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Pathogenic BRCA1 mutations may be necessary but not sufficient for tissue genomic heterogeneity: Deep sequencing data from ovarian cancer patients

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HIGHLIGHTS

- Intra-patient tissue genomic heterogeneity (t-HET) is common in ovarian cancer (OVCA).
- t-HET coexists with pathogenic *BRCA1* & *TP53* mutations in tissues; such mutations do not necessarily coexist with t-HET.
- Pathogenic *BRCA1* mutations are shared in t-HET; *BRCA2* and *TP53* are not.
- Disrupted *BRCA1&2* are positively associated with t-HET; disrupted *TP53* is not.
- t-HET may reflect tissue plasticity against *BRCA1* and affect OVCA patient outcome.

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ABSTRACT

Background. Tissue genomic heterogeneity (t-HET) in patients with epithelial ovarian cancer (OVCA) is related to tissue plasticity, i.e., flexibility to adapt to adverse molecular environments. Here, we interrogated the presence and clinical relevance of OVCA t-HET.

Methods. We applied high-depth (>2000×) sequencing on 297 paraffin tissue samples (fallopian tubes, ovaries, intra-abdominal metastases) from 71 treatment-naïve patients who subsequently received first-line platinum-based chemotherapy. Based on tissue mutation patterns, we distinguished tissue genotypes into: no mutation (33/297 samples; 11.1%), stable (173; 58.2%) and unstable (91; 30.7%). We profiled genotypes per patient and assessed t-HET in 69 patients. Predicted pathogenic mutations refer to germline and/or tissues.

Results. Among all 71 patients, 46 (64.8%) had pathogenic BRCA1 mutations and 15 (21.7%) had BRCA1/2 disruption (i.e., pathogenic mutations with position-LOH). We classified 29 patients with t-HET (42%), all with pathogenic BRCA1; t-HET was observed in 64% with such mutations ($p < 0.001$). As opposed to non-t-HET, matched tissues in t-HET shared pathogenic BRCA1 ($p < 0.001$) but not BRCA2 and TP53. Germline BRCA1 mutations in tissues exhibited position-LOH; heterozygous status; or, partial loss of the inherited allele accompanied by additional clonal mutations. Patients with t-HET had worse outcome (log-rank $p = 0.048$ [progression-free]; $p = 0.037$ [overall survival]), including 12/15 patients with disrupted BRCA1/2 and 3 BRCA1 carriers with partial germline loss in tissues.

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Conclusions. Pathogenic BRCA1 mutations appear necessary but may not be sufficient for the establishment of t-HET. t-HET may be associated with worse outcome, including in patients with disrupted BRCA1/2, which is usually considered as a favourable marker. OVCA t-HET may need to be addressed for treatment decisions.

1. Introduction

Intra-tumor (or, intra-patient) genomic heterogeneity is tightly bound to so-called genomic plasticity, i.e., the ability of tumors to continuously adapt in adverse molecular environments [1–5]. Plasticity involves the continuous generation of new fit clones and the involution of less fit or incompetent ones and is therefore a dynamic process, whereas heterogeneity represents the static end-state of the same concept. Tissue geno/phenotypes may change during lifetime and during the course of a disease, with or without treatment [4]. Attempts to study plasticity, based on probabilistic modeling of tumor evolutionary dynamics over time remain currently arbitrary [2, 3]. Conversely, when examining multiple tissue samples or single cells from the same individual, we can observe and describe genomic heterogeneity, which is concrete; however, we can only infer genomic plasticity from heterogeneity; plasticity remains abstract.

Plasticity and the resulting intra-patient heterogeneity in high-grade serous ovarian cancers (OVCA) are linked to high genomic instability, which in turn is attributed to TP53 mutations and to defects in the homologous recombination repair (HRR) pathway [6–9]. Clone selection pressure in the context of plasticity has retrospectively been proven in OVCA by studying tumor genomics before and after chemotherapy [7,10–12] or treatment with PARP-inhibitors (PARPi's) [11,13–15]. In the prospective setting, however, the translation of the accumulated OVCA genomic data into clinical-grade biomarkers is restricted to the guidelines-driven application of BRCA1/2 mutation testing for cancer prevention (germline mutations) and for the selection of PARPi's (germline and tumor mutations) [16,17]. OVCA tissue plasticity is currently not addressed except after treatment failure. Moreover, heterogeneity and especially plasticity are hard to demonstrate in the regular diagnostic setting governed by the analysis of one representative sample, while surrogate markers for this important biological parameter are currently not available.

The primary objective of this study was to interrogate the presence and clinical relevance of genomic heterogeneity and inferred plasticity in tissues from patients with OVCA. For this purpose, we investigated multiple cancerous and non-cancerous tissue samples from treatment-naïve patients with high-depth panel sequencing. We classified tissue genotypes into 3 distinct classes of genomic integrity: no-mutation; stable; unstable. For demonstrating intra-patient genomic heterogeneity, we evaluated whether the same genotype class was retained among matched tissue samples from the same patient. Particularly for inferring plasticity, we traced the fate of germline mutations in the tissues of affected patients, where possible. We also examined the observed intra-patient genotype patterns in association with top mutated genes in OVCA, i.e., *BRCA1*, *BRCA2* and *TP53*, in order to investigate the potential utility of such mutation markers in this assessment of heterogeneity. Finally, we addressed the impact of intra-patient genomic heterogeneity on the outcome of the examined patients.

2. Methods

2.1. Patients and tissues

In this translational research study, we retrospectively analyzed biological material from 71 OVCA patients who underwent debulking surgery followed by standard paclitaxel–carboplatin chemotherapy and were monitored over a period of 10 years (2004–2014) at the Department of Medical Oncology, Papageorgiou Hospital, Faculty of Medicine, Aristotle University of Thessaloniki (AUTH). The available 376 formalin-fixed paraffin-embedded (FFPE) tissue blocks with surgical material (ovarian tumor, intra-abdominal metastatic sites, fallopian tube epithelium) were retrieved from the affiliated Pathology Department. All patients provided signed informed consent for research use of their material; the study was approved by the AUTH Bioethics Committee (Approval #79/10.6.2014) and by the Papageorgiou Hospital Institutional Review Board (193rd Meeting decision, 15.1.2014). Detailed patient demographic, clinicopathological, treatment and outcome data were retrieved from the Hellenic Cooperative Oncology Group (HeCOG) data office, Athens, Greece. Histological review, tissue processing for molecular studies and targeted NGS genotyping of FFPE and available matched whole blood DNA samples were performed at the Laboratory of Molecular Oncology (MOL; Hellenic Foundation for Cancer Research/HeCOG/AUTH). Germline DNA testing for patients with available whole blood samples was performed at the Molecular Diagnostics & Cytogenetics Laboratory (MDCL), National Center for Scientific Research 'Demokritos', Athens, Greece. The study is outlined in Fig. 1. Patient demographic and clinicopathological characteristics are shown in Table 1.

2.2. Tissue processing and DNA extraction

The steps for sample processing are shown in Fig. 1A and described in detail in Supplementary methods. Briefly, low-density tissue microarrays (TMA) were constructed from 349 paraffin blocks. Each tissue sample (fallopian tube epithelium [salpinx], primary tumor [ovary], metastatic site) was represented by 2 × 1.5 mm diameter cores. Tumor cell content (TCC%) and ciliated epithelium from fallopian tube fimbriae were evaluated on hematoxylin & eosin stained TMA core sections. TCC% was ≥60% in 75% of the samples and did not differ between primary and metastatic tumor samples (Mann-Whitney $p = 0.230$). In the group of fallopian tube samples the epithelium was morphologically normal or close to normal in the TMA cores; in whole sections from the same specimens, however, dysplastic changes and one in situ carcinoma were occasionally observed (Table 1).

