Ioncopy: an R Shiny app to call copy number alterations in targeted NGS data

Jan Budczies1,4*, Nicole Pfarr2,4, Eva Romanovsky1, Volker Endris3, Albrecht Stenzinger3,4 and Carsten Denkert1,4

Abstract
Background: Somatic copy number alterations (CNAs) contribute to the clinically targetable aberrations in the tumor genome. For both routine diagnostics and biomarkers research, CNA analysis in a single assay together with somatic mutations is highly desirable.

Results: Ioncopy is a validated method and easy-to-use software for CNA calling from targeted NGS data. Copy number and significance of CNA are estimated for each gene in each sample. Copy number gains and losses are called after multiple testing corrections controlling FWER or FDR.

Conclusions: Ioncopy facilitates calling of CNAs in a cohort of tumors tissues with or without using normal (germline) DNA controls.

Keywords: Copy number alterations, NGS, Amplicon sequencing, R Shiny

Background
In addition to conventional somatic mutations such as point mutations and small indels, clinically relevant genetic alterations in tumors include large-scale somatic aberrations such as translocations and copy number alterations (CNAs). As classical example of an actionable CNA, testing for HER2 amplifications revolutionized breast cancer care, since the FDA approved Herceptin for the treatment of metastatic and later for early breast cancer [1]. Both the numbers of patients and genes commonly tested for amplifications in routine diagnostics keep continuously growing. For example, amplified HER2, MET and FGFR1 are potential drug targets in lung and other cancers [2–4]. MDM2 is used in diagnostics of liposarcoma [5] and has recently been described to be associated with hyperprogression of metastatic cancer after immune therapy [6].

Gene amplifications are usually tested using in situ hybridization (ISH) based assays. On the other hand, targeted NGS, e.g. using the IonTorrent S5 or the Illumina MiSeq platform, is the today's mainstay for routine testing of mutations. Therefore, integrating mutation analysis and CNA detection in a single assay would be highly desirable for both routine diagnostics and biomarker research.

Calling of CNAs from targeted sequencing data has shown to be feasible by several authors after publication of the first larger study [7]. Sensitivities obtained for HER2 amplification calling in breast cancer range between 89% and 93% at specificities between 98% and 100% [8–10]. Methodically, all approaches use DNA sequencing coverages as input and rely on calling coverage outliers after data normalization. Most of the current algorithm requires sequencing of paired tumor and normal (germline) DNA samples [11] or utilize a normal DNA references for normalization [10, 12, 13]. Outlier detection relies on simple thresholding [7, 9, 14] or more sophisticated methods of p-value estimation [8, 10] or bootstrap based estimation of confidence intervals [15].

Ioncopy is a method for calling of CNAs from targeted NGS data validated before [8], which can be run in two modes either using only the cohort of interest as input (and outlier detection by internal statistics) or making use of an additional reference cohort (e.g. of normal tissue or blood samples). The extended version presented here includes a GUI for easy data upload and straightforward CNA analysis.

Implementation
Ioncopy is an R Shiny app that can be operated without knowledge of the R programming language [16]. Input file
format, analysis parameters and the formats of the output files are described in the user manual (Additional file 1).

Amplicon coverages of the cohort of interest (and optionally additionally of a reference cohort) are used as input. Coverage data are uploaded as one or several tab-separated files. Data procession and CNA calling are performed as follows: 1. Sample normalization: Each sample is scaled with the median of its amplicon coverages. 2. Amplicon normalization: If reference coverages are available, each amplicon is scaled by its median coverage in the reference data. If no reference coverages are available, each amplicon is scaled by its median coverage in the target data. Multiplication by two (corresponding to two alleles) is performed to obtain estimates of copy numbers (CNs). 3. Significance assessment for CNAs in each amplicon and each sample: First, a normal distribution centered around CN = 2 with variance estimated from the median average deviation (mad) is fitted to the distribution of CNs for each amplicon. Then, a p-value is calculated for each amplicon and each sample assessing the degree of being an outlier to normal distribution. 4. In the mode “gene-wise”, the p-values of all amplicons interrogating a gene are summarized to a single p-value using Fisher’s method [17] and CNs are summarized by taking the amplicon average. 5. Either, no multiple testing corrections, multiple testing corrections with respect to samples, multiple testing corrections with respect to genes or multiple testing corrections with respect to samples and genes are done. Then, p-values are corrected to control either family-wise error rate (FWER) or false discovery rate (FDR) using the Bonferroni or the Benjamini-Hochberg method respectively. 6. CNAs (gains and losses) are called when passing the significance threshold (corrected \( p < 0.05 \)).

