Somatic mutation profiles in primary colorectal cancers and matching ovarian metastases: Identification of driver and passenger mutations

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Abstract

The mutational profiles of primary colorectal cancers (CRCs) and corresponding ovarian metastases were compared. Using a custom-made next generation sequencing panel, 115 cancer-driving genes were analyzed in a cohort of 26 primary CRCs and 30 matching ovarian metastases (four with bilateral metastases). To obtain a complete overview of the mutational profile, low thresholds were used in bioinformatics analysis to prevent low frequency passenger mutations from being filtered out. A subset of variants was validated using Sanger and/or hydrolysis probe assays. The mutational landscape of CRC that metastasized to the ovary was not strikingly different from CRC in consecutive series. When comparing primary CRCs and their matching ovarian metastases, there was considerable overlap in the mutations of early affected genes. A subset of mutations demonstrated less overlap, presumably being passenger mutations. In particular, primary CRCs showed a substantially high number of passenger mutations. We also compared the primary CRCs and matching metastases for stratifying variants of six genes (KRAS, NRAS, BRAF, FBXW7, PTEN and PIK3CA) that select for established (EGFR directed) or future targeted therapies. In a total of 31 variants 12 were not found in either of the two locations. Tumours thus differed in the number of discordant variants between the primary tumours and matching metastases. Half of these discordant variants were definitive class 4/5 pathogenic variants. However, in terms of temporal heterogeneity, no clear relationship was observed between the number of discordant variants and the time interval between primary CRCs and the detection of ovarian metastases. This suggests that dormant metastases may be present from the early days of the primary tumours.

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Introduction

Next-generation sequencing (NGS) provides the ability to determine the mutational profiles of tumours in a rapid and cost-effective manner [1,2]. Previous NGS experiments showed that distinct parts of the same tumour show different mutation profiles (spatial intra-tumour heterogeneity; ITH) [3,4]. Additionally, primary tumours and their metastases can differ in their mutational pattern, thereby showing temporal heterogeneity [5,6]. Determining the concordance between primary tumours and metastases is of interest for choosing the optimal treatment, ie, targeted therapies that are directed against variants present in the primary tumour but not in metastases will not be effective. Studies investigating the overlap and differences between the mutational profiles of primary tumours and matched metastases at specific locations are mostly lacking.

In this study, we selected colorectal tumours (colorectal cancer – CRCs) that metastasized to the ovaries. CRCs frequently metastasize to the liver and the lung, whereas ovarian metastases are sparse [7]. Ovarian metastasis occurs in approximately 3.4% of women diagnosed with a colorectal malignancy [8]. However, in up to 38% of cases, ovarian metastasis
because primary tumours and metastases are clonally related, it is possible to study the overlap of mutations and the effect of analysis settings.

Materials and methods

Medical consent

The present study was approved by the Medical Ethical Committee of the Leiden University Medical Center (protocol P01-019). Informed consent was obtained according to protocols approved by the LUMC Medical Ethical Committee (02-2004). Patient samples were handled according to the medical ethics guidelines described in the Code for Proper Secondary Use of Human Tissue established by the Dutch Federation of Medical Sciences (www.federa.org; accessed July 2014).

Sample selection and DNA isolation

Twenty-six CRCs, all diagnosed as adenocarcinomas, that metastasized to the ovaries were selected together with their 30 matching ovarian metastases. In four cases, metastases to both ovaries were included. The samples were obtained from the archives of the LUMC Pathology Department (period 1985–2010; n = 13) and from PALGA (the nationwide Dutch network and registry of histopathology and cytopathology; n = 13) [25]. The MMR proteins were not stained; however no class 5 (pathogenic) CTNNB1 variants, characteristic of most sporadic MSI-H cancers, were found. Lynch cancer cases were not included. So, most likely, the CRC cohort consisted primarily of microsatellite stable (MSS)-BRAF mutation negative cases (24/26) and a subset of MSS-BRAF mutation positive cases (2/26). The tissue taken for analysis was enriched for tumour cells after the evaluation of haematoxylin and eosin (H&E)-stained slides. Based on this evaluation, 0.6- or 2.0-mm tissue punches were taken from the selected tumour foci in the FFPE block using a tissue microarrayer (Beecher Instruments, Sun Prairie, WI, USA). In cases where the tumour cells were more dispersed, micro-dissection was performed on ten unstained 10-μm sections to achieve the highest tumour percentage (at least 50%). Prior to DNA isolation, the tissue was deparaffinized in xylene and washed in 70% ethanol. DNA was isolated using the NucleoSpin Tissue Genomic DNA Purification Kit (Machery-Nagel, Düren, Germany) according to the manufacturer’s instructions.
Construction of the gene list for target-enriched NGS