FFPE DNA was extracted with the QIAamp® DNA Mini kit (Qiagen, Hilden, Germany) from 8 μm TMA core sections. Samples with >2 ng/μl DNA as measured with Qubit (Thermo – Fisher, Waltham, MA) were processed for NGS library construction.

2.3. NGS genotyping and technical evaluation

For genotyping, we used a custom Ampliseq panel (IAD75668_167; Applied Biosystems/Thermo-Fisher) targeting a total area of 36.8 Kb including coding regions in 40 genes on both FFPE and available germline DNA samples (Supplementary Table S1) that were analyzed in a Proton sequencer (Ion Torrent/Thermo-Fisher). Details for panel design, method, technical evaluation of samples and variants, including approaches to eliminate FFPE artifacts, and technical comparisons between normal and tumor FFPE samples are provided in Supplementary methods. By applying previously published criteria for variant filtering and sample eligibility [18] at higher stringency levels we obtained 20,717 variants for analysis. Out of 310 sequenced samples 297 (95.8%) were informative corresponding to a median 5 (mean: 4.3) samples per patient; we compared genotypes from ≥2 samples in 69/71 patients, and salpinx-ovary-metastasis genotypes in 32 patients (Fig. 1A). The

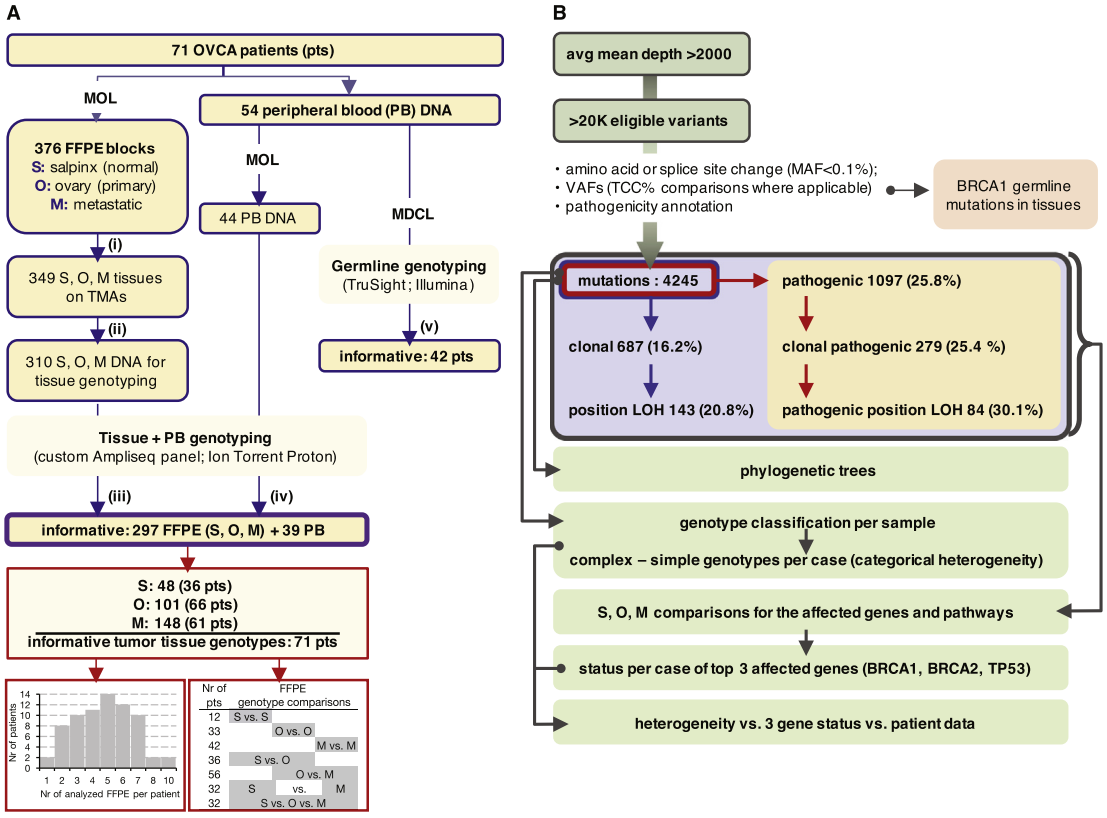


Fig. 1. Study outline. A: REMARK diagram on sample processing. MOL: Laboratory of Molecular Oncology; MDCL: Molecular Diagnostics & Cytogenetics Laboratory; (i): 27 paraffin blocks were excluded because of inadequate tissue; (ii) 39 DNA samples failed quality control and were not submitted for NGS; (iii) 13 FFPE samples (4.2% of sequenced FFPE) yielded non-informative NGS results; (iv) 1/44 PB samples was non-informative with the custom panel and 4 proved mismatched to the tissue samples; (v) 9/54 (16.7%) PB samples were non-informative with TruSight and 4 proved mismatched to the tissue samples. Informative FFPE were available for all 71 patients (≥2 samples in 69 patients). Tissue genotypes were compared for matched samples from the same (S vs. S; O vs. O; M vs; M) and among anatomical compartments (S vs. O; O vs. M; S vs. M). B. Outline of NGS data annotation and analysis. The large group of all mutations and the subgroup of pathogenic mutations were processed for presence, clonality and position-LOH (copy neutral) in all genes and pathways for comparisons among the three sample groups per patient, and among patients. Focus was paid on the three more frequently mutated genes and their associations with tissue genotypes and intra-patient/intra-tumoral heterogeneity. Percentages for clonal mutations and position LOH correspond to the immediately upper level, e.g., pathogenic position-LOH 30.1% was observed for clonal pathogenic mutations but it was only 7.6% of pathogenic mutations and 3.4% of all mutations. S, O, M as in A.

detailed origin of informative samples is shown in Supplementary Fig. S1. Informative samples had on average 2307 mean depth and 122 high quality variants; detailed technical characteristics are presented in Supplementary Fig. S2.

2.4. Bioinformatics, tissue genotype classes and heterogeneity

Details on bioinformatics analysis and on the rationale for the assessment of heterogeneity are provided in Supplementary methods. As outlined in Fig. 1B, we analyzed all non-synonymous coding and splice-site variants with minor allele frequencies (MAF) <0.1%; further, we evaluated predicted pathogenic mutations, based on combined variant characterization by Ion Reporter v5.6 and ANNOVAR for Clinical Significance (ClinVar), COSMIC ID and combined FATHMM/FATHMM-MKL scores. The term pathogenic mutation corresponds to all predicted pathogenic variants independent of their origin (germline, tissue or both). As clonal mutations we defined those with variant allele frequency (VAF) ≥25% [19]; in comparison, shared mutations were those observed in different samples from the same patient irrespectively of clonality. We assessed clonality and potential loss of the wild-type allele at variant position (position-LOH) by examining variant allele frequencies (VAFs) and TCC% where applicable. Mutations at low frequencies were occasionally shared in matched samples; such mutations were retrieved via manual inspection of the Integrative Genome Viewer (IGV) software and were prominent in fallopian tube samples. This observation led us to include all available mutations for the generation of mutation profile maps (R-software) and disease phylogenetic trees (PHYLIP, v3.69).