The intra-gene inconsistency (IGI) is a quality measure for a sample \( s \) that is calculated from its amplicon coverages \( CN(a, s) \) by

\[
IGI(s) = \sqrt{\frac{1}{n(g)-1} \sum_{a \in A(g)} (CN(a, s) - CN(g, s))^2}
\]

Therein, \( G \) denotes the set of all genes, \( n(g) \) the number and \( A(g) \) the set of amplicons interrogating the gene \( g \). \( CN(g, s) \) is the CN estimate for gene \( g \) calculated as average of \( CN(a, s) \) over the amplicons \( a \) interrogating the gene \( g \). Higher IGI corresponds higher intra-gene variance of CNs and is a technical issue related to inferior sample quality in the most cases. The IGI for each sample is included in the sample-centered output file of CNAs (CNAs samples: list). Hierarchical clustering in the heatmap display is performed using Manhattan distance and the average linkage method.

**Results and discussion**

As use case, we analyze 184 breast carcinoma sequenced on an Ion Torrent PGM using a 154-amplicon-panel and published before [8]. After loading of the ioncopy package and starting of the app by runIoncopy(), data upload and analysis can be performed GUI-based and without knowledge of the R language (Fig. 1). First, the

![Fig. 1 Screenshot of the Ioncopy GUI. The left panel functions to upload the matrix of sequencing coverages (amplicons x samples) and to start the analysis. In the middle panel, the user chooses gene-wise or amplicon-wise analysis and a method to correct CNA p-values for multiple testing. The appearance of the overview heatmap can be modified using the lower part of the middle panel. The right panel functions as result area and offers download of tables including calculated copy numbers, significances and CNA calls.](image)
coverage matrix of the breast cancer cohort (Additional file 2) is uploaded as target coverage file. Upload of a reference coverage matrix is optional and left out here. After hitting “Go!” Ioncopy estimates CNs and significances of CNAs for each gene in each sample. In the middle panel of the GUI, it can be chosen to perform the analysis either amplicon-wise or gene-wise. Multiple testing corrections and CNA calling can be performed in a more or a less stringent ways. In the gene-wise mode, the user can chose to call only CNAs that are supported by two or more calls of amplicons or by 50% (or another selectable percentage) of the amplicons located in the gene under consideration.

Operating Ioncopy in the gene-wise analysis mode, 396 (4.5%) gains and 80 losses (0.9%) are detected for FWER control at \( p = 0.05 \) taking into account multiple testing for both genes and samples. Out of these, 351 (4.0%) gains and 59 (0.7%) losses are supported by calls of at least two amplicons. The analysis mode of FWER control can be relaxed to FDR control at 5%, which leads to detection of 856 (9.7%) gains and 295 (3.3%) losses. Also, it is possible to run Ioncopy without multiple testing corrections, which is recommendable only for usage as search test together with validation of CNA candidates by an independent confirmatory test.

Called CNAs can be downloaded as matrix genes × samples, as gene-focused list or as sample-focused list. The latter includes IGI as a sample quality measure that is calculated from the CNs as described in the Implementation section. High IGI corresponding to inconsistency of CNs within genes and inferior coverage data quality, which is often detected in samples with low DNA quality (data not shown).

