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ARTICLE

Deriving tumor purity from cancer next generation sequencing data: applications for quantitative ERBB2 (HER2) copy number analysis and germline inference of BRCA1 and BRCA2 mutations

Stephanie E. Siegmund[1], Danielle K. Manning[1], Phani K. Davineni[1] and Fei Dong[1]✉

Tumor purity, or the relative contribution of tumor cells out of all cells in a pathological specimen, influences mutation identification and clinical interpretation of cancer panel next generation sequencing results. Here, we describe a method of calculating tumor purity using pathologist-guided copy number analysis from sequencing data. Molecular calculation of tumor purity showed strong linear correlation with purity derived from driver *KRAS* or *BRAF* variant allele fractions in colorectal cancers ($R^2 = 0.79$) compared to histological estimation in the same set of colorectal cancers ($R^2 = 0.01$) and in a broader dataset of cancers with various diagnoses ($R^2 = 0.35$). We used calculated tumor purity to quantitate *ERBB2* copy number in breast carcinomas with equivocal immunohistochemical staining and demonstrated strong correlation with fluorescence in situ hybridization ($R^2 = 0.88$). Finally, we used calculated tumor purity to infer the germline status of variants in breast and ovarian carcinomas with concurrent germline testing. Tumor-only next generation sequencing correctly predicted the somatic versus germline nature of 26 of 26 (100%) pathogenic *TP53*, *BRCA1* and *BRCA2* variants. In this article, we describe a framework for calculating tumor purity from cancer next generation sequencing data. Accurate tumor purity assessment can be assimilated into interpretation pipelines to derive clinically useful information from cancer genomic panels.

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INTRODUCTION

Over the past decade, massively parallel next generation sequencing (NGS) of cancer specimens has been rapidly adopted to become standard of care in many oncology practices. NGS has replaced single gene assays in many molecular pathology laboratories[1]. As of 2017, 76% of oncologists in the United States in a nationally representative survey reported using NGS tests to guide treatment decisions for patients with advanced disease, to determine eligibility for clinical trials, and to prescribe off-label therapy[2].

Cancer panel NGS assays have been validated in multiple academic and commercial laboratories[3–6], and these comprehensive assays are capable of detecting many types of genetic alterations within cancer genomes, including sequence alterations (nucleotide substitutions, insertions and deletions), structural variants and copy number alterations[7]. Identifying different types of alterations usually requires multiple informatics tools[8,9]. As a result, single nucleotide variants and copy number alterations are commonly assessed independently in validation and in the clinical report. Clinical practices vary among laboratories in the reporting of higher complexity findings, including tumor purity, variant allele fraction, copy number analysis and clinical variant interpretation[10].

Pathological evaluation of tumor tissue is an important pre-analytical consideration for cancer molecular testing. Most cancer NGS assays are validated to detect somatic mutations at variant allele fraction of as low as 5–10% and generally require tissue specimens containing at least 20% tumor nuclei. While most laboratories conduct histological tumor purity assessment, there is a need for standardization in clinical practice[11]. Tumor purity can also be used to guide analysis of somatic copy number variation and is necessary if quantitative copy number analysis is performed. It has been shown previously that computational assessment of tumor purity can help refine NGS analysis, including such parameters as germline mutation inference and tumor mutational burden[12,13]. While several tools exist to calculate tumor purity based on the sequencing data, these generally rely on paired tumor-normal samples or require whole genome or whole exome sequencing[14–17].

In this article, we describe methods to analyze panel sequencing data and achieve a more comprehensive analytical and clinical interpretation of an unpaired tumor specimen. We demonstrate that NGS data can be used to quantitate tumor purity, which can in turn be used to improve quantitative copy number analysis and to infer the somatic or germline status of pathogenic variants.

MATERIALS AND METHODS

Cancer next generation sequencing

Next generation sequencing (NGS) was performed using OncoPanel, a hybrid-capture based targeted sequencing assay[5]. Pre-analytical histologic

[1]Department of Pathology, Brigham and Women's Hospital, Boston, MA, USA. ✉email: fdong1@bwh.harvard.edu

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examination was performed for all cases prior to sequencing. A tumor-enriched region of interest was outlined on a hematoxylin and eosin-stained slide, and a histological estimation of tumor purity was recorded. Sequencing required at least 20% tumor nuclei in the region of interest. Deoxyribonucleic acid was isolated after macro-dissection of the corresponding tissue from unstained slides. A panel of 447 cancer-associated genes was enriched using solution-based hybrid capture (Agilent SureSelect; Agilent Technologies; Santa Clara, CA). Massively parallel sequencing was performed using Illumina HiSeq2500 (Illumina, Inc., San Diego, CA). Single nucleotide variants were detected using Mutect version 2. Insertion and deletion variants were detected using GATK version 4. Copy number analysis was visualized with RobustCNV version 2.0.1. Germline polymorphism variant allele fractions were visualized using a laboratory developed tool.

