Prognostic impact of copy number alterations and tumor mutational burden in carcinoma of unknown primary

Tilmann Bochtler1,2,3 | Timothy Wohlfromm1 | Thomas Hielscher4 | Damian Stichel5,6 | Maria Pouyiourou1,3 | Bianca Kraft1 | Olaf Neumann7 | Volker Endris7 | Andreas von Deimling5,6 | Albrecht Stenzinger7 | Alwin Krämer1,3

1Clinical Cooperation Unit Molecular Hematology/Oncology, German Cancer Research Center (DKFZ) and Department of Internal Medicine V, University of Heidelberg, Heidelberg, Germany
2Department of Medical Oncology, National Center for Tumor Diseases (NCT), University of Heidelberg, Heidelberg, Germany
3Department of Internal Medicine V, University of Heidelberg, Heidelberg, Germany
4Division of Biostatistics, German Cancer Research Center (DKFZ), Heidelberg, Germany
5Institute of Neuropathology, University of Heidelberg, Heidelberg, Germany
6Clinical Cooperation Unit Neuropathology, German Cancer Research Center (DKFZ), Heidelberg, Germany
7Institute of Pathology, University of Heidelberg, Heidelberg, Germany

Correspondence
Alwin Krämer, Clinical Cooperation Unit Molecular Hematology/Oncology, German Cancer Research Center (DKFZ) and Department of Internal Medicine V, University of Heidelberg, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany.
Email: a.kraemer@dkfz.de

Abstract
Introduction: Chromosomal aberrations are known to drive metastatic spread, but their profile is still elusive in carcinoma of unknown primary (CUP). Therefore, the aim of this study was to characterize the chromosomal aberration pattern in CUP depending on histological and clinical features and to assess its prognostic impact together with chromothripsis, tumor mutational burden (TMB), microsatellite instability (MSI), and mutational profiles as potential prognostic biomarkers.

Methods: Chromosomal aberrations and chromothripsis were detected by methylation-based copy number variation (CNV) analysis, whereas TMB and MSI were calculated based on large next-generation sequencing (NGS) panels. Putative primaries were assigned by consensus between two independent oncologists.

Results: CNV losses varied depending on putative primaries and were more abundant in patients harboring TP53 mutations and/or deletions 17p. CNV loss was prognostically adverse in localized CUP treated with surgery and/or radiotherapy, but not in disseminated poor-risk CUP treated with palliative chemotherapy. CNV loss also worsened the prognosis in squamous cell CUP. Chromothripsis was detected in 18/59 (30.5%) patients without prognostic effect. TMB was highest in cases with MSI, squamous cell histology, and with lung, anal or cervical putative primaries.

Conclusion: Overall, CNV, chromothripsis, TMB, and MSI profiles in CUP are reminiscent of biological characteristics known from other cancer entities without a unifying CUP-specific signature. Markedly, high-level CNV loss is an adverse predictive biomarker in localized but not disseminated chemotherapy-treated CUP. This implies that chromosomal losses drive CUP progression, but also increase susceptibility to chemotherapy, with both effects apparently leveling out in disseminated CUP.

Keywords
carcinoma of unknown primary, chromosomal instability, chromothripsis, copy number variations, mutational profile, prognosis, tumor mutational burden
1 | INTRODUCTION

Carcinoma of unknown primary (CUP) designates a cancer entity where no primary tumor is detectable in spite of histologically proven metastatic spread and a comprehensive clinical work-up. Several next-generation panel sequencing-based studies have elucidated the mutational landscape in CUP. These studies have consistently demonstrated that the mutational spectrum in CUP is diverse, with TP53, KRAS, CDKN2A/B, MYC, PIK3CA, ARID1A, ERBB2, and NOTCH1 genes most frequently harboring alterations. With respect to prognosis CDKN2A/B deletion and KRAS activation have been identified as adverse prognostic markers.

In contrast, the pattern of chromosomal aberrations in CUP has only been assessed in small studies. These have shown that approximately 70% of CUP cancers harbor an aneuploid karyotype with particularly complex and unbalanced cytogenetic patterns well in excess of those observed in cancers with a known primary. Hereby, the karyotypic complexity has been deemed a reflection of the aggressiveness of metastatic growth in CUP and has been reported to confer adverse outcome. While chromosomal regions 4q31, 6q15, 10q25, and 13q22 were most frequently involved in adenocarcinomas, region 11q22 was most frequently involved in non-adenocarcinomas. Cytogenetic aberrations have also been used for cytogenetics-based putative primary prediction.

It is surprising that the role of chromosomal instability is still quite elusive in view of CUP as the prototypic metastatic disease and chromosomal instability as a known driver of metastatic spread. Therefore, it was the aim of this study to decipher the chromosomal aberration pattern in CUP, also with respect to clinical parameters including histology, CUP stage (localized vs. disseminated disease), subtype, putative primary and TP53 mutational/del17p13 status, as well as to assess the prognostic impact of chromosomal aberrations. Additionally, we have complemented the chromosomal aberration profiles with large panel sequencing-based tumor mutational burden (TMB) data to obtain a comprehensive genetic profile.

2 | PATIENTS AND METHODS

2.1 | Patient selection

This study included a consecutive series of 74 CUP patients for whom Trusight panel sequencing is available. In 59 of these 74 patients, material for CNV and chromothripsis profiles by methylation analysis was available as well.