Heatmaps are generated to display the estimated CNs or the detected CNAs (Fig. 2). In the breast cancer data, \( \text{ERBB2, GRB7, STARD3 and MED1} \), all located in the chromosomal region 17q12 and typically co-amplified in HER2+ breast cancer, cluster tightly together. Several options are available to influence the appearance of the heatmaps. For example, sample clustering can be spared to preserve the natural order of the input data what can be helpful for technical quality control and exclusion of batch effects.

A limitation of NGS based CNA detection is tumor purity, as detection sensitivity decreases significantly when the tumor cell content falls below 50% [8]. For low tumor content samples, spatial-dissolved assays such as FISH should be preferred for CNA detection instead of bulk tissue based detection methods.

Conclusions
Ioncopy is a freely available and easy-to-use method for calling CNAs from targeted NGS data.

Availability and requirements
Project name: Ioncopy.
Project home page: https://cran.r-project.org/package=ioncopy
Operating system: Platform independent.
Programming language: R Shiny.
License: GNU GPL 3.
Restrictions to use by non-academics: none.

Additional files

| Additional file 1: | Ioncopy user manual. Comprehensive description of the input file format, of the analysis parameters and of the format of the output files. (PDF 1600 kb) |
| Additional file 2: | Breast cancer example data set. Coverage matrix of 152 amplicons (48 genes) in 184 breast cancer samples. (XLS 196 kb) |
Abbreviations
CN: Copy number; CNA: Copy number alterations; FDR: False discovery rate; FWER: Family-wise error rate; IG: Intra-gene inconsistency

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Availability of data and materials
The breast cancer data presented as use case are available in the extdata directory of the ioncopy R package.

Authors’ contributions
JB conceived the ioncopy method for calling CNAs and implemented it in R. ER contributed to software implementation as R Shiny app and to data analysis. NP, VE, AS and CD contributed to refinement of the Ioncopy method and to the discussion of the results. JB worked out the critical revision of the manuscript and the software with contributions by ER. All authors approved the final version of the manuscript.

Ethics approval and consent to participate
Generation and analysis of the data presented as use case was approved by the ethics board of the Charité Hospital (Reference number EA1/139/05 Amendment 2008).

Competing interests
The authors declare that they have no competing interests.

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Author details
1Institute of Pathology, Charité Universitätsmedizin Berlin, Berlin, Germany.
2Institute of Pathology, Technical University Munich (TUM), Munich, Germany.
3Institute of Pathology, University Hospital Heidelberg, Heidelberg, Germany.
4German Cancer Consortium (DKTK), Berlin, Munich and Heidelberg partner sites, Germany.