Tumor purity calculation

Relative copy number variation was calculated and visualized using RobustCNV. Briefly, the relative read contribution of each gene exon in the tumor specimen, or the proportion of reads mapping to the exon compared to all reads, was compared to relative read contribution of the same exon in a non-neoplastic control. To improve copy number detection, systematic bias and GC bias correction was implemented to increase the signal to noise ratio via a two-step process. In step one, systematic bias was removed by fitting a robust regression model with iteratively re-weighted least squares, as implemented in the MASS package in R. The model was then used to estimate expected values for each gene exon. The log2(observed/predicted) value was then calculated for each gene exon. In step two GC bias was removed through Local Polynomial Regression (loess) where the previously normalized values were fitted against GC content. The resulting model was then used to generate a set of predicted values which were subtracted from the observed values. Relative read contributions from each gene exon was plotted in log2 scale, with relative copy number gains displayed as values above 0, and relative copy number losses displayed as values below 0. Manual copy number calls were made by human reviewers via an interactive user interface portal. In validation, copy number detection by NGS achieved 86% sensitivity and 98% specificity compared to array comparative genomic hybridization[5].

The cancer panel incidentally captured germline polymorphisms, which were detected by MuTect and plotted for evaluation concurrent with copy number changes. Pathologist copy number interpretation was performed akin to analysis of microarray data in accordance with technical standards[18,19].

The tumor purity was calculated by the following formula, where T represents tumor purity, X represents the median log2 ratio of a one-copy (haploid) state, and Y represents the median log2 ratio of a two-copy (diploid) state. These reference states were selected by the pathologist and correspond to a whole chromosome or chromosome region or arm.

$$T = 2 - 2\left(2^{X-Y}\right)$$

Absolute copy number estimation was performed as follows, where A represents absolute copy number, Z represents the median log2 ratio of gene of interest, Y represents the median log2 ratio of the diploid copy number state, and T represents calculated tumor purity.

$$A = \frac{2(2^{Z-Y} + T - 1)}{T}$$

Examples of pathologist-guided copy number estimation and tumor purity calculation are provided in Supplemental File 1.

To infer the germline status of pathogenic variants in *BRCA1*, *BRCA2* and *TP53*, expected variant allele fractions were modeled for both germline and somatic scenarios at the observed copy number state (either one copy deletion or copy number neutral loss of heterozygosity). A probability density function was constructed for the expected number of mutant reads given the observed target coverage with normal approximation of the binomial distribution. The variant was inferred to be germline if the probability of the observed allele fraction in the expected germline scenario is greater than that in the expected somatic scenario.

Case selection

All sequencing results were reviewed by a board-certified molecular pathologist (F.D.). Cases were excluded if there were no identifiable copy number changes due to low tumor purity or have highly complex copy number changes precluding determination of one-copy and two-copy states.

To validate tumor purity calculations, 12 colorectal adenocarcinomas with driver oncogenic mutations in *KRAS* or *BRAF* were identified[20]. Analysis was limited to cases with mutations at non-amplified diploid loci, and the driver mutations were assumed to represent de novo mutations involving all tumor cells and absent in non-neoplastic cells. For *KRAS* or *BRAF* mutations involving one of two tumor alleles, tumor purity was estimated as follows, where T represent tumor purity and X represents driver mutation variant allele fraction, and compared to tumor purity calculation based on copy number variants:

$$T = 2X$$

Analysis of absolute copy number quantitation included 19 invasive breast carcinomas with equivocal (2+) expression of *ERBB2* (HER2) by immunohistochemistry. *ERBB2* fluorescence in situ hybridization (FISH) was performed in accordance to the 2018 American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) guidelines[21]. By FISH, tumors with *ERBB2* copy number ≥4.0 and *ERBB2*/CEP17 ratio ≥2.0 were considered positive. Tumors with *ERBB2* copy number <4.0 and *ERBB2*/CEP17 ratio <2.0 were considered negative. By NGS, tumors with *ERBB2* copy number ≥4.0 and *ERBB2*/*NF1* ratio ≥2.0 were considered positive. Tumors with *ERBB2* copy number <4.0 and *ERBB2*/*NF1* ratio <2.0 were considered negative.