We used all available mutations for the classification of tissue genotypes. As previously described [18] and as shown in Supplementary Fig. S3, based (i) on the described diversity in the number of mutations (range 0–94) and mutated genes (range 0–29) among samples; and (ii) on the observation that some samples carried a high number of mutations in relatively few genes, we assessed the difference (N mutations − N mutated genes) and distinguished three genotype classes: (a) no-mutation (self-explanatory); (b) stable, if (N mutations − N mutated genes) was <8; unstable, if (N mutations − N mutated genes) was ≥8. We profiled multisite tissue genotypes in individual patients for the assessment of intra-patient genomic heterogeneity.

Based on pathogenic clonal mutations and position-LOH we presumed aberrant (disrupted) BRCA1, BRCA2 and TP53 function, as described previously [20].

2.5. Germline genotyping

As described in Supplementary methods and Supplementary Table S2, 15 patients were initially identified with germline mutations. All were validated with Sanger sequencing (primers available upon request) in the same blood samples and in the available tissue samples from 11 patients. Tissue validation failed in 4 patients, for whom tissue and germline DNA did not match, as proven with microsatellite ID-testing. For these patients we processed tissue data only. Thus, TruSight-tested germline results were considered for 42 patients; in

Table 1
Patient characteristics.

Age (years)		Performance status	
			46
Mean	58.4	0	(64.8)
			22
Median	59	1	(31.0)
Range	31.5–80.6	2	3 (4.2)
	N (%)	Histological type	
			14
Age (binary)		Endometrioid[a]	(19.7)
>60	37 (52.1)	Mucinous	4 (5.6)
			53
≤60	34 (47.9)	Serous[a,b,c]	(74.7)
Menopausal status at diagnosis		Histological grade	
Pre	18 (25.3)	I	2 (2.8)
			17
Post	53 (74.7)	II	(23.9)
			51
Family history		III	(71.8)
No	38 (53.5)	IV	1 (1.4)
Yes	33 (46.5)	Ovarian tumor manifestation	
			19
Previous other cancer		Unilateral	(26.7)
			52
No	68 (95.8)	Bilateral	(73.3)
		Germline mutations in cancer	
Yes[d]	3 (4.2)	predisposing genes	
			11
Stage at diagnosis		Positive[e]	(15.5)
IIb	1 (1.4)	Negative	31
			(43.7)
IIc	1 (1.4)	Not tested/non-info	29
			(40.8)
III	2 (2.8)		
IIIa	3 (4.2)		
IIIb	4 (5.6)		
IIIc	44 (62.0)		
IV	16 (22.5)		

[a] Differential diagnosis included fallopian tube origin in 3 cases.
[b] Differential diagnosis included peritoneal origin in 3 cases.
[c] Tubal in situ carcinoma in one patient.
[d] 3 patients had breast cancer 20, 18 and 3 years before OVCA diagnosis.
[e] 26.2% carriers among informative patients.

39 of them germline DNA was also sequenced with the Ampliseq panel parallel to FFPE samples (Fig. 1A).

2.6. Statistics

Mutation numbers were examined as continuous and categorical variables. In order to avoid as much as possible small sample bias, we did not interpret statistical results for categories with ≤15 members (20% of the patient cohort); we evaluated Pearson's, Fisher's exact, and Kruskal-Wallis 2-sided results at a level of significance <0.05. For this study, follow-up was updated in December 2016. We considered progression-free (PFS) and overall survival (OS) from the date of treatment start until event, last contact or loss from follow-up. We also classified response to platinum chemotherapy based on the interval between the dates of last platinum-based chemotherapy and disease progression [21]: refractory — platinum-resistant (progression within 6 months); intermediate sensitivity (6–<12 months); platinum-sensitive (≥12 months).

3. Results

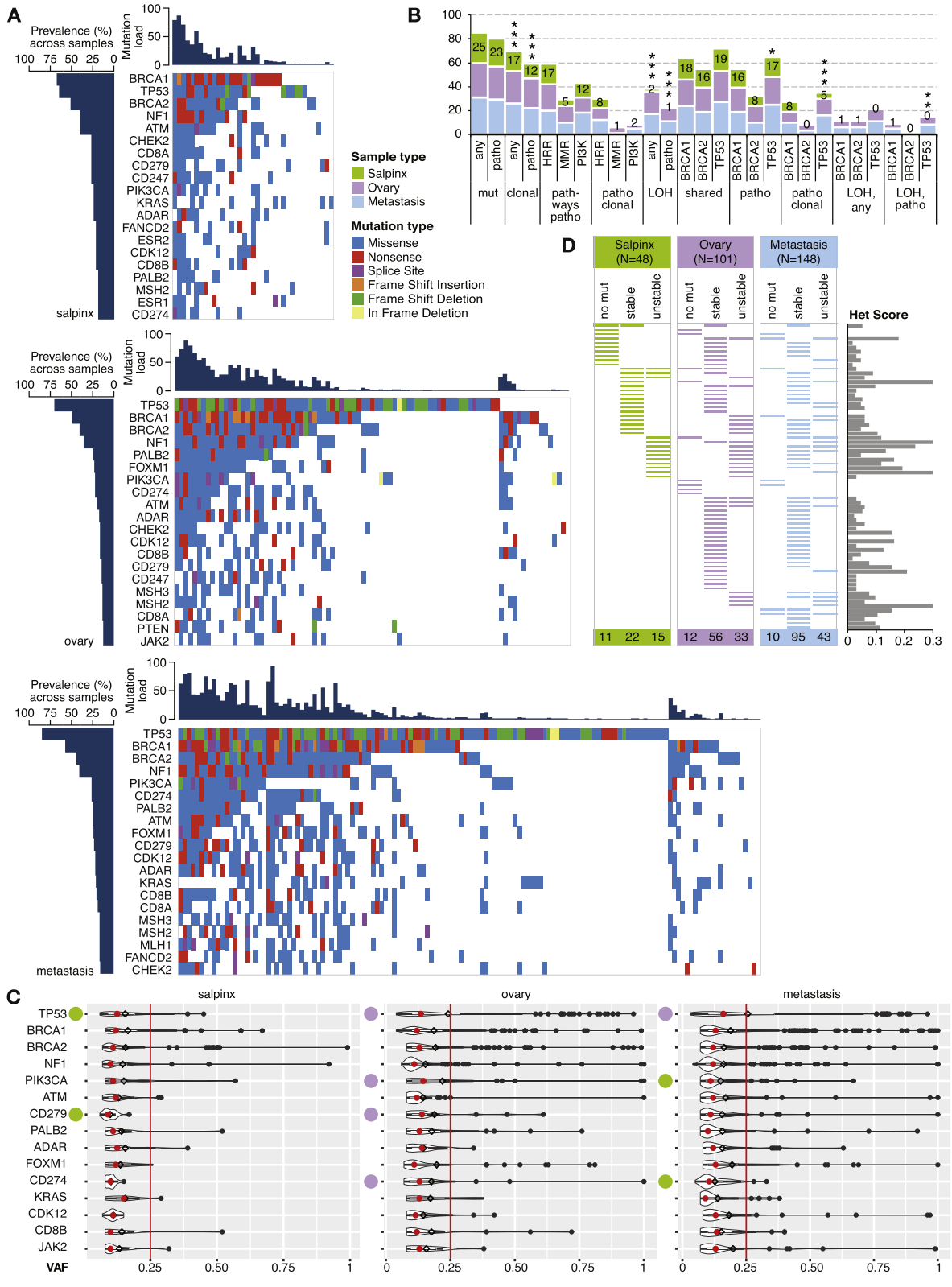
Patient demographic and clinicopathological characteristics are shown in Table 1; 53 (75%) patients had FIGO stage III disease; 43 out of 53 (81%) patients with serous, 8 out of 14 with endometrioid and 1 out of 4 with mucinous carcinomas had bilateral ovarian manifestation. Out of 11 germline mutation carriers, 8 had inherited a pathogenic mutation in BRCA1 and one each in BRCA2, CHEK2 and BLM (Supplementary Table S2).