A gene list was compiled based on the most frequently mutated genes in COSMIC and the mutated genes lists described in the literature, resulting in a selection of 115 genes targeting 0.015% of the human genome (486,013 bp) [26–28]. See supplementary material, Table S1 for an overview of the genes included.

Sample library preparation

Library preparation was performed according to the HaloPlex protocol (Agilent, Santa Clara, CA, USA). In short, 225 ng of FFPE-DNA was fragmented using eight pairs of restriction enzymes. Hereafter, the customized probe library was added and hybridized to the targeted fragments. Additionally, a sample barcode sequence was incorporated in this step. Next, the targeted fragments were purified and amplified. The enriched, barcoded samples were sequenced on an Illumina HiSeq 2000. See supplementary material, Table S2 for the coverage numbers of the CRCs and the matching metastases to the ovaries.

Data analysis

Adaptors, barcodes and enzyme footprints were removed from the sequenced reads using SureCall software (Agilent Technologies, Santa Clara, CA), after which the reads were aligned to the human genome (hg19) using the Burrows-Wheeler aligner (BWA, version 0.7.5a) [21]. The Genome Analysis Toolkit (GATK, version 2.5) was used for realignment around the indels and base quality recalibration [22]. Duplicate removal was not performed due to the nature of hybridization-extension used to capture the target DNA regions. SNP and indel calling were carried out using VarScan software (version v2.3.6) with the following arguments: minimum read depth = 8, minimum number of reads with the alternative allele = 2, minimum base quality = 15, and minimum variant allele frequency = 0.10. VarScan somatic mode was used to analyze the primary vs. metastasis pairs.

Variants were functionally annotated using ANNOVAR [23,24]. We then selected variants more likely to have a deleterious effect. This was achieved by focusing on splicing and exonic variants (excluding synonymous) and removing the variants that were present with a frequency higher than 1% in the 1000 Genomes project (http://www.1000genomes.org/; data from April 2012) and/or in the NHLBI Exome Sequencing Project (http://evs.gs.washington.edu/EVS/; data from January 2013) because they are more likely to be germline. Variants in only the primary tumour or the metastasis were visually inspected to identify false discordant calls, ie, variants that are in fact present in both the primary tumour and the metastasis but that failed the 10% minimum threshold variant allele in one of the tissues.

Validation target-enriched NGS

Validation of the variants detected using target-enriched NGS was obtained via allele-specific qPCR of hotspot mutations and classic DNA Sanger sequencing. Allele-specific qPCR was performed to confirm the status of KRAS, BRAF and PIK3CA mutation hotspot loci [29]. TP53 (exons 5–8) and APC (mutation cluster region; exon 15) were analyzed via Sanger DNA sequencing. Sequences were analyzed with Mutation Surveyor (Bioke, Leiden, The Netherlands).

Results

Patient characteristics

In total, 26 primary CRCs and 30 matching ovarian metastases were tested (in four cases, both left and right ovarian metastases were included). The average age at CRC diagnosis was 56 years (range 28–84). In nine cases (35%), the ovarian metastases were synchronously present at the time of CRC diagnosis (cut-off point 6 months). In the other 17 cases, the ovarian metastases were diagnosed on average 2.9 years later (range 0.5–13). See Table 1 for details.

Data analysis

An average of 409 unfiltered variants were identified per sample with a standard deviation of 201. Regarding only variants more likely to have a deleterious effect (see supplementary materials), the numbers of variants per sample decreased to an average of 37 and a standard deviation of 30. The number of variants detected in this study was higher than the average
The number of variants observed in comparable sequencing efforts targeting a comparable number of bases [30,31]. The variant calling parameters were set at relatively low values to prevent passenger mutations from being filtered out. However, because the primary CRCs and the ovarian metastases were analyzed in pairs, when in doubt the variant could be manually compared with the matched sample. If the tumours had been analyzed without matched metastases, the parameters would have been higher to remove false positive variants. For more details, see Table 2 and supplementary material, Table S3 for all variants detected.