To infer germline status from tumor sequencing, nine cases of invasive breast carcinoma and six cases of high grade serous carcinoma of the ovary harboring *BRCA1* or *BRCA2* mutations were identified. Each *BRCA1*, *BRCA2,* and *TP53* variant was classified based on tumor-only NGS data as germline or somatic. All 15 patients received concurrent germline testing for *BRCA1*, *BRCA2* and *TP53* as a component of clinical care via a targeted sequencing panel. Clinical germline testing results were collected by chart review. The tumor-only NGS classification was compared to germline panel testing results for each variant.

RESULTS

Calculation of tumor purity from NGS data

We first calculated tumor purity for a clinical dataset of tumors sequenced by NGS. We identified 265 NGS cases consecutively reported by a single pathologist. Of these 265 cases, 135 (51%) were excluded due to failed sequencing ($n = 4$, 2%), having a flat copy number profile indicating tumor purity below level of detection of copy number alterations ($n = 85$, 32%), or having complex number profile precluding reliable identification of the diploid copy number state ($n = 46$, 17%). Cases with flat copy number profiles are enriched for specimens of borderline adequacy, defined as a histological estimate of 30% or fewer tumor nuclei. 43 of 85 (51%) cases with flat copy number profiles were of borderline adequacy compared to 24 of 130 (18%) cases that underwent successful tumor purity analysis (Fisher's exact $p < 0.001$). Cases with complex copy number profiles had a similar rate of borderline adequacy (11 of 46, 24%) compared to cases that underwent successful tumor purity analysis (Fisher's exact $p = 0.52$).

TP53 variants were identified by sequencing in 142 cases in the cohort. Specimens excluded due to a flat copy number profile had a mean *TP53* variant allele fraction of 18% (standard deviation 12%), which was significantly lower than the mean *TP53* variant allele fraction observed in specimens that were successfully analyzed for tumor purity (mean *TP53* allele fraction 52%, standard deviation 19%, *t*-test $p < 0.001$). The mean *TP53* variant allele fraction for cases excluded due to complex copy number changes was 48% (standard deviation 20%), which was not significantly different from that of cases that were successfully analyzed (*t*-test $p = 0.35$). These findings demonstrated that specimens with low tumor purity were likely to exhibit flat copy number profiles, limiting the utility of methodologies dependent on copy number changes in this scenario.