3.1. Distinct genotype characteristics in fallopian tubes, ovaries and metastatic sites

Excluding the above 11 germline mutations, the Ampliseq panel revealed 4245 mutations in 39 genes; out of all mutations 2602 were unique and 1097 were pathogenic (Fig. 1B). These were distributed in 265 out of 297 (89.2%) tissue samples from 65 out of 71 (92%) patients. Most mutations (84%) were detected at low frequencies albeit at high coverage; for mutations with VAFs <25%, median and mean position coverage were 1365 and 1334, respectively, while mean and median variant coverage were 150 and 153.6, respectively. Samples without mutations were not technical failures, since their performance characteristics (average mean depth and uniformity 3289.8 and 84.8, respectively) did not differ from those in samples with mutations (average mean depth and uniformity 2818.2 and 84.3, respectively; Mann-Whitney $p = 0.402$ and $p = 0.997$, respectively).

In all tissue sample groups, top genes with point mutations or small indels were BRCA1, TP53, BRCA2 and NF1 (Fig. 2A; Supplementary Tables S3 and S4). In comparison to fallopian tube samples, ovarian tumors and metastases had a higher mean number of mutations per sample (19.6 vs. 20.9 and 35.4, respectively; Mann-Whitney $p = 0.042$); of pathogenic mutations per sample (3.9 vs. 8.6 and 6.5, respectively; $p = 0.049$), and were more frequently mutated (Pearson's $p = 0.014$) (Fig. 2B; Supplementary Table S5). Mutations, pathogenic or not, exhibited significantly lower VAFs in fallopian tube samples compared to ovarian tumors and metastases (p's < 0.001) (Fig. 2B and C). Compared to fallopian tubes, clonal TP53 mutations were more frequent in ovarian tumors and metastatic sites (Fig. 2C, Supplementary Table S5), which also applied for CD279; compared to metastatic sites, clonal CD274 and PIK3CA mutations were more frequent in tumors (Fig. 2C). Although interesting, the latter findings were not further pursued due to the small number of involved patients.

Fig. 2. Mutation patterns in tissues from patients with ovarian cancer. A: Tissue mutations mapped per site of sample origin. Mutation profiles of 37 FFPE from fallopian tube epithelia (salpinx), 89 from ovarian tumors and 139 from metastatic sites are shown. Maps include all mutations (pathogenic, of unknown significance and benign). Because samples were read at very high depth, tissues appeared overloaded with mutations in multiple genes. In patients with mutated tissues, the mutation rate (prevalence) of top affected genes was: TP53 87.7%; BRCA1 70.8%; BRCA2 46.2%; NF1 41.5%. B: Comparison of mutation incidence in the three anatomical sample groups. Results from the 32 patients with matched sample trios (salpinx–ovary–metastases) are shown. Piled bars correspond to the number of patients with alterations for the indicated categories (X-axis) in the fallopian tubes (salpinx), in ovarian tumors and in metastatic sites. Numbers in the piled bars are shown for fallopian tubes only due to space limit but can be inferred for the remaining categories. ***: Pearson's p values < 0.001; **: p values < 0.01–0.001; *: p value < 0.05–0.01. C. Comparison of mutation VAFs in the 15 most frequently altered genes in fallopian tube epithelia, ovarian tumors and metastatic samples. Within plots, red dots and open diamonds indicate VAF median and mean values per gene, respectively. Green and purple dots indicate low and high VAFs, respectively, for statistically significant comparisons. Vertical red lines: 25% VAFs. In comparison to the noncancerous fallopian tubes (salpinx), mutations in TP53 and in CD279 (PDCD1 or PD1), exhibited significantly higher VAFs in ovarian tumors ($p = 0.005$ and $p = 0.010$, respectively), while VAFs of TP53 mutations were significantly higher in metastatic sites as well ($p < 0.001$). In comparison to ovarian tumors, mutations in PIK3CA and in a further immune checkpoint, CD274 (PDL1), were present at lower VAFs in metastatic sites ($p = 0.031$ and $p = 0.034$, respectively), although tumor cell content did not differ between these sites. D. Genotype heterogeneity in tissues from patients with OVCA. Tissue genotypes were classified into no-mutation, stable and unstable. Each row represents tissue genotypes from the same patient; each cell represents the presence of genotype class in one or more (up to 7) samples per patient. Different genotype classes coexisted in 29 patients (complex or heterogeneous disease), while in the remaining cases single-class genotypes were present (simple or non-heterogeneous disease). For comparison, the continuous values of the heterogeneity score (HET score) are shown on the right (range 0–0.3). Numbers at the bottom of the chart: sum of samples with the corresponding genotype class per tissue origin.



Bilateral disease, pathogenic BRCA1 mutations including those in the BRCA1 C-terminal (BRCT) domain and pathogenic TP53 mutations were more frequent in serous than in endometrioid cancer (Supplementary Table S6), in line with previous observations [22,23]. Pathogenic TP53 mutations and TP53 position-LOH were more frequent in metastatic samples from serous compared to endometrioid cancers (Pearson's $p = 0.015$ and $p = 0.009$, respectively). None of the 4 patients with mucinous carcinomas carried pathogenic BRCA1 mutations, while one of them had pathogenic BRCA2 and TP53; none had clonal mutations or position-LOH in these genes, in line with a recent report [24].

Table 2
Associations of clinicopathological and mutation parameters with intra-patient tissue heterogeneity status, as indicated by heterogeneous and non-heterogeneous genotype patterns in patients with ovarian cancer.