Diagnosis of CUP was made according to European Society of Medical Oncology (ESMO) guidelines. In cases where a relapsed antecedent malignancy could possibly be mistaken for CUP, we performed comparative panel sequencing of both tumors as previously described in order to exclude misdiagnoses. Localized CUP was defined as single-site or oligometastatic disease amenable to surgery and/or radiochemotherapy in curative intent. Favorable subtypes were defined by either a solitary metastatic deposit or by analogy to well-defined chemo-sensitive cancers in tune with ESMO guidelines. Based on clinical and immunohistochemical features patients were independently classified for their putative primary by two experienced oncologists, with putative primary tumors being only registered in case of consensus. Putative primary tumor groups comprised distinct favorable subtypes recognized in the ESMO guidelines, namely colon (adenocarcinoma with CK7-, CK20+, CDX2+ immunohistochemistry [IHC] and metastatic spread compatible with colon cancer), head and neck (squamous cell carcinomas with predominant cervical lymph node metastases), breast (adenocarcinoma with predominant axillary lymph node metastases in females) and inner genitals (serous pelvic/peritoneal adenocarcinoma in females), but also upper gastrointestinal tract (adenocarcinoma with leading peritoneal carcinosis and/or abdominal wall infiltration with compatible IHC and without colonic profile), lung (CK7+ adeno- or squamous cell carcinomas with a metastatic pattern suggestive of lung cancer, that is, mediastinal lymph nodes, predominant tumor burden within the chest and distant metastatic sites typical of lung cancers) and anal/cervix (squamous cell carcinomas with pelvic masses and or inguinal lymph node metastases), as well as fully enigmatic cases where no or no unanimous assignment could be made.

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of Heidelberg University (S-638/2016).

2.2 | Determination of CNVs, chromothripsis, NGS sequencing, TMB calculation, and MSI status

DNA was extracted using a Maxwell 16 Research System (Promega), followed by quantification using the QuBit 2.0 DNA High Sensitivity Kit (Thermo Fisher Scientific) as described previously.

For copy number variation (CNV) quantification (gain, loss, and total) we applied DNA methylation analysis as described by Capper et al. using the Infinium MethylationEPIC 850k BeadChip as described previously. The amount of CNV is given in million DNA integrity was assessed using the Genomic DNA Analysis Kit (Thermo Fisher Scientific) on a StepOnePlus qPCR system (Thermo Fisher Scientific). Libraries were sequenced on a NextSeq 500 instrument (Illumina) to a mean
coverage of \(10^9\) using high-output cartridge and v2 chemistry. All assays were performed according to the manufacturers’ protocols. Processing of raw sequencing data and variant calling was carried out using the TruSight Oncology 500 Local App (version 1.3.0.39). Called variants were verified by visual inspection in the Integrative Genomics Viewer.

Only variants with an allele frequency above 2% and a minimum coverage of greater than 100 were considered.

The TSO500 panel (Illumina) was also used for calculation of TMB and microsatellite instability (MSI). TMB values are presented as number of mutations per megabase, with cutoffs for TMB low, intermediate and high set at <6, 6–12, and \(\geq 12\) Mut /Mb, respectively. MSI was determined based on the 130 homopolymer MSI markers within the Trusight panel with the threshold for MSI set at >20% instable sites.

### 2.3 Statistical analysis

Pairwise associations between genomic and clinicopathological factors were assessed with Fisher’s exact test, Cochran–Armitage trend test, Wilcoxon test, Kruskal–Wallis test, and Spearman’s correlation coefficient depending on the type of variables. Values of \(p\) were adjusted for multiple testing using Holm’s correction. For patients with biopsy at first diagnosis, prognostic impact of genomic factor on overall survival (OS) and progression-free survival (PFS) was assessed using log-rank test and Cox regression models. OS was defined as time from initial diagnosis to death from any cause, PFS was defined as time from initial diagnosis to disease progression, relapse or death from any cause, whichever occurred first. Subgroup effects were assessed based on a Cox model with interaction term. Log-transformation of TMB and square-root transformation of sum of CNV gains/losses was selected based on model AIC. Optimal cut-point was selected using maximally-selected log-rank statistic. Impact on response to chemotherapy

### TABLE 1 Clinical characteristics of the study cohort

| Age | Median (range) (years) | 62.7 (37.1–84.4) |
| --- | --- | --- |
| Gender | Male/female | 32/42 (43.2%/56.8%) |
| ECOG status | Median (range) | 1 (0–3) |
| CUP stage and histology | Localized | 22 (29.7%) |
| | Squamous cell carcinoma | 6 (27.3%) |
| | Adenocarcinoma | 15 (68.2%) |
| | Adenosquamous carcinoma | 1 (4.5%) |
| Disseminated | 52 (70.3%) |
| | Squamous cell carcinoma | 6 (11.5%) |
| | Adenocarcinoma | 39 (75.0%) |
| | Undifferentiated carcinoma | 7 (13.5%) |
| CUP subtype according to ESMO guidelines | Favorable | 32 (43.2%) |
| | Unfavorable | 42 (56.8%) |
| Favorable CUP subtypes (other than localized) | CUP with a colorectal IHC (CK20+, CDX2+, CK7–) or molecular profile | 12 (16.7%) |
| | SCC involving non-supraclavicular cervical lymph nodes | 1 (1.4%) |
| | Serous papillary peritoneal carcinoma in females | 1 (1.4%) |
| | Isolated axillary nodal metastases in females | 1 (1.4%) |
| Metastatic sites (baseline involvement at first diagnosis) | Number (median, range) | 2 (1–7) |
| Lymph node | 45 (60.8%) |
| Lung | 22 (29.7%) |
| Bone | 19 (25.7%) |
| Liver | 19 (25.7%) |
| Peritoneum | 16 (21.6%) |
| Pleura | 9 (12.2%) |
| Brain | 5 (6.8%) |
| Adrenal | 4 (5.4%) |
| Bone marrow | 3 (4.1%) |
| Other | 26 (35.1%) |
| Putative primary | Enigmatic | 38 (51.4%) |
| | Colon | 31 (43.9%) |
| | Lung | 9 (12.2%) |
| Upper GI (gastro-intestinal) | 6 (8.1%) |
| Anal/cervix | 4 (5.4%) |
| | Inner genitals | 3 (4.1%) |

(Continues)
was analyzed with logistic regression models. Software R was used to perform analyses.