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References
1. Romond EH, Perez EA, Bryant J, Suman VJ, Geyer CE Jr, Davidson NE, Tan-Chiu E, Martinus S, Paik S, Kaufman PA, et al. Tastuzumab plus adjuvant chemotherapy for operable HER2-positive breast cancer. N Engl J Med. 2005;353(16):1673–84.
2. Takáts K, Pirazzoli V, Arcila ME, Nebhan CA, Song X, de Stanchina E, Ohashi K, Janjigian YY, Spitzler PJ, Melnick MA, et al. HER2 amplification: a potential mechanism of acquired resistance to EGFR inhibition in EGFR-mutant lung cancers that lack the second-site EGFR790M mutation. Cancer Discov. 2012;2(10):922–33.
3. Bean J, Brennan C, Shi J, Li JY, Yiely G, Vale A, Wang L, Chitale D, Motto N, Szoke J, Broderick S, et al. MET amplification occurs with or without T790M mutations in EGFR mutant lung tumors with acquired resistance to gefitinib or erlotinib. Proc Natl Acad Sci U S A. 2007;104(52):20932–7.
4. Helsten T, Ekin S, Arthur E, Tomson BN, Carter J, Kurzrock R. The FGFR gene landscape in Cancer: analysis of 4,833 tumors by next-generation sequencing. Clin Cancer Res. 2016;22(1):259–67.
5. Nakayama T, Toguchida J, Wadayama Y, Kanoe H, Kotoura Y, Sasaki MS. MDM2 gene amplification in bone and soft-tissue tumors: association with tumor progression in differentiated adipose-tissue tumors. Int J Cancer. 1995;64(5):342–6.
6. Forsschner A, Niessner H, Moller Y, Horak P, Frohlich M, Warsow G, Stenzinger A, Frohling S, Grimm H, Klumpp B, et al. Genomics of immunotherapy-associated Hyperprogressors-letter. Clin Cancer Res. 2017;23(20):6374–5.
7. Endri V, Penzel R, Warth A, Muckenhuber A, Schirmacher P, Stenzinger A, Weichert W. Molecular diagnostic profiling of lung cancer specimens with a semiconductor-based massive parallel sequencing approach: feasibility, costs, and performance compared with conventional sequencing. J Mol Diagn. 2013;15(6):765–75.
8. Budczies J, Pfarr N, Stenzinger A, Treue D, Endri V, Ismael F, Bangemann N, Blohmer JU, Dietel M, Loibl S, et al. Ioncopy: a novel method for calling copy number alterations in amplicon sequencing data including significance assessment. Oncotarget. 2016;7(11):15326–47.
9. Pfarr N, Penzel R, Endri V, Lier C, Flechtenmacher C, Volckmar AL, Kirchner M, Budczies J, Leichsenring J, Herpel E, et al. Targeted next-generation sequencing enables reliable detection of HER2 (ERBB2) status in breast cancer and provides ancillary information of clinical relevance. Genes Chromosomes Cancer. 2017;56(4):255–65.
10. Ross DS, Zehir A, Cheng DT, Benajide R, Nafa K, Hechtman JF, Janjigian YY, Weigelt B, Razavi F, Hyman DM, et al. Next-generation assessment of human epidermal growth factor receptor 2 (ERBB2) amplification status: clinical validation in the context of a hybrid capture-based, comprehensive solid tumor genomic profiling assay. J Mol Diagn. 2017;19(2):244–53.
11. Hoogstraat M, Hinrichs JW, Beselink NJ, Radersma-van Loon JH, de Voijf CM, Peeters T, Nijman U, de Weger RA, Voest EE, Willems SM, et al. Simultaneous detection of clinically relevant mutations and amplifications for routine cancer pathology. J Mol Diagn. 2015;17(1):10–8.
12. Pritchard CC, Salipante SJ, Koehler K, Smith C, Scroggins S, Wood B, Wu D, Lee MK, Dintzis S, Adey A, et al. Validation and implementation of targeted capture and sequencing for the detection of actionable mutation, copy number variation, and gene rearrangement in clinical cancer specimens. J Mol Diagn. 2014;16(1):56–67.
13. Grasso C, Butler T, Rhodes K, Quist M, Neff TL, Moore S, Tomlins SA, Reisig E, Beadling C, Andersen M, et al. Assessing copy number alterations in targeted, amplicon-based next-generation sequencing data. J Mol Diagn. 2015;17(1):53–63.
14. Pfarr N, Penzel R, Klauschen F, Heim D, Brandt R, Kazdal D, Resinghaus M, Herpel E, Schirmacher P, Warth A, et al. Copy number changes of clinically actionable genes in melanoma, non-small cell lung cancer and colorectal cancer—a survey across 822 routine diagnostic cases. Genes Chromosomes Cancer. 2016;55(11):821–33.
15. Oliveira C, Wolf T. CMPanelizer: reliable CNV detection in targeted sequencing applications. R package version 1.8.0. 2016; https://bioconductor.org/packages/CMPanelizer.
16. Budczies J, Romanovsky E. Ioncopy: calling copy number alterations in amplicon sequencing data. R package version 2.0.1. 2017; https://cran.r-project.org/package=ioncopy.
17. Fisher RA. Statistical methods for research workers. Edinburgh: Statistical Methods for Research Workers; 1925.