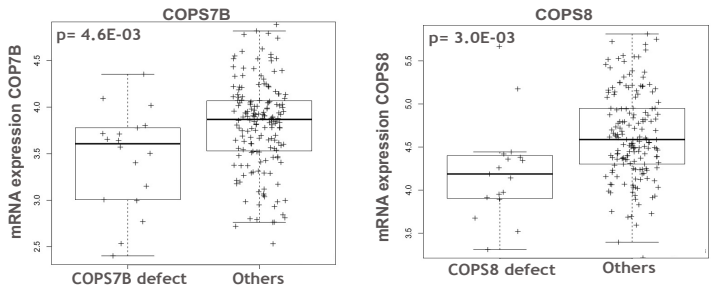

Figure 1

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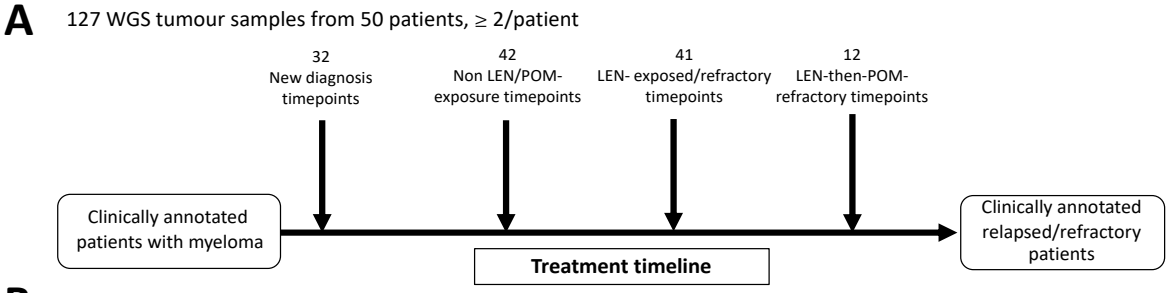
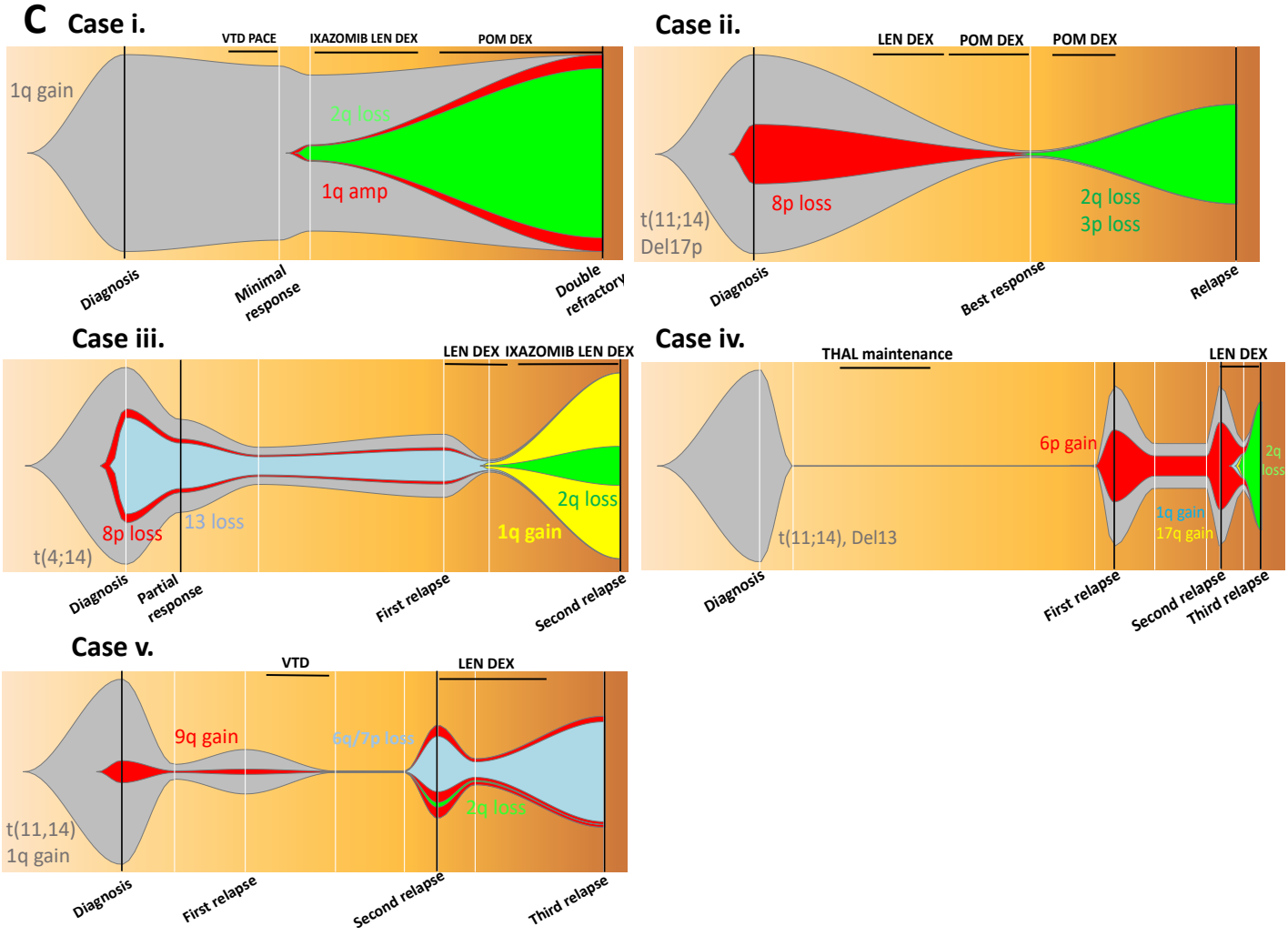