## RESULTS

### 3.1 Patients

Patient characteristics are shown in Tables 1 and S1. Adenocarcinoma was the prevailing histologic entity (73.0%), but the study cohort also included squamous cell (16.2%) and undifferentiated carcinoma cases (9.5%). Favorable CUP subtypes were represented by 32/74 (43.2%) patients. The median number of organs with metastatic deposits was 2 (range 1–7, average 2.4) at first diagnosis. Among the 64 patients for whom TMB analysis was performed from a pretreatment biopsy, 52 events and 36 deaths occurred during follow-up, leading to a PFS and OS of 6.1 and 13.2 months, respectively.

### 3.2 CNV profiles

Figure 1 depicts the cumulative CNV profile of 59 CUP patients for whom CNV data were available. The median amount of CNV gains and losses was 168 Mb (range: 0–1217) and 282 Mb (range: 0–1983), respectively. The amounts of CNV gains and losses were significantly associated with each other, with patients with many CNV gains typically displaying many CNV losses as well ($p < 0.001$). On an individual chromosomal arm level, gains of 1q and 8q as well as losses of 3p and 6q were most frequent. With respect to histology, squamous cell carcinomas displayed higher levels of CNV gains and losses, although statistical significance was not reached ($p = 0.12$ and $p = 0.22$, respectively; Figure 2 and Table S2). In squamous cell CUP, chromosome arm 3p was most frequently deleted and 3q most frequently gained (Figure S1).

The amounts of CNV gains and losses were not significantly different between localized versus disseminated CUP ($p = 0.50$ and $p = 0.39$, respectively) (Figure 2 and Table S2). However, when CUP cases were broken down by their clinically assigned putative primaries, the amounts of CNV gains and losses differed markedly among different primaries, with a high amount of CNVs in lung and cervical/anal cancers, a low amount in colon and upper GI cases and a widespread with intermediate median in elusive cases ($p = 0.10$ for CNV gain and $p = 0.14$ for CNV loss; see Figure 2 and Table S2).

CNV losses were increased in tumors harboring deleterious TP53 mutations and/or del17p as shown in Figure 2 and Table S2. In detail, CNV loss was highest in cases with del17p irrespective of TP53 mutational status [median 537 Mb (63–1339) and 713 Mb (282–1983), respectively], was intermediate in TP53 mutated cases without del17p [299 Mb (4–1390)] and was lowest in cases harboring neither del17p nor TP53 mutation [128 Mb (0–662); $p = 0.001$]. MSI was associated with a lower level of CNV gains ($p = 0.07$) and losses ($p = 0.05$).

### 3.3 Chromothripsis

Among the 59 patients evaluable for chromothripsis 18/59 (30.5%) were chromothripsis-positive, with nine of the positive patients displaying one single chromothriptic chromosome and another nine at least two (range: 2–27). Significantly more CNV losses were observed in chromothripsis-positive versus negative patients ($p = 0.009$). Importantly, the association of chromothripsis with del17p or TP53 mutation fell short of statistical significance ($p = 0.16$). Markedly, chromothripsis was equally frequent in favorable versus unfavorable CUP subtypes [7/24 (29.2%) vs. 11/35 (31.4%), $p = 1$] as well as in localized versus disseminated CUP [6/19 (31.6%) vs. 12/40 (30.0%), $P = 1$; see Table S2].

### 3.4 TMB values

TMB values displayed a wide heterogeneity ranging from 0 to 77.58 mutations/Mb with a median of 4.71 mutations/Mb. When grouped into low (<6 mutations/Mb), intermediate (≥6 to <12 mutations/Mb) and high (≥12 mutations/Mb), 45 patients (60.8%) fell into the TMB-low, 18 (24.3%) into the TMB-intermediate and 11 (14.9%) into the TMB-high category. The two tumor samples with the highest TMB...
values (77.58 and 59.52 mutations/Mb, respectively) were the two MSI cases ($p = 0.02$; see Figure 2 and Table S2). When TMB values were analyzed by histologic subgroups, CUP cases with squamous cell carcinoma histology displayed a statistically significantly higher TMB load (median 9.0 mutations/Mb, range 3.13–57.91 mutations/Mb) as compared to adenocarcinoma and undifferentiated carcinoma histology (median 4.70 mutations/Mb, range 0–77.58 mutations/Mb; $p = 0.002$; see Figure 2 and Table S2). TMB values were about equally high in unfavorable versus favorable CUP ($p = 0.70$; see Figure 2 and Table S2), while there was a trend for higher TMB in localized CUP (7.44 [1.57–57.91] mutations/Mb, $p = 0.07$). When patients were grouped according to their clinicopathologically putative primary, the TMB load was highest in cases with a presumed cervical/anal (median 13.38 mutations/Mb [3.91–29.77]) or pulmonary primary (median 8.61 mutations/Mb [1.57–13.33]), whereas the TMB load was lower in cases with suspected colonic (median 3.92 mutations/Mb [0–77.58]) or upper gastrointestinal (median 2.35 mutations/Mb [1.57–4.71]) primary and intermediate in cases with enigmatic primary (median 4.70 mutations/Mb [0–59.52]; overall $p = 0.04$; see Figure 2 and Table S2).