	Genotypes				*p* value
	het	%	non-het	%	
Genotype classes per patient					
noMUT (NM)	0		4		n.a.
Stable (St)	0		24		
Unstable (US)	0		4		
NM & St	0		8		
St & US	23		0		
NM & St & US	6		0		
Salpinx, all mutations					
N patients	20		7		0.0403
Mean	29.6		17.1		
Median	21		2		
Range	2–87		0–79		
Ovary, all mutations					
N patients	26		31		<0.0001
Mean	46.2		5.9		
Median	47.5		2		
Range	3–116		0–40		
Metastases, all mutations					
N patients	29		29		<0.0001
Mean	64		10.6		
Median	38		5		
Range	3–184		0–78		
Histological type					
Endometrioid	4	13.8	10	25.0	0.1356
Mucinous	0	0.0	3	7.5	
Serous	25	86.2	27	67.5	
Response to 1st line platinum-based chemotherapy					
Non-responder[a]	9	31.0	12	30.0	0.0858
Poor responder	10	34.5	5	12.5	
Responder	8	27.6	14	35.0	
Super responder[b]	2	6.9	9	22.5	
BRCA1, pathogenic					
No	0	0.0	24	60.0	<0.0001
Yes	29	100.0	16	40.0	
BRCA2, pathogenic					
No	5	17.2	34	85.0	<0.0001
Yes	24	82.8	6	15.0	
TP53, pathogenic					
No	0	0.0	12	30.0	0.0004
Yes	29	100.0	28	70.0	
BRCA1, clonal pathogenic					
No	8	27.6	33	82.5	<0.0001
Yes	21	72.4	7	17.5	
BRCA2, clonal pathogenic					
No	24	82.8	38	95.0	0.1133
Yes	5	17.2	2	5.0	
TP53, clonal pathogenic					
No	9	31.0	15	37.5	0.4616
Yes	20	69.0	25	62.5	
BRCA1, position LOH					
No	20	69.0	37	92.5	0.0113
Yes	9	31.0	3	7.5	
BRCA2, position LOH					
No	22	75.9	39	97.5	0.0066
Yes	7	24.1	1	2.5	
TP53, position LOH					
No	21	72.4	20	50.0	0.1379
Yes	8	27.6	20	50.0	
Shared pathogenic mutations					
No	1	3.4	13	32.5	0.0051
Yes	28	96.6	27	67.5	
Shared pathogenic BRCA1					
No	9	31.0	35	87.5	<0.0001
Yes	20	69.0	5	12.5	
Shared pathogenic BRCA2					
No	23	79.3	36	90.0	0.338
Yes	6	20.7	4	10.0	
Shared pathogenic TP53					
No	12	41.4	17	42.5	1
Yes	17	58.6	23	57.5	

Notes: Where not indicated, the number of patients is 69; het: heterogeneous; non-het: non-heterogeneous; n.a.: not applicable.
[a] Including 2 patients with mucinous carcinomas.
[b] Including 1 patient with mucinous carcinoma.

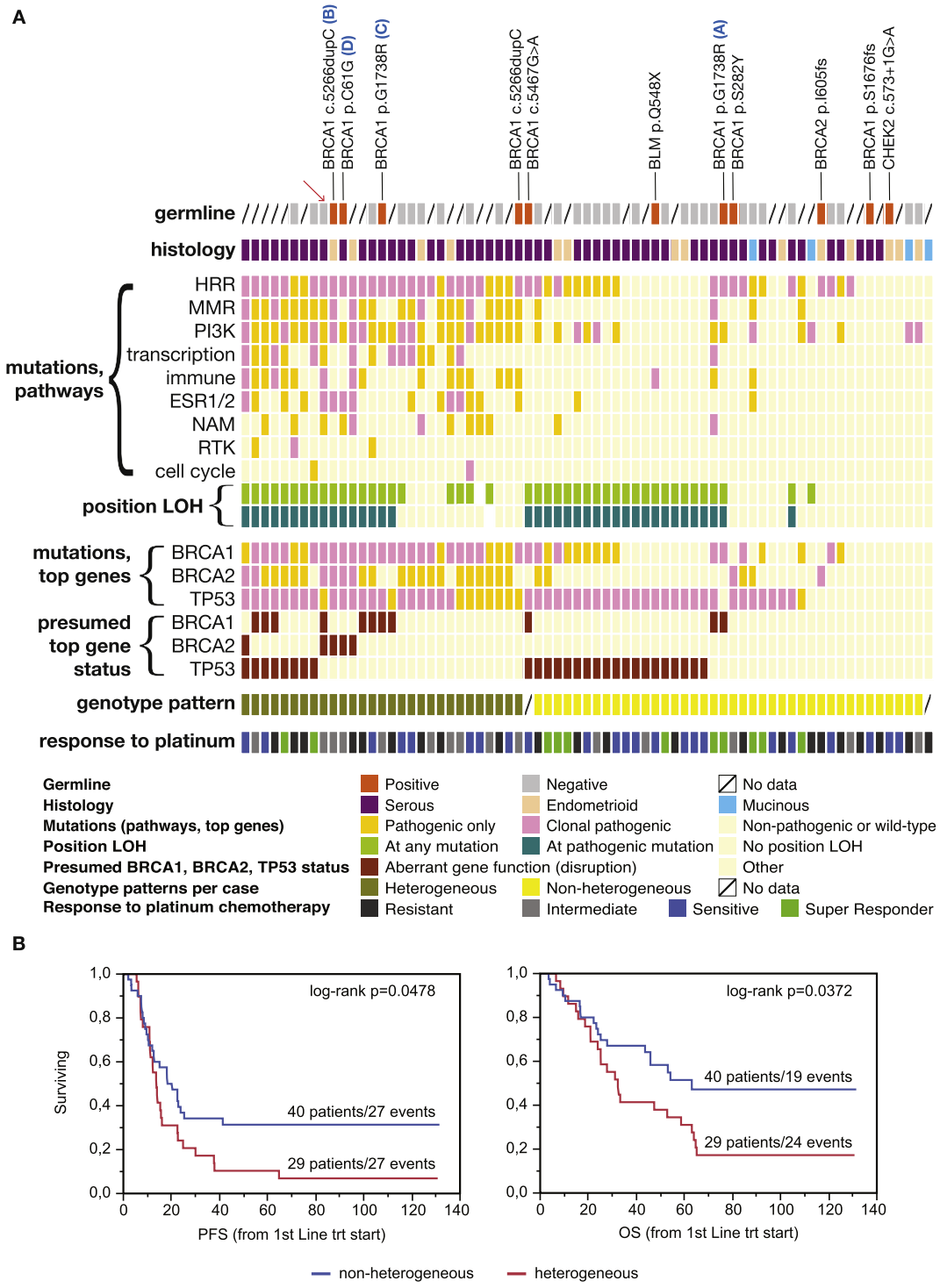
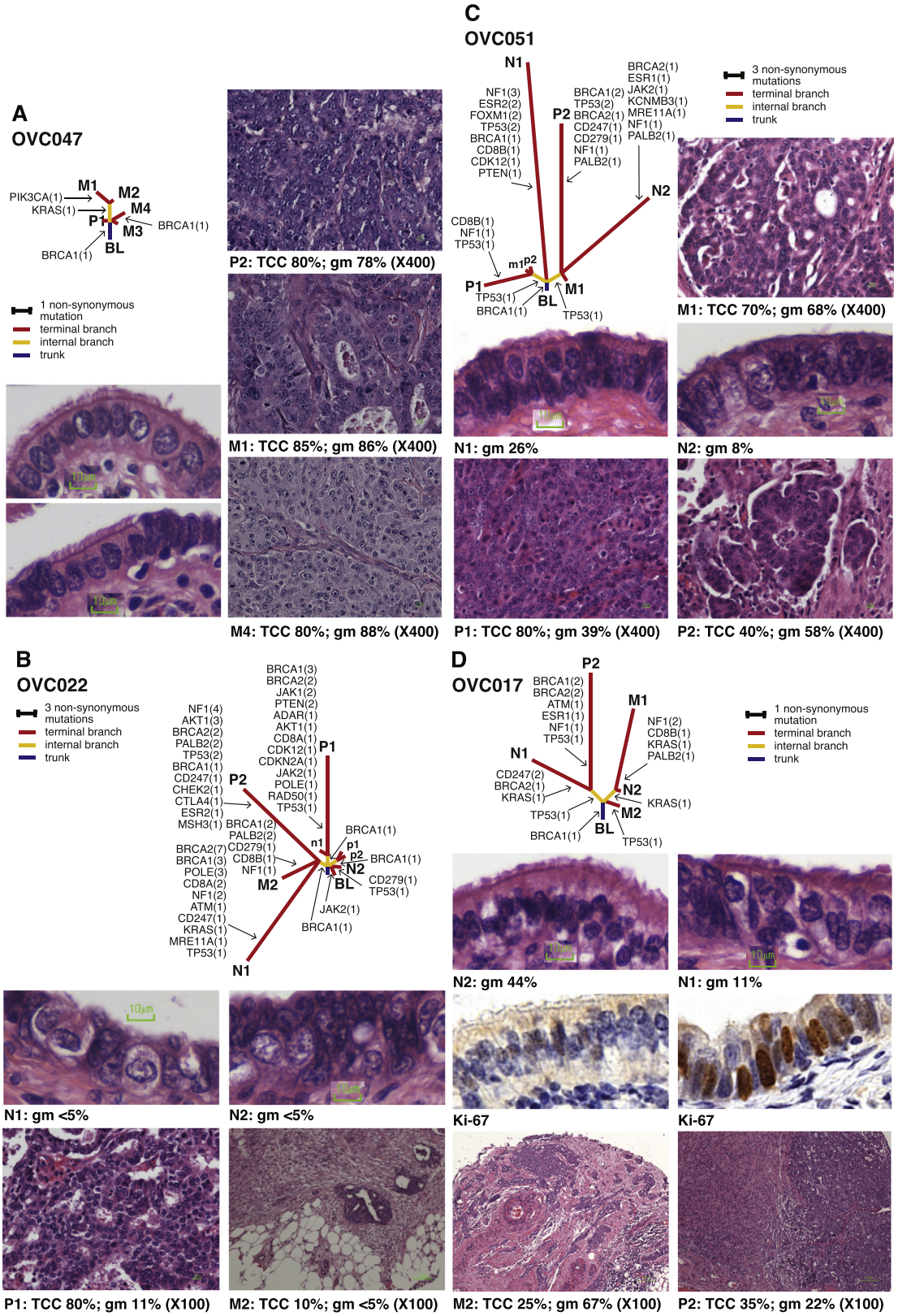