Mutation profile of primary CRCs and their metastases to the ovaries

An overview of the number of gene variants per case, varying from 9 to 113, can be observed in Table 2 and supplementary material, Table S3. Supplementary material, Figure S1A shows an overview of the number of variants per gene. APC, FAT4, NOTCH1, CACNA1B, STAB1 and TP53 showed the highest numbers of variants in the primary CRC samples. The most frequently affected gene was APC. Variants in APC were identified in 19 out of 26 samples (73%). FAT4 showed variants in 15 out of 26 analyzed (58%). NOTCH1 carried variants in 58%. In 54% of the cases, variants were observed in CACNA1B, STAB1 and TP53.

In the COSMIC database, the PIK3CA, FBXW7 and SMAD4 genes were reported to be mutated in CRC with frequencies of ~23%, ~20% and ~26%, respectively [32]. In this cohort, only two variants in PIK3CA (8%), five in FBXW7 (15%) and six in SMAD4 (23%) were found. To ensure that the low number of mutations in PIK3CA was not a sequencing artifact, the coverage was checked. The average coverage for PIK3CA was 155×; (median 113×; with a range of 1.4–1401). However, one of the hotspot positions in PIK3CA (ES542) showed less coverage than other parts of PIK3CA. Only one sample showed more than 20 reads at position E542 in PIK3CA. To investigate this mutation hotspot

Table 2. Overview of paired analysis of primary CRCs and matching metastases to the ovaries

|       | Total | Total Selected | Concordant | Discordant | Unique in CRC | Unique in metastasis |
|-------|-------|----------------|------------|------------|---------------|----------------------|
| 1     | 228   | 22             | 16         | 6          | 4             | 2                    |
| 2     | 147   | 15             | 9          | 6          | 2             | 4                    |
| 3     | 365   | 15             | 13         | 2          | 2             | 0                    |
| 4 Left| 788   | 20             | 11         | 9          | 6             | 3                    |
| 4 Right| 804  | 22             | 10         | 12         | 8             | 4                    |
| 5     | 162   | 28             | 3          | 25         | 24            | 1                    |
| 6     | 160   | 41             | 3          | 38         | 37            | 1                    |
| 7     | 204   | 33             | 10         | 23         | 5             | 18                   |
| 8     | 256   | 24             | 8          | 16         | 13            | 3                    |
| 9 Left| 854   | 113            | 12         | 101        | 96            | 5                    |
| 9 Right| 808  | 109            | 11         | 98         | 88            | 10                   |
| 10    | 251   | 42             | 6          | 36         | 34            | 2                    |
| 11    | 187   | 9              | 7          | 2          | 1             | 1                    |
| 12    | 242   | 25             | 7          | 18         | 2             | 16                   |
| 13    | 286   | 37             | 6          | 31         | 19            | 12                   |
| 14    | 242   | 10             | 5          | 5          | 1             | 4                    |
| 15 Left| 1136 | 42             | 10         | 32         | 25            | 7                    |
| 15 Right| 1003 | 36             | 9          | 27         | 22            | 5                    |
| 16    | 238   | 15             | 7          | 8          | 5             | 3                    |
| 17 Left| 892  | 56             | 10         | 46         | 27            | 19                   |
| 17 Right| 781  | 47             | 8          | 39         | 21            | 18                   |
| 18    | 134   | 16             | 7          | 9          | 5             | 4                    |
| 19    | 136   | 9              | 5          | 4          | 3             | 1                    |
| 20    | 315   | 76             | 11         | 65         | 55            | 10                   |
| 21    | 206   | 15             | 3          | 12         | 13            | 3                    |
| 22    | 209   | 68             | 13         | 55         | 55            | 0                    |
| 23    | 229   | 55             | 7          | 48         | 35            | 13                   |
| 24    | 378   | 25             | 6          | 19         | 16            | 3                    |
| 25    | 238   | 9              | 4          | 5          | 3             | 2                    |
| 26    | 395   | 61             | 10         | 51         | 43            | 8                    |
| Total | 12274 | 1095           | 247        | 848        | 669           | 179                  |