Patients with an inactivating TP53 mutation displayed higher TMB values than patients with wild-type TP53 (5.50 mutations/Mb [1.57–77.58] vs. 3.91 mutations/Mb [0–59.52]; $p = 0.03$), whereas del17p had no impact on TMB ($p = 0.92$). No statistically significant effects were detected for any other genes covered by the Trusight panel. Also, no statistically significant effects were observed for the group of DNA damage response (DDR) alterations (BRCA1/2, ATM, ATR, CHEK2, ...

![Figure 2](image-url)  
**FIGURE 2** Distribution of CNV loss, CNV gain, and TMB depending on histology, local versus disseminated disease, favorable versus unfavorable risk, putative primary, microsatellite instability as well as the TP53 mutational and del17p status (patients grouped as TP53 mutation or chr17p loss harbored only either the TP53 mutation or the chr17p loss). The bar is representing the respective median value.
TABLE 2  Prognostic effect of clinical and genetic baseline parameters on progression-free survival, overall survival, and remission to first-line chemotherapy in univariate analysis

| Parameter                                      | Progression-free survival | Overall survival | Partial remission (PR) to first-line chemotherapy |
|-----------------------------------------------|---------------------------|-----------------|--------------------------------------------------|
|                                               | Hazard ratio [95% CI]     | p-value         | Hazard ratio [95% CI]     | p-value         | Odds ratio [95% CI]     | p-value         |
| Higher age [per 1 year]                       | 1.02 [0.99–1.05]         | 0.20            | 1.03 [1.00–1.07]         | 0.09            | 1.03 [0.94–1.12]         | 0.58            |
| Female gender                                 | 0.91 [0.52–1.59]         | 0.74            | 0.93 [0.48–1.79]         | 0.82            | 2.75 [0.47–15.96]        | 0.26            |
| ECOG 1 versus 0 <1 versus 0                   | 1.92 [1.02–3.62]         | 0.04            | 2.72 [1.22–6.07]         | 0.01            | 0.45 [0.10–2.10]         | 0.31            |
| Squamous cell histology                       | 3.33 [1.52–7.29]         | 0.003           | 5.93 [2.27–15.45]        | <0.001          | not estimable            |                 |
| Metastatic organs b ≥4                        | 2.00 [1.06–3.80]         | 0.03            | 2.80 [1.22–6.42]         | 0.01            | 1.40 [0.20–9.87]         | 0.74            |
| Localized CUP                                 | 1.52 [1.52–1.52]         | <0.0001         | 1.52 [1.52–1.52]         | <0.001          | 1.52 [1.52–1.52]         | 0.80            |
| Favorable CUP                                 | 2.01 [1.34–3.04]         | 0.003           | 2.01 [1.34–3.04]         | 0.003           | 2.01 [1.34–3.04]         | 0.94            |
| CNV gain                                      | 0.75 [0.51–1.12]         | 0.16            | 0.75 [0.51–1.12]         | 0.16            | 0.75 [0.51–1.12]         | 0.09            |
| CNV loss f                                    | 1.09 [0.60–1.99]         | 0.42            | 1.09 [0.60–1.99]         | 0.42            | 1.09 [0.60–1.99]         | 0.36            |
| Chromothripsis                                | 0.99 [0.49–1.99]         | 0.97            | 1.01 [0.44–2.31]         | 0.98            | 1.01 [0.44–2.31]         | 0.26            |
| Number Chromothriphic chr. f                  | 0.95 [0.73–1.24]         | 0.72            | 0.94 [0.68–1.30]         | 0.71            | 0.94 [0.68–1.30]         | 0.12            |
| TMB d                                         | 0.78 [0.61–1.00]         | 0.05            | 0.85 [0.65–1.10]         | 0.22            | 1.30 [0.71–2.71]         | 0.43            |
| TMB group Intermediate versus low             | 0.58 [0.30–1.13]         | 0.11            | 0.70 [0.32–1.51]         | 0.36            | 0.90 [0.08–9.97]         | 0.93            |
| High versus low                               | 0.84 [0.37–1.93]         | 0.69            | 0.40 [0.12–1.34]         | 0.14            | 3.60 [0.55–23.64]        | 0.18            |

Note: Among the 74 patients, only the 64 patients with biopsy at first diagnosis were considered for prognostic analyses. TMB was available in all patients, whereas CNV profile was available in 59 patients (among them 49 at first diagnosis). PR to first line chemotherapy could be assessed in 34 patients. Abbreviations: chr: chromosome; CNV, copy number variation; ECOG, Eastern Cooperative Oncology Group performance status; TMB, tumor mutational burden. Bold denotes statistically significant effects. 

bDue to small patient numbers of patients evaluable for PR only value for ECOG ≥1 versus 0 given.

cOnly metastases present at first diagnosis counted.

dSquare root.

PALB2, FANCA, FANCC, FANCG, FANCI, FANCL, PARP1, RAD21, RAD51C, RAD51D, and RAD54L as a whole (P = 0.30, Padj = 1).