Fig. 3. Genomic characteristics and clinical associations of OVCA intra-patient heterogeneity. A. BRCA1/2 and TP53 mutation portraits were related to histology, intra-patient genomic heterogeneity and response to platinum chemotherapy. Mutations are presented for pathogenicity, clonality and position-LOH; for BRCA1 and TP53, for which multiple mutations were occasionally present in tissues from the same patients, the affected gene domains have been added. Mucinous carcinomas were negative for pathogenic BRCA1 mutations, as expected. BRCA1 pathogenic, clonal pathogenic and LOH were observed in 46 (64.8%), 28 (39.4%) and 12 (17%) patients, respectively. In the same order for BRCA2, 30 (42.2%), 7 (9.9%) and 8 (11.2%) patients were affected. Lastly, for TP53, 59 (83.1%), 45 (63.4%), and 28 (39.4%) were affected. BRCA1 was completely disrupted (pathogenic clonal mutation and LOH) in 12 (17%), BRCA2 in 7 (9.9%) and TP53 in 25 (35.2%) of the patients. Complete disruption of BRCA1 and BRCA2 (red arrow) was identified in 1 patient with heterogeneous disease and poor outcome. In this patient, BRCA1 was found in 2/4 metastases, additionally to BRCA2 in the ovarian tumor and in the remaining metastases. No patient had complete disruption of all three genes. Note that all patients with heterogeneous disease had pathogenic tissue BRCA1 ($p < 0.001$). With respect to outcome, 26/29 patients with heterogeneous disease relapsed in <5 yrs, 11 of them in <6 months from last platinum treatment. Blue suffixes after germline mutation names: cases shown in Fig. 4. B. Intra-patient genomic heterogeneity was associated with worse patient outcome. Log-rank tests are shown for the 69 patients with data available for heterogeneity. Compared to non-heterogeneous disease, patients with heterogeneous disease suffered more relapses (93.1% vs. 76.5%) and deaths (82.7% vs. 47.5%), in shorter time (median PFS 13.8 vs. 19.4 months; median OS 32.3 vs. 63.1 months).

3.2. Intra-patient tissue genotype heterogeneity in the presence of OVCA

Analysis of eligible mutations in matched tissues allowed for the creation of phylogenetic trees in 63 patients (presented for all patients in Supplementary Fig. S4). We identified ancestral clones in 57 patients: BRCA1 mutations in 29 and TP53 in 37, coexisting in 16 patients; BRCA2 in 7, coexisting with BRCA1 and/or TP53 in 3; KRAS ancestry in the 2 mucinous carcinomas; and, no ancestral clones in 8 patients. Phylogenetic tree branches reflected the individual sample genotype classes that are described in Supplementary Fig. S5: no-mutation genotypes



(33/297 samples; 11.1%), stable (173/297; 58.2%), and, unstable (91/297; 30.7%). The distribution of these tissue genotype classes did not differ in the three anatomical compartments.

We assessed intra-patient genomic heterogeneity by using (a) a categorical approach, i.e., profiling the presence of the above tissue genotypes in the 69 patients with ≥2 tissue samples, and (b) a previously described continuous heterogeneity score [25] (Fig. 2D). The concept for the categorical classification of heterogeneity is presented in detail in Supplementary methods. Briefly, no-mutation, stable and unstable genotypes were considered to result from different mutational processes in each case. Thus, patients with no-mutation, stable and unstable genotypes in their tissues or with stable and unstable genotypes were considered to have complex, i.e., heterogeneous disease. Patients with the same genotype class in multiple tissue samples or with combinations of no-mutation and stable genotypes were considered to have simple, i.e., non-heterogeneous disease (Table 2, Supplementary Fig. S5). No-mutation and stable genotypes were grouped together because mutations in single genes may have been missed in the former with the applied limited panel. Twenty-nine patients were classified with heterogeneous and 40 with non-heterogeneous disease. Of note, the 4 patients with only unstable genotypes in multiple samples were also classified as non-heterogeneous. The combination of no-mutation and unstable genotypes without the stable class was not observed (0/69 patients).

3.3. Associations between intra-patient genomic heterogeneity and mutation patterns in BRCA1/2 and TP53 might provide surrogates with clinical relevance

The number of examined samples appeared to be a confounding factor for calling intra-patient disease heterogeneity with either our categorical approach or the continuous score; in addition, heterogeneous disease was significantly more frequent among the 32 patients with informative samples from all anatomical sites compared to all other patients, a fact that was not significantly associated with the number of examined samples per patient in this subgroup (Supplementary Fig. S6). Heterogeneous disease was significantly associated with pathogenic mutations in the HRR, mismatch repair (MMR), PI3K, and immune-response-related pathways, and with the entire pathology spectrum of BRCA1/2 (all p's < 0.001). Heterogeneous disease was not associated with TP53 mutation clonality and position-LOH (TP53 disruption). Importantly, in heterogeneous disease, pathogenic BRCA1 mutations were frequently shared in matched samples ($p < 0.001$) but BRCA2 and TP53 were not (Table 2).