B

	LEN or POM exposed/ refractory timepoint	No LEN or POM refractory timepoint
Total patients (n)	31	19
Acquire new 2q37 loss	5 (16%)	0
2q37 loss pre-existent	0	0



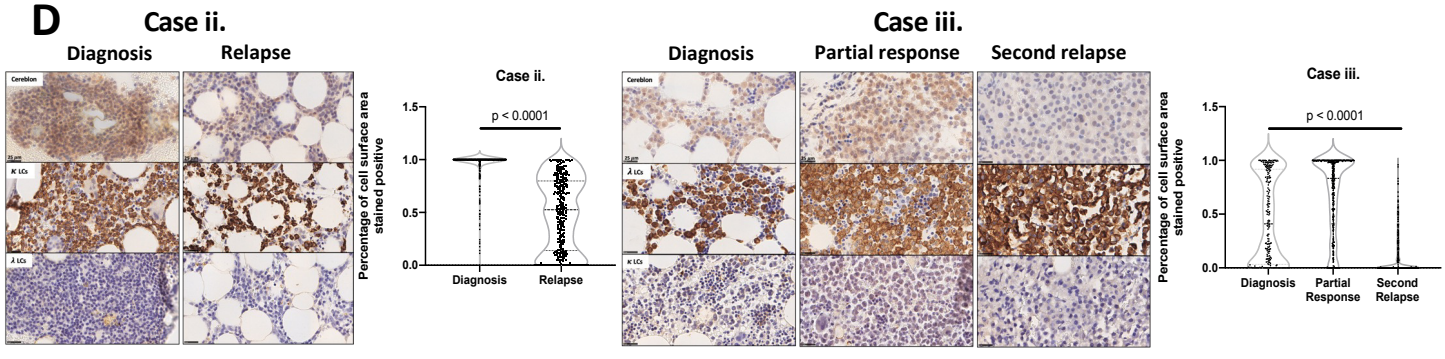


Figure 2

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