3.5  Prognostic impact of CNV, chromothripsis, and TMB

A higher amount of CNV gains conferred superior PFS (HR 0.72 95% CI [0.51–1.02], p = 0.07) as well as OS (HR 0.66 [0.44–0.97], p = 0.03; see Table 2). To the contrary, CNV losses had no statistically significant prognostic impact in the overall group (PFS: HR 1.01 [0.70–1.46], p = 0.95; OS: HR 0.98 [0.64–1.50], p = 0.91). In a subgroup analysis CNV losses appeared to worsen the prognosis in localized CUP (PFS: HR 1.99 [0.70–5.14], p = 0.18), but had the opposite effect in disseminated CUP (PFS: HR 0.71 [0.41–1.14], p = 0.16, Pcomp = 0.08) (see Forest Plot Figure 3). With regard to histology, CNV losses significantly worsened the prognosis in squamous cell (PFS: HR 4.54 [1.31–166.6], p = 0.016), but not in adeno- or undifferentiated CUP (PFS: HR 0.92 [0.60–1.37], p = 0.7, Pcomp = 0.006). The latter effect also translated into worsened OS in squamous cell CUP (OS HR 3.76 [1.15–258.0], p = 0.03, Pcomp = 0.007). In contrast, chromothripsis had no prognostic effect.

High TMB values were associated with improved PFS (HR 0.78 [0.61–1.00], p = 0.05), whereas statistical significance was not reached for OS (HR 0.85 [0.65–1.10], p = 0.22) and response to chemotherapy in disseminated CUP (OR 1.39 [0.71–2.71], p = 0.34). In order to discern prognosis best, an optimal TMB cutoff of 2.42 mutations/Mb was determined by maximally selected log-rank statistic for both PFS and OS (p = 0.09 and p = 0.14, respectively; see Figure S2). In multivariate analysis incorporating the clinical factors age, gender, performance status (ECOG), histology, favorable versus unfavorable CUP subgroups, and number of organs with metastatic deposits, high TMB was not significantly associated with EFS or OS anymore (HR 0.86 [0.64–1.21], p = 0.33 and p = 0.91 [0.66–1.25], p = 0.55, respectively; see Table 3).

Overall, 10/74 (13.5%) patients were treated with immune checkpoint inhibitors. This group, which is shown in detail in Table S3, was very heterogenous with respect to compound, histology, treatment line, and rationale to administer checkpoint inhibitor treatment. In detail, the reason to administer immune checkpoint inhibitor treatment was MSI (n = 1), high PD-L1 (n = 1), a putative HNSCC (n = 1),
lung \( (n = 1) \) or kidney \( (n = 1) \) primary and physician’s choice due to platin refractory disease, exhaustion of other options and / or poor health \( (n = 5) \). Overall, 4/10 (40%) patients achieved partial remission and another 2/10 (20%) stable disease. Although small numbers and the heterogeneity of the patient set preclude statistical analysis, responding patients appeared to skew for higher TMB values.

**FIGURE 3** Showing in a Forrest Plot the prognostic impact of CNV loss with respect of PFS and OS in the subgroups male versus female, favorable versus unfavorable CUP, local versus disseminated disease and the different histologies. The hazard ratio (HR) and the 95% confidence interval (CI) are given for each subgroup. Confidence values out of the graphic range are represented by arrows.

**TABLE 3** Prognostic impact of TMB in multivariate analysis together with clinical baseline parameters

| Parameters                              | Progression-free survival | Overall survival |
|-----------------------------------------|---------------------------|-----------------|
|                                        | Hazard ratio [95% CI]     | Hazard ratio [95% CI] | p-value | p-value |
| Higher age                              | 0.98 [0.95–1.01]          | 0.95 [0.91–0.99] | 0.23    | 0.02    |
| Female gender                           | 1.08 [0.59–1.98]          | 1.06 [0.52–2.17] | 0.79    | 0.88    |
| ECOG 1 versus 0                         | 1.51 [0.72–3.16]         | 2.24 [0.93–5.41] | 0.27    | 0.07    |
| >1 versus 0                             | 4.17 [1.54–11.29]        | 26.12 [6.56–103.99] | 0.005   | <0.001  |
| Squamous cell carcinoma                 | 0.39 [0.13–1.16]         | 0.27 [0.06–1.16] | 0.09    | 0.08    |
| Favorable CUP                           | 0.40 [0.16–0.97]         | 0.14 [0.04–0.42] | 0.04    | <0.001  |
| Metastatic organ number (2, 3)\( ^a \)  | 1.30 [0.54–3.15]         | 1.42 [0.50–4.03] | 0.56    | 0.51    |
| \( \geq 4 \)\( ^a \)                    | 1.44 [0.51–4.05]         | 2.50 [0.79–7.90] | 0.49    | 0.12    |
| High TMB\( ^b \)                        | 0.86 [0.64–1.16]         | 0.91 [0.66–1.25] | 0.33    | 0.55    |

Abbreviations: ECOG, Eastern Cooperative Oncology Group performance status; TMB, tumor mutational burden. Bold denotes statistically significant effects.

\( ^a \)Only metastases present at first diagnosis counted.

\( ^b \)Values are processed logarithmically.