The observed clinicopathological and mutational associations are landscaped in Fig. 3A. Heterogeneous disease was further associated with clonal pathogenic mutations in the MMR ($p < 0.001$) but not in the PI3K pathway. In the entire cohort, 15/69 (21.7%) patients carried disrupted BRCA1/2 in their tissues and 27 (40%) disrupted TP53. Triple disruption was not observed; BRCA1/2 and TP53 disruption coexisted in only 2, while BRCA1 and BRCA2 in only 1 case. The latter patient had disrupted BRCA2 in the ovarian tumor, while disrupted BRCA1 was acquired in 2/4 metastatic sites. Most endometrioid carcinomas (10/14) were within the non-heterogeneous group; only 2/14 had TP53 disruption.

Although the present cohort was small, we examined the presence of intra-patient genomic heterogeneity in association with response to platinum chemotherapy, progression-free (PFS) and overall survival (OS). Among all 71 patients, there were 22 resistant to treatment; 15 intermediate responders; and 23 sensitive to treatment. In addition, we classified 11 super-responders (15.5% of the cohort) without events for >5 years; 5 of these were without event for >10 years (PFS and OS range: 60.9–131.5 months). Age, histological type and grade, disease stage, germline status, and bilateral disease did not differ between super-responders and the remaining patients. Most super-responders (9 out of 11) and patients sensitive to platinum chemotherapy (14/22) had non-heterogeneous disease (Table 2 and Fig. 3A). Of note, the 2 super-responders with heterogeneous disease had TP53 disruption only. The median follow-up for the present series was 71.5 months (range: 2.2–131.5 months). In this period, 56 (78.9%) patients experienced disease progression (median PFS: 15.3 months) and 44 had died of disease (median OS: 47.4 months). Intra-patient heterogeneity was associated with worse PFS and OS (Fig. 3B). Excluding 4 patients with mucinous carcinomas yielded similar results. HETscore was not associated with patient outcome, either as a continuous or as a categorical variable (JMP v.10 partitioning cut-off).

3.4. Germline mutations in tissues

Five out of 11 germline mutation carriers (4 out of 8 BRCA1 carriers) had heterogeneous disease but these patients were too few for statistics to be applied. On a case by case evaluation, carriers randomly had heterogeneous disease. Tissue VAFs were available in 6 BRCA1 mutation carriers (1 p.C61G, 2 p.G1738R, 2 c.5266dupC, 1 c.5467G>A). We observed the expected BRCA1 disruption in tissues from 3 patients only; two patients had BRCA2 position-LOH, while in one no BRCA1/2 LOH was identified (Fig. 3). It was further noticed that in 3 out of the above 6 patients, the germline mutation was present at VAFs lower than expected in some or all matched tissue samples, while alternative pathogenic mutations were present at clonal frequencies (Supplementary Table S3). This phenomenon was observed in samples from all anatomical sites and was partly or entirely present in fallopian tube tissues from the 3 patients with available such samples (Fig. 4). In comparison to patients with overt BRCA1 disruption (Fig. 4A), fallopian tube epithelia from samples with underrepresentation of the germline mutation exhibited disorientation, loss of cilia (Fig. 4B–D) and, in one case, high proliferation rate (Fig. 4D). Of note, matched tissues in two patients exhibited all patterns of germline mutation load: heterozygous VAF around 50%, underrepresentation, and position-LOH (Fig. 4C and D). Patients with underrepresented germline mutation in tissues had heterogeneous disease (Fig. 3 and corresponding phylogenetic trees in Fig. 4).

With respect to outcome, there was only one super-responder among the 11 germline carriers (BRCA1 p.G1738R with disrupted BRCA1 in tissues [Fig. 4A], PFS and OS 75.3 months, alive without any

Fig. 4. Case examples with underrepresented germline allele in tissues Microphotographs showing fallopian tube epithelia (N), ovarian tumors (P) and metastases (M) from 4 BRCA1 carriers, one with position-LOH at the germline mutation in all examined samples (A) and three with underrepresented germline mutation load in tissues (B–D). Phylogenetic trees are shown in each case in comparison to the indicated germline mutation (gm) status in the samples (% corresponds to mutant allelic frequency [VAF]). Magnifications for fallopian tube epithelia are 400×; all others, as indicated. A–D: Case order corresponds to the suffixes described in Fig. 3. A. OVC047, 51 y.o., serous carcinoma, BRCA1 p.G1738R (c.5212G>A), simple genotype pattern (non-heterogeneous); simple evolution tree. The normal tissue sample was non-informative for genotyping but tumor and metastatic samples exhibited position-LOH for the germline mutation. Note the well preserved ciliated fallopian tube epithelium in N. The patient had no event after 75.3 months from 1st line treatment stop (super-responder). B. OVC022, 66 y.o., endometrioid carcinoma, BRCA1 p.Q1777fs (c.5266dupC), complex genotype pattern (heterogeneous), very complex evolution tree. The germline mutation was underrepresented in all examined samples. Note aberrant orientation and multilayering in the fallopian tube epithelium (N). The patient progressed within 9 months from last platinum treatment (intermediate responder). C. OVC051, 40 y.o., serous carcinoma, BRCA1 p.G1738R (c.5212G>A), heterogeneous, complex evolution tree. In comparison to the fallopian tube with clonal germline mutation (N1), the one with underrepresented germline mutation load (N2) shows morphological changes similar to those in (B). Tumors had germline VAF at the expected range, while position-LOH was inferred for the metastatic site. The patient had the same germline mutation as in (A) but progressed within 7.2 months from last platinum treatment (intermediate responder). D. OVC017, 49 y.o., serous carcinoma, BRCA1 p.C61G (c.181T>G); heterogeneous; complex evolution tree. Again, the germline mutation was underrepresented in one fallopian tube (N1) and the ipsilateral tumor (P2), where BRCA2 disruption was noticed. Clonal germline mutation was present in the contralateral fallopian tube and position-LOH in the metastases. In comparison to the morphologically normal N2, the aberrant epithelium in N1 was highly proliferative, as indicated with Ki67 labelling. Again, the patient progressed within 6.7 months from last platinum chemotherapy (intermediate responder).

event); the BLM, CHEK2 and 2 BRCA1 carriers were considered as platinum-sensitive (progressed within 20.0–45.5 months from the date of last treatment); additional 5 BRCA1 carriers were intermediate responders (progressed within 6.7–9.1 months), while the only one BRCA2 carrier was platinum-resistant (progressed at 5.3 months).

4. Discussion

To address OVCA intra-patient genomic heterogeneity, we analyzed panel genotypes from multiple matched non-cancerous and cancerous tissues in one of the largest to-date patient series in this context. The herein captured heterogeneity reflects tissue genomic plasticity, i.e., the ability of tissues to adapt for fitness with clonal evolution and involution, which are site-specific and not universal within the same individual [2–4,7]. We developed an approach for describing changes in tissue genomic stability states within the same patient. We demonstrate that this heterogeneity is present in 42% of treatment-naïve OVCA and, among these, in 48% of serous OVCA, in line with published wide -omics data for these tumors [1,9,12,26–29]. Heterogeneity may also be present in endometrioid OVCA, which are less often related to TP53 pathology compared to serous, but are otherwise similar with respect to genotypes and germline status; our genotype and heterogeneity data support a common origin for serous and endometrioid histological types [9,24,30] unrelated to mucinous OVCA [24].