Shown are number of variants, number of selected variants [see Data analysis in supplementary materials], number of concordant and discordant variants, number of unique variants in the primary CRCs and in the metastases to the ovaries.
position, a TaqMan assay was performed. An additional mutation (p.E542K, c.1624G>A) was found in two cases leading to a mutation frequency in 15% of PIK3CA (4/26). Thus, in this cohort of CRCs, no differences were noted in mutation frequency for these driver genes. The most frequently affected genes in the ovarian metastasis were APC, TP53, CACNA1B and FAT4 (supplementary material, Figure S1B). Although the gene lists in supplementary material, Figures S1A and 1B slightly appear to contradict

Table 3. Concordant and discordant variants

Illustration of concordant and discordant variants. Black boxes indicate concordant variants (present in both the primary colorectal tumour as the metastasis to the ovary). Dotted boxes indicate discordant variants (present only in the primary colorectal tumour or in the metastasis to the ovary).

the gene lists that are normally reported to be mutated in CRC, the census genes show comparable mutation frequencies.

Concordance analysis of genes that select for targeted therapy

We compared the presence of stratifying mutations in the primary CRCs versus the ovarian metastases that (potentially) select for established (EGFR directed) or future targeted therapies. These genes comprise KRAS, NRAS, BRAF, FBXW7, PTEN and PIK3CA. MTOR, TSC1 and TSC2 were not covered in our gene panel. KRAS was discordant in 3 of 12 mutated cases; NRAS was not discordant (0/4); BRAF was discordant in 4/6 mutated cases; FBXW7 was discordant in 3/3 mutated cases; PTEN was discordant in 1/3 mutated cases and lastly PIK3CA was discordant in 1/3 mutated cases. Overall nine gene variants (2×KRAS, 3×BRAF, 3×FBXW7, 1×PIK3CA) that were present in the primary CRCs were not found in the metastases. Three gene variants (of KRAS, BRAF and PTEN) that were identified in the metastases were not found in the primary CRCs. Half of the 12 discordant variants were class 4/5 pathogenic variants, see supplementary material, Table S5.

Concordance analysis between primary CRCs and matching ovarian metastases: Effect of time intervals

All genes were analyzed for concordant and discordant variants (see Table 3). In cases of discordant variants, we investigated whether this was caused by an absence of the variant in the primary CRC or the metastasis. There were no variants that were called discordant due to an absence of reads in the matching sample at the position of that specific variant. The total number of discordant variants was 848. The average number of discordant variants per tumour pair was 28. Most discordant variants were caused by presence in the primary CRC tumours and absence in the metastases. Thus, primary CRCs demonstrate a large cohort of passenger mutations of which only a minor part is present in the matching metastases. The known driver genes were (as expected) mostly concordant. The numbers of discordant variants were more or less comparable between cases with the exception of one case (case number 9), which showed a remarkably high number of unique variants in the primary CRC (see Table 2).

Next, we plotted the time intervals between primary CRCs and metastases versus the amount of unique variants in the metastases. No correlation was
observed (Figure 1). For example, case 8 had a long interval of 13 years between the primary tumour and the metastasis; however, only 3 of 16 discordant variants were unique to the metastasis in the ovary.

Separate ovarian metastases of the same primary tumour show evidence for different metastasizing patterns

In four cases, both left and right ovarian metastases of the same CRC were sequenced. Two of the cases (numbers 4 and 17) showed a limited number of additional variants that were shared by both metastases but were not present in the primary CRC. In these cases, both ovaries were likely affected by the same metastasizing clone. In the other two cases (9 and 15), the additional variants that were present in both metastases were not observed in the primary tumour and showed no overlap with each other. In these cases, the metastases to both ovaries are most likely to be independent events originating from different subclones with their own specific mutational profiles. See supplementary material, Table S3 for details.

Discussion and Conclusion

Using the analysis of 115 cancer-driving genes, we compared the mutation profiles of primary CRC and matching ovarian metastases. Mutations could be grossly classified into three categories: mutations that are (1) ubiquitous (present in both the primary tumour and the metastasis), (2) restricted to the primary tumour or (3) only found in the metastasis. We show that loosening filter settings and manual inspection of mutation positions reveal a substantially larger overlap in mutation profiles. Many (driver) mutations are present in both the primary tumour and the metastasis, although sometimes only in a limited number of tumour cells. This could explain the dissimilarities in the mutational status of \textit{KRAS} and \textit{EGFR} in CRCs and hepatic metastases reported earlier [5].