In this study, we present a comprehensive analysis of frequency, distribution, and prognostic as well as predictive impact of chromosomal CNVs, chromothripsis, TMB, and mutational spectra as genomic aberration types in CUP.
CNVs are recognized as a proxy for chromosomal instability. Chromosomal instability is known to drive cancer progression by increased intratumoral heterogeneity and subsequent better adaptability of malignant cells. Accordingly, chromosomal instability has been associated with metastasis, treatment resistance, and poor prognosis in many cancer types. On the other hand, an exceedingly high degree of chromosomal instability is known to impair cell viability, implying that an ideal degree of chromosomal instability exists for cancer cells. Also, cancer cells with high-level chromosomal instability are less able to compensate for cytotoxic stress and therefore display a higher vulnerability to chemotherapy, as exemplified in breast cancer, both in the experimental setting and in clinical trials. In the experimental setting, paclitaxel has been shown to exert its anti-cancer effect by increasing chromosomal instability over a maximally tolerable threshold. Clinically, breast cancer patients with the highest degree of chromosomal instability showed improved outcome when treated with chemotherapy and patients with high-level chromosomal instability showed improved response to taxanes.

In a recent study, we have demonstrated that high-level CNV loss predicts for adverse prognosis in localized CUP. This adverse prognostic effect of CNV loss in localized CUP was confirmed in the current study. To the contrary, CNV loss had a slightly beneficial effect on PFS in disseminated CUP treated with chemotherapy. Whereas localized CUP per se is associated with favorable prognosis and a median overall survival of 52.5 months after local ablative treatment, disseminated CUP has an extremely poor prognosis with a median overall survival of only 9 months. As chromosomal instability is believed to drive metastasis, high-level CNV loss might forecast the development of additional metastases and thereby poor prognosis in single-site/oligometastatic CUP after local ablative treatment only. On the other hand, disseminated CUP cases with high-level CNV loss might cope worse with cytostatic treatment, which pushes the chromosomal instability level beyond the “right” equilibrium, especially as taxanes are a standard component of CUP chemotherapy regimens. Also, as these cases suffer from disseminated disease already, the impact of chromosomal instability on metastatic progression might have been leveled out at this disease stage.

A detrimental prognostic effect of CNV loss both on PFS and OS was found in CUP with squamous cell carcinoma histology. As squamous cell carcinomas displayed higher levels of DNA copy number aberrations and 6/12 (50%) squamous cell carcinoma cases overlap with the single-site CUP cohort, the adverse prognostic effect of high CNV loss in the local and squamous cell groups seems closely intertwined. In conclusion, our CNV data imply that the prognostic impact of CNV loss heavily relies on treatment, metastatic state, and histology and should be analyzed in the context of these clinical data.

Chromothripsis was another candidate as a prognostic marker in CUP. We observed a high chromothripsis rate in CUP in the 30% range, which was expected in view of its aggressive clinical course and dismal prognosis. However, chromothripsis did not confer a significantly inferior prognosis in our cohort.

High TMB levels conferred a tendency for better prognosis, although statistical significance was not reached in multivariate analysis incorporating the essential patient characteristics and established prognostic factors. Conclusions with respect to OS are also limited by the rather small number of OS events. Given the limited access to immune checkpoint inhibitors in our study cohort, the overall prognostic effect of TMB was only mildly affected by the potential beneficial effect of immune checkpoint inhibition in TMB-high patients. Thus, similar to CNV alterations, TMB as a biomarker seems also heavily dependent on the treatment modality and is rather predictive than prognostic.

Our data also allow for further insight into CUP pathophysiology. The question whether CUP represents a distinct cancer entity or rather a heterogenous group of biologically different cancer types which merely share the lack of an identifiable primary is still controversially discussed. As an obvious limitation, the putative primary assignment based on (immuno)histology and clinical picture in our study was explorative and included putative primary subsets not validated by retrospective cohorts. Nevertheless, genomic patterns characteristic of other cancer entities were observed in our CUP cohort. First, the spectrum of TMB values varied widely, ranging from 0 to 77.58 mutations/Mb. Cases with MSI showed the highest TMB values, and cases with squamous cell histology or a putative lung cancer primary displayed above average values, as expected. Thus, the distribution of TMB values in CUP is reminiscent of patterns established in other cancer entities. Second, the CNV profiles encountered in CUP similarly reproduced known patterns from other cancer entities, for example, the high frequency of chromosome 3p deletions in the overall cohort and chromosome 3q gains in the squamous cell carcinoma subgroup. Therefore, genomically CUP appears to represent a microcosm of different carcinoma types. It is a contentious debate whether the lack of a unique CUP-specific genomic signature on the one hand, and the presence of CNV and TMB spectra that are reminiscent of different defined carcinoma types on the other hand is a justified argument against a unifying pathophysiology in CUP.

5 | CONCLUSIONS

From a genetic perspective, this study gives biological insights into the enigmatic phenomenon of CUP. Similar levels of chromosomal aberrations in local versus disseminated CUP argue in favor of a “primary metastatic cancer” model with early metastatic spread. Strong parallels of chromosomal and TMB signatures between CUP and other cancer entities argue against a distinct genetic CUP profile.

With respect to biomarkers, CNV loss confers adverse prognosis in local but not disseminated CUP. High CNV loss levels might therefore drive CUP progression from localized to disseminated disease on the one hand, but on the other hand also confer higher sensitivity to chemotherapy.