We demonstrate that the presence of heterogeneity in treatment-naïve OVCA is associated with adverse outcome after standard 1st line chemotherapy. Similarly, a high clonal expansion score was previously associated with worse outcome in 14 OVCA patients [12]. Of note, the as yet published heterogeneity scores [2,3,5,8,12,25] integrate the presence and frequency of shared pathogenic alterations for the identification of actionable drivers and their preservation among tissues from the same patient. The present rather agnostic assessment of heterogeneity exploits our previous genotype classification with a small panel [18] and bears similarities to the tumor mutational burden for predicting response to immunotherapy [31]. Tissue heterogeneity, as examined here, was based on intra-patient differences of tissue mutational patterns. Plasticity may be inferred as the critical, presumably genome-state altering leap between stable and unstable genotypes, driven by different underlying mutational processes. In this context, an interesting finding from our profiling was that mutation-zero and unstable did not co-occur in the absence of stable genotypes; this might indicate a stepwise transition from one extreme to the other. Taking into account the size of the panel; having excluded artificial hypermutation and lack of mutations; assuming that the observed genotypes represent adaptations to underlying functional perturbations; and, considering that 88% of the profiled patients had entirely or partially stable tissue genotypes, this finding indicates that the mechanism underlying the stable genotype is essential for the evolution of OVCA. Further, although heterogeneity calls were associated with the number of examined samples and anatomical sites, which is reasonable, this was not always the case. It thus seems possible that plasticity is a disease property the expression of which, in the form of heterogeneity, depends on the opportunities for selection pressure in space and time. We show that the usually blamed pathogenic TP53 and BRCA1 mutations are necessary but not sufficient in this direction. Additional work is needed to reveal surrogate markers of plasticity, which remains a major challenge in clinical practice.

We inferred position-LOH indicative of disrupted gene function, which may also reflect HRR deficiency (HRD), in 17% of the patients for BRCA1 and in 6.5% for BRCA2; overall, 21% BRCA1/2 disruption; and, mutual exclusiveness of disrupted BRCA1 and BRCA2. All these features are within the reported range and patterns for BRCA1/2 alterations in OVCA [6,8,20,23,32]. In addition, most probably due to high reading depth, we observed an almost 64% prevalence of tissue pathogenic BRCA1 mutations in our patients. The majority were of low frequency and may have not necessarily derived from tumor cells, as we have shown in nasopharyngeal carcinomas [18]; or, these may have been locally imported by infiltrating immune cells in the context of clonal hematopoesis [33]. Such low-VAF pathogenic BRCA1 mutations will probably not be encountered in the clinical setting, since tissue diagnostic NGS is usually performed at lower depth (400–800×) than applied here (>2000×). In any case, the presence of pathogenic mutations is not synonymous with BRCA1 disruption, in line with previous statements [20] [8], and it is not synonymous with HRD, as also previously suggested [8] [34]. Other than expected [21], herein, pathogenic BRCA1/2 mutations either germline and/or in tissues were overall not associated with benefit from platinum-based treatment, probably due to the small size of the patient cohort. Whether tissue pathogenic BRCA1 mutations will prove beneficial for patients with OVCA in terms of predicting response to PARP-inhibitors remains to be seen.

Additional questions on the role of BRCA1 in stage III–IV OVCA arise from our findings in tissues from BRCA1 germline mutation carriers. Despite our case-by-case approach, the multiplicity of examined matched samples allows for formulating hypotheses. We observed variability in the germline mutation load in tissue samples among patients, and within the same individual, i.e., underrepresentation with partial loss, retention of the normal allele, and position-LOH. Particularly in samples with partial loss, additional pathogenic BRCA1 mutations and/or BRCA2 disruption were present. The phenomenon was not linked to specific mutations. This condition may be paralleled to the reversion of the germline mutation upon platinum-based treatment [7,10,15] and PARP-inhibition [14,15,35], which has recently been coined as tumor-suppressor tolerance [13]. Retention of the normal allele has been described in a tissue- and gene-specific manner, leading to haploinsufficiency [36]. Haploinsufficiency in experimental breast cancer models may lead to elimination of the mutated allele in tissue cell lineages [37], as a defense mechanism against senescence and genomic instability [38]. Restoration of HRR may take place in pre-treatment tissues, as shown with the functional RAD51-foci assay [39]. In addition, (partial) loss of the germline mutation has been described in ovarian and breast tumors with MSK-IMPACT [40]. Although partial elimination of the germline mutation in tissues appears provocative, our findings support that the evolutionary principles described in post-treatment cancer-related tissues apply in the pre-treatment setting as well, in the context of plasticity for fitness [2,4]. The extent and clinical impact of this phenomenon are currently unknown, since the usually applied analysis algorithms do not take this possibility into account. Interestingly, tissue plasticity against inherited pathogenic BRCA1 may be associated with adverse outcome and seems worth considering in clinical practice.

To put our findings into context, it appears that pathogenic BRCA1 mutations in tissues of patients with OVCA are a lot more frequent when analyzed with high-depth sequencing than when investigated in the context of routine testing for therapy selection. Pathogenic BRCA1 are much more frequent than TP53 and BRCA2 in fallopian tube epithelia; they may even be clonal in these tissues. Clonal pathogenic BRCA1 are preserved as ancestral clones, indicating their driver role in disease development. However, BRCA1 disruption is not a universal phenomenon in tissues from OVCA patients with pathogenic BRCA1 mutations, including germline mutation carriers, in line with previous observations [8,36]. Overall, BRCA1/2 pathology seems to serve both as a driver and a surrogate for different operating insults in cells and tissues. This is compatible with the reported multiple mutational signatures associated with BRCA1 and HRD [8,41] and with the herein presented association of BRCA1 pathology with tissue heterogeneity. Tissue pathogenic BRCA1 mutations are always present in heterogeneous disease but not all such mutations coexist with this feature. In patients with heterogeneous disease and BRCA1 disruption, the reported favourable prognostic/predictive impact of BRCA1 pathology [20,21] may be lost, which is important for the clinical relevance of this marker. Whether BRCA1, BRCA2 and HRR dysfunction and/or

deficiency are cause or consequence of tissue genomic heterogeneity needs to be clarified.

In conclusion, we show that tissue genomic heterogeneity is frequently encountered in patients with serous and endometrioid OVCA. Pathogenic BRCA1 mutations, which are detected at a high rate with deep sequencing in the tissues of OVCA patients, appear to be essential but not sufficient for the development of tissue heterogeneity. Plasticity of OVCA genomes is indicated by other closely related phenomena, such as tissue BRCA1/2 restoration of constitutional BRCA1/2 disruption or acquisition of BRCA1/2 disruption via additional subclonal events in tissues. Importantly, the resulting heterogeneity may be associated with suboptimal efficiency of platinum-based therapy, even in the presence of germline and/or somatic pathogenic BRCA1/2 mutations that are considered as favourable prognosticators in OVCA. If validated in larger studies, the herein presented assessment of tissue genomic heterogeneity and its associations with BRCA1/2 pathology may aid in increasing precision of treatment interventions in patients with OVCA.

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NGS experiments and validations: KP
Bioinformatics: IT, EG, IV, VK
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Patient data and biologic material: GF, GL, IE, AP, BT
Patient data management: GF, KM
Manuscript writing: VK, SL, IT, EG, KP; GF
Final approval of manuscript: all authors

Conflict of interest statement

Dr. Sotirios Lakis reports personal fees from NEO New Oncology, personal fees from BioNTech Diagnostics, outside the submitted work.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygyno.2018.11.016.

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