Primary CRCs and their metastases showed considerable concordance for driver genes. In contrast to the classic driver genes, we identified a subset branch type of genes that displayed substantially less overlap [33]. The primary CRCs show substantially more passenger mutations than the ovarian metastases of CRC. It could be speculated that the large number of passenger mutations in the primary CRCs displays a large number of subclones that are spatially present. In this model certain subclones within primary tumours are most capable of homing into different target organs and even surviving adjuvant therapy. The other subclones do not contribute to the metastasizing process. Vignot \textit{et al} observed a similar pattern in lung tumours and their metastases [6].

We analyzed the number of variants in a temporal context (with metastasis occurring synchronously or metachronously with intervals of up to 13 years). It is assumed that new mutations will arise as time passes between the detection of the primary tumour and the metastasis, leading to more discordant gene variants. For synchronous metastases, the mutation profile is expected to be a comparable reflection of the mutation profile of

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Correlation between the number of discordant variants plotted against the time between the primary tumour and the metastases to the ovaries. The number of discordant variants is plotted on the y-axis (in bold). The time between the primary tumour and the metastases is plotted on the x-axis (in bold). The individual cases (1–26) are displayed in italics.}
\end{figure}
the primary tumour. However, no correlation between the number of variants and the time interval between primary CRC and matching metastasis was observed. Apparently, the underlying biology driving each individual tumour is more important than the actual intervals between the primary tumour and the metastasis in our cohort. Recent publications indicate differing mutational burdens in different cancer types [34,35]. These differences can for example occur as a result of highly mutagenic influences (smoking, sunburn, asbestos, etc), through the inactivation of DNA repair systems or the activation of APOBEC deaminases (apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like) [36]. Interestingly, the primary CRC in case number 9 carried a remarkably high number of passenger mutations (see Table 2), possibly caused by an underlying hypermutability deficit. An explanation to keep in mind is that the input DNA could have been of poor quality, leading to false positive variants. This option appears less likely in our study because the DNA quality was checked at case selection. An explanation for comparable mutation profiles between the primary tumour and the metastasis in cases where there is a long period between primary and metastasis detection is that metastases arise early during the development of the disease and are dormant for a period before they present clinically.

CRCs have been extensively characterized at the molecular level. The genes most frequently mutated in CRC are APC, TP53, KRAS, PIK3CA and SMAD4 [37]. All other genes are mutated in less than 10% of samples. In our series, APC and TP53 are frequently mutated (in 73 and 58% of the cases). The prevalence of other mutations in our series is comparable with the mutational profiles described in the literature and the COSMIC database [32]. The initial low frequency of PIK3CA mutations could be attributed to a low coverage of one of the PIK3CA hotspots. After performing an additional TaqMan analysis, the mutation frequency of PIK3CA was 15%, equal to data in available databases and previous studies (on average 15%) [37–39].

The link between mutation profiles and metastasizing patterns has been analyzed before. KRAS mutations in CRC were found to be associated with lung metastases [21]. CRCs with a wild type KRAS status showed more frequent liver and distant lymph node metastases [22]. KRAS mutation status is not informative in predicting peritoneal or ovarian metastases. Additionally, BRAF mutations are claimed to correlate with higher rates of peritoneal metastasis, distant lymph node metastasis, and lower rates of lung metastasis [40]. However, the number of reports on this topic are limited; thus, the metastasizing pattern in correlation with specific mutation profiles of CRC is not completely understood.

CRCs showing metastases to the ovaries did not show a specific profile. Besides, no specifically mutated gene in the metastases that could for example explain the ‘homing capacity’ of circulating tumour cells was identified.