ACKNOWLEDGMENT

We thank the entire technical staff of the Center for Molecular Pathology for excellent technical assistance. Open access funding enabled and organized by Projekt DEAL.
CONFLICT OF INTEREST
Tilmann Bochtler and Alwin Krämer work as study oncologists for the CUPISCO trial which is sponsored by Roche and have received reimbursement for study-related travels as well as remuneration for their work as study oncologists for the benefit of their employer. Alwin Krämer has also received research funding from BMS. Volker Endris: advisory board and lecture fees from AstraZeneca and ThermoFisher. Albrect Stenzinger: Advisory Board and/or Speaker’s Bureau: AlGnostics, AstraZeneca, Bayer, BMS, Illumina, Janssen, MSD, Novartis, Pfizer, Roche, Seattle Genetics, Takeda, Thermo Fisher; Research Grants: Bayer, BMS, Chugai, Incyte. The remaining authors have no conflict of interest.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID
Tilmann Bochtler https://orcid.org/0000-0003-4162-7881
Albrecht Stenzinger https://orcid.org/0000-0003-1001-103X
Alwin Krämer https://orcid.org/0000-0001-8232-9982

REFERENCES
1. Varadhachary GR, Raber MN. Carcinoma of unknown primary site. N Engl J Med. 2014;371:757-765.
2. Fizazi K, Greco FA, Pavlidis N, et al. Cancers of unknown primary site: ESMO clinical practice guidelines for diagnosis, treatment and follow-up. Ann Oncol. 2015;26(Suppl 5):v133-v138.
3. Bochtler T, Kramer A. Does cancer of unknown primary (CUP) truly exist as a distinct cancer entity? Front Oncol. 2019;9:402.
4. Ross JS, Wang K, Gay L, et al. Comprehensive genomic profiling of carcinoma of unknown primary site: new routes to targeted therapies. JAMA Oncol. 2015;1:40-49.
5. Loffler H, Pfarr N, Kriegsmann M, et al. Molecular driver alterations and their clinical relevance in cancer of unknown primary site. Oncotarget. 2016;7:44322-44329.
6. Tothill RW, UJ, Mileskin L, et al. Massively-parallel sequencing assists the diagnosis and guided treatment of cancers of unknown primary. J Pathol. 2013;231:413-423.
7. Gatalica Z, Millis S, Vranic S, et al. Comprehensive tumor profiling identifies numerous biomarkers of drug response in cancers of unknown primary site: analysis of 1806 cases. Oncotarget. 2014;5:12440-12447.
8. Gatalica Z, Xiou J, Swensen J, Vranic S. Comprehensive analysis of cancers of unknown primary for the biomarkers of response to immune checkpoint blockade therapy. Eur J Cancer. 2018;94:179-186.
9. Clynick B, Dessauvagie B, Sterrett G, et al. Genetic characterisation of molecular targets in carcinoma of unknown primary. J Transl Med. 2018;16:185.
10. Bochtler T, Reiling A, Endris V, et al. Integrated clinicomolecular characterization identifies RAS activation and CDKN2A deletion as independent adverse prognostic factors in cancer of unknown primary. Int J Cancer. 2020;146:3053-3064.
11. Hedley DW, Leary JA, Kirsten F. Metastatic adenocarcinoma of unknown primary site: abnormalities of cellular DNA content and survival. Eur J Cancer Clin Oncol. 1985;21:185-189.
12. Pantou D, Tsarouha H, Papadopoulou A, et al. Cytogenetic profile of unknown primary tumors: clues for their pathogenesis and clinical management. Neoplasia. 2003;5:23-31.
13. Speel EJ, van de Wouw AJ, Claessen SM, et al. Molecular evidence for a clonal relationship between multiple lesions in patients with unknown primary adenocarcinoma. Int J Cancer. 2008;123:1292-1300.
14. Kompisaros K, Penteroualdakis G, Pavlidis N. Exploring the biology of cancer of unknown primary: breakthroughs and drawbacks. Eur J Clin Invest. 2013;43:491-500.
15. Vikesa J, Moller AK, Kaczkowski B, et al. Cancers of unknown primary origin (CUP) are characterized by chromosomal instability (CIN) compared to metastasis of known origin. BMC Cancer. 2015;15:151.
16. Motzer RJ, Rodriguez E, Reuter VE, Bosl GJ, Mazumdar M, Chaganti RS. Molecular and cytogenetic studies in the diagnosis of patients with poorly differentiated carcinomas of unknown primary site. J Clin Oncol. 1995;13:274-282.
17. Ilson DH, Motzer RJ, Rodriguez E, Chaganti RS, Bosl GJ. Genetic analysis in the diagnosis of neoplasms of unknown primary tumor site. Semin Oncol. 1993;20:229-237.
18. Kolling S, Ventre F, Geuna E, et al. “Metastatic cancer of unknown primary” or “primary metastatic cancer”? Front Oncol. 2019;9:1546.
19. Bakhoun SF, Swanton C. Chromosomal instability, aneuploidy, and cancer. Front Oncol. 2014;4:161.
20. Bakhoun SF, Ngo B, Laughney AM, et al. Chromosomal instability drives metastasis through a cytosolic DNA response. Nature. 2018;553:467-472.
21. Bochtler T, Endris V, Leichsenring J, et al. Comparative genetic profiling aids diagnosis and clinical decision making in challenging cases of CUP syndrome. Int J Cancer. 2019;145:2963-2973.
22. Capper D, Jones DTW, Sill M, et al. DNA methylation-based classification of central nervous system tumours. Nature. 2018;555:469-474.
23. Pouyliourou M, Wohlfirrm T, Kraft B, et al. Local ablative treatment with surgery and/or radiotherapy in single-site and oligometastatic carcinoma of unknown primary. Eur J Cancer. 2021;157:179-189.