The use of targeted therapy has become standard practice in advanced CRC; therefore it is important to determine the mutational landscape in various tumour locations within the same patient [41]. Depending on the type of targeted therapy, effectiveness will depend on the absence or presence of certain gene variants preferably in all tumour locations. We now present the first study that compares mutational profiles of ovarian metastases with their matching primary CRCs. Furthermore, druggable or stratifying mutations that select for targeted therapy can be present in one region of the tumour but absent in another, a phenomenon known as ITH. In this study we compared the primary tumour with one metastatic site. However, only one region of both the primary tumours and the metastases were investigated. When analyzing six different genes that select for current or future targeted therapies we found remarkable differences in KRAS, NRAS and BRAF mutational status that can select for EGFR directed therapy [42]. We identified differences in 7 of 22 variants when comparing primary tumours and matching metastases. These variants were identified in 20 cases, as in two cases both KRAS and BRAF mutations were identified, probably in distinct clones. Five of these seven were not found in the metastases, two of seven not in the primary tumours. Two of these seven variants were known class 5 pathogenic KRAS variants (see supplementary material, Table S5). Also gene variants (of PIK3CA, PTEN, FBXW7) that potentially could select for mTOR pathway(s) directed therapies showed remarkable differences. Will there be any benefit to a patient if only a minor fraction of the tumour mass carries a druggable mutation? Or what will be the benefit if the druggable mutation is present in the primary tumour, but not in the metastases? For CRC, data from actual studies testing these variables are mostly lacking. On the other hand previous studies have shown that KRAS pathogenic variations are often concordant between primary tumours and matching metastases. In our study KRAS, NRAS and BRAF mutational status did overlap between primary tumours and matching metastasis in 15/22 cases. In order to avoid the unrealistic goal of testing all tumour sites of every individual tumour it has been proposed that testing circulating free tumour DNA in plasma might be an alternative approach to pursue.

Limitations of our study are the restricted number of genes we investigated. Whole exome/genome sequencing might reveal differences that were not found with our 115-gene panel. Secondly, more extensive molecular characterization of tumours that also includes the...
analysis of transcriptome, methylome, microRNA, and proteome profiles could potentially show alterations that would explain why a small subset of CRCs show metastasis to ovarian sites.

Finally, the comparison of metastases to both left and right ovaries in individual cases revealed mutations that were shared by both metastases but were not identified within the matching primary tumours in two cases. The latter could suggest that separate metastases of the same primary tumour can have more overlap with each other than with the primary tumour.

In conclusion, this study showed a high concordance rate between CRCs and corresponding ovarian metastases for driver genes but less overlap for passenger genes. Although gene variants currently known to be clinically relevant were largely concordant between primary CRCs and matching metastases to the ovaries, there was a subset of cases that showed differences. The clinical relevance of mutations that are present in only a small percentage of tumour cells needs to be clarified. The number of discordant variants could likely be better explained by intra-tumour characteristics than by the time interval between the primary tumour and metastasis. CRCs metastasizing to the ovaries did not show a specific mutation profile in comparison to consecutive series of CRC, nor did the ovarian metastases.

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Author contributions

Conception/Design: SC, GF, TvW, HM. Financial support: All authors have nothing to disclose. Collection and/or assembly of data: SC, DR, RvE, MS. Data analysis and interpretation: SC, DR. Manuscript writing: SC, DR, TvW, HM. Final approval of manuscript: SC, TvW, HM.

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SUPPLEMENTARY MATERIAL ONLINE
Table S1. List of genes included in the custom-made gene panel (n = 115). The total number of genes is shown
Table S2. Coverage for (2A) primary CRC tumours and (2B) metastases to the ovaries. Total reads, mean coverage and percentage of bases covering more than 1x, 10x, 20x and 30x is shown for (2A) primary CRCs and (2B) metastases to the ovaries
Table S3. List of mutations in primary CRC tumours and metastases to the ovaries; and mutation profiles. In the first column the variants present in the primary colorectal tumour are shown. In the second column the variants present in the metastases to the ovaries are shown. Discordant variant are shown in italics
Table S4. Overlap and differences in mutation profiles of primary CRCs and matching metastases. A number of studies comparing the mutational profile of primary CRCs and matching metastases are shown. Due to the low number of studies a clear pattern cannot be observed
Table S5. List of discordant variants detected in KRAS, BRAF, FBXW7 and PTEN and their pathogenicity class
Figure S1A. Overview of number of variants per gene in primary CRCs metastasizing to the ovaries. Genes with 5 or more variants are displayed
Figure S1B. Overview of number of variants per gene in metastases to the ovaries from primary CRCs. Genes with 5 or more variants are displayed