24. Rausch T, Jones DT, Zapatka M, et al. Genome sequencing of pediatric medulloblastoma links catastrophic DNA rearrangements with TP53 mutations. Cell. 2012;148:59-71.
25. Kazdai D, Endris V, Allgauer M, et al. Spatial and temporal heterogeneity of panel-based tumor mutational burden in pulmonary adenocarcinoma: separating biology from technical artifacts. J Thorac Oncol. 2019;14:1935-1947.
26. Robinson JT, Thorvaldsdottir H, Winckler W, et al. Integrative genomic viewers. Nat Biotechnol. 2011;29:24-26.
27. Ronellenfitsch MW, Harter PN, Kirchner M, et al. Targetable ERBB2 mutations identified in neurofibroma/scwannoma hybrid nerve sheath tumors. J Clin Invest. 2020;130:2488-2495.
28. Hothorn T, Lausen B. On the exact distribution of maximally selected rank statistics. Comput Stat Data Anal. 2003;3:121-137.
29. Cahlil DP, Kanzler KW, Vogelstein B, Lengauer C. Genetic instability and darwinian selection in tumours. Trends Cell Biol. 1999;9: M57-M60.
30. Bakhoun SF, Compton DA. Chromosomal instability and cancer: a complex relationship with therapeutic potential. J Clin Invest. 2012;122:1138-1143.
31. Rangel N, Forero-Castro M, Rondon-Lagos M. New insights in the cytogenetic practice: Karyotypic chaos, non-clonal chromosomal alterations and chromosomal instability in human cancer and therapy response. Genes (Basel). 2017;8:155.
32. Scrivano CM, Wang J, Esbona K, et al. Chromosomal instability sensitizes patient breast tumors to multipolar divisions induced by paclitaxel. Sci Transl Med. 2021;13:eabd4811.
33. Zasadil LM, Andersen KA, Yeum D, et al. Cytotoxicity of paclitaxel in breast cancer is due to chromosome missegregation on multipolar spindles. Sci Transl Med. 2014;6:229ra43.
34. Birkbak NJ, Eklund AC, Li Q, et al. Paradoxical relationship between chromosomal instability and survival outcome in cancer. Cancer Res. 2011;71:3447-3452.
35. Jamal-Hanjani M, A’Hern R, Birkbak NJ, et al. Extreme chromosomal instability forecasts improved outcome in ER-negative breast cancer: a prospective validation cohort study from the TACT trial. Ann Oncol. 2015;26:1340-1346.
36. Roylance R, Endesfelder D, Gorman P, et al. Relationship of extreme chromosomal instability with long-term survival in a retrospective analysis of primary breast cancer. Cancer Epidemiol Biomarkers Prev. 2011;20:2183-2194.
37. Lee J, Hahn S, Kim DW, et al. Evaluation of survival benefits by platinums and taxanes for an unfavourable subset of carcinoma of unknown primary: a systematic review and meta-analysis. Br J Cancer. 2013;108:39-48.
38. Golfinopoulos V, Pentheroudakis G, Salanti G, Nearchou AD, Ioannidis JPA, Pavlidis N. Comparative survival with diverse chemotherapy regimens for cancer of unknown primary site: multiple-treatments meta-analysis. Cancer Treat Rev. 2009;35:570-573.
39. Stephens PJ, Greenman CD, Fu B, et al. Massive genomic rearrangement acquired in a single catastrophic event during cancer development. Cell. 2011;144:27-40.
40. Maher CA, Wilson RK. Chromothripsis and human disease: piecing together the shattering process. Cell. 2012;148:29-32.
41. Hellmann MD, Callahan MK, Awad MM, et al. Tumor mutational burden and efficacy of Nivolumab monotherapy and in combination with Ipilimumab in small-cell lung cancer. Cancer Cell. 2018;33:853-861.e4.
42. Vanderwaide A, Spetzler D, Xiao N, Gatalica Z, Marshall J. Microsatellite instability status determined by next-generation sequencing and compared with PD-L1 and tumor mutational burden in 11,348 patients. Cancer Med. 2018;7:746-756.
43. Buchhalter I, Rempel E, Endris V, et al. Size matters: dissecting key parameters for panel-based tumor mutational burden analysis. Int J Cancer. 2019;144:848-858.
44. Klemperer SJ, Fabrizio D, Bane S, et al. Tumor mutational burden as a predictive biomarker for response to immune checkpoint inhibitors: a review of current evidence. Oncologist. 2020;25:e147-e159.
45. Chalmers ZR, Connelly CF, Fabrizio D, et al. Analysis of 100,000 human cancer genomes reveals the landscape of tumor mutational burden. Genome Med. 2017;9:34.
46. Yarchoan M, Albacker LA, Hopkins AC, et al. PD-L1 expression and tumor mutational burden are independent biomarkers in most cancers. JCI insight. 2019;4:e126908.
47. Zabarovsky ER, Lerman MI, Minna JD. Tumor suppressor genes on chromosome 3p involved in the pathogenesis of lung and other cancers. Oncogene. 2002;21:6915-6935.
48. Kettunen E, el Rifai W, Bjorkqvist AM, et al. A broad amplification pattern at 3q in squamous cell lung cancer—A fluorescence in situ hybridization study. Cancer Genet Cytoenet. 2000;117:66-70.

SUPPORTING INFORMATION
Additional supporting information may be found in the online version of the article at the publisher’s website.

How to cite this article: Bochtler T, Wohlfromm T, Hielscher T, et al. Prognostic impact of copy number alterations and tumor mutational burden in carcinoma of unknown primary. Genes Chromosomes Cancer. 2022;61(9):551-560. doi:10.1002/gcc.23047