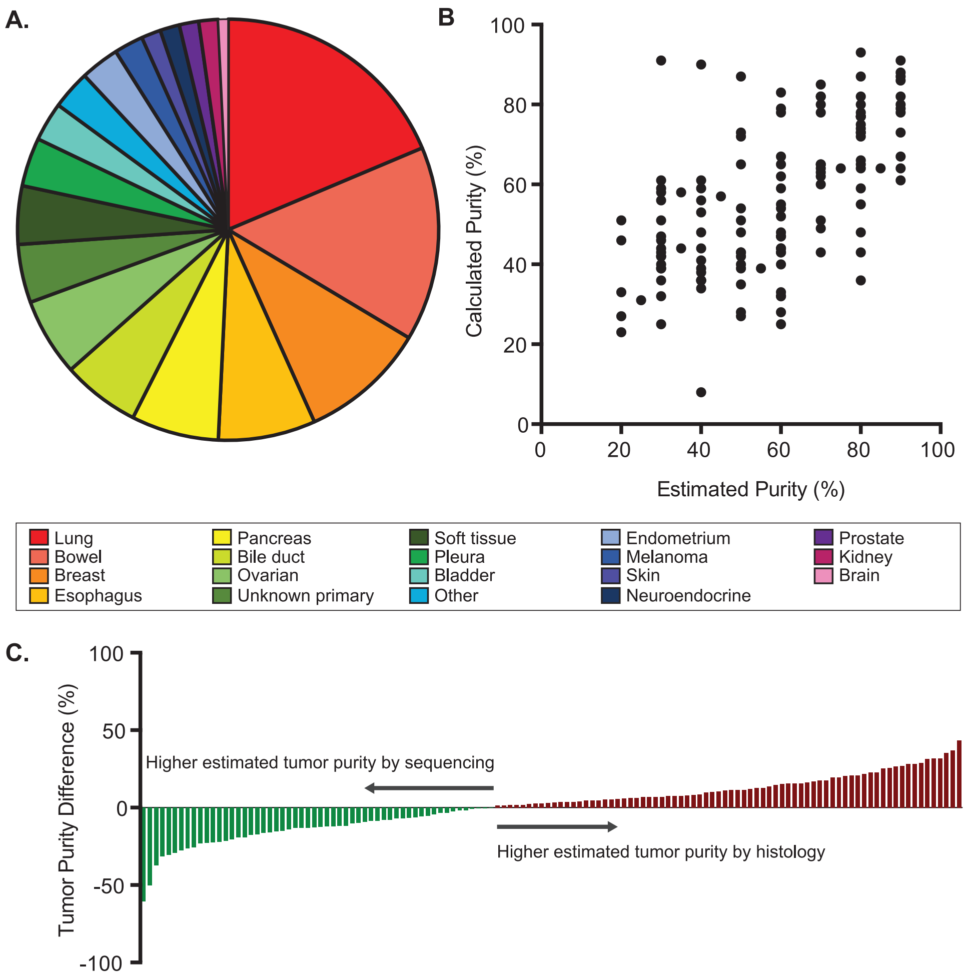


Fig. 1 Tumor purity estimation by histology versus calculation based on next generation sequencing (NGS). A Pie chart of primary sites for 130 tumors, representing the spectrum of clinical solid tumor specimens sequenced at the institution. **B** Tumor purity based on histologic estimation versus tumor purity based on calculation by NGS. **C** Difference in tumor purity based on histology and sequencing in cancer specimens.

The successfully analyzed dataset included 130 tumors representing the spectrum of clinical specimens sequenced at our institution (Fig. 1A). Calculated tumor purity was compared to estimated tumor purity by histology, and these variables showed a weak positive association ($R^2 = 0.35$, 95% Confidence Interval (CI) = 0.47–0.69, Fig. 1B). Calculated tumor purity was within 10% of histologic estimation in 45% (59 of 130) of tumors and within 20% histologic estimation in 74% (96 of 130) of tumors (Fig. 1C). In this cohort, histology review estimated higher tumor purity compared to the calculated tumor purity in 57% of cases. Of note, the actual value may be higher since specimens with lowest tumor purity with flat copy number profiles were excluded from this calculation.

To further validate our method of tumor purity calculation, we analyzed the calculated tumor purity based on copy number alterations in 12 colorectal adenocarcinomas with driver oncogenic mutations in *KRAS* or *BRAF* (Table 1). Pathogenic pathway-activating somatic mutations in the RAS-MAPK pathway genes have been strongly implicated as causative events of colorectal tumor evolution[22–24]. Calculated tumor purity based on copy number alterations was compared to tumor purity based on driver mutation allele fraction, demonstrating a strong positive correlation ($R^2 = 0.79$, 95% CI = 0.64–0.97, Fig. 2A). In contrast, there was poor correlation between histologic estimation and calculated tumor purity by copy number alterations ($R^2 = 0.01$, 95% CI = −0.51–0.63, Fig. 2B) or between histologic estimation and calculated tumor purity by driver mutations ($R^2 = 0.05$, 95% CI = −0.41–0.70).

Finally, we assessed interobserver variability between two experienced reviewers (D.K.M. and F.D.) across 12 specimens. The calculated tumor purity between the two observers was linearly correlated ($R^2 = 0.83$, 95% CI 0.72–0.98, Fig. 3). The median absolute difference in tumor purity between the two observers was 3.5%.

Absolute copy number quantitation

To demonstrate the clinical utility of calculating tumor purity, we used calculated tumor purity to quantitate unknown copy number states. We selected the clinically relevant target gene *ERBB2*, which is amplified in a subset of breast carcinoma and serves as both a prognostic biomarker and a target for *ERBB2* inhibitor therapies[25]. Current clinical guidelines according to the 2018 ASCO/CAP call for *ERBB2* gene evaluation by a combination of immunohistochemistry and FISH[21]. Carcinomas that are equivocal (2+) for ERBB2 protein expression by immunohistochemistry represent a diagnostic dilemma, as these tumors demonstrate a broad range of copy number states in *ERBB2*[26].

We evaluated 19 invasive breast carcinomas with equivocal (2+) ERBB2 expression by immunohistochemistry, including 12 cases that were positive for *ERBB2* amplification and 7 that were negative for *ERBB2* amplification by FISH (Table 2). First, we quantitated

Table 1. Tumor purity calculation in colorectal cancers by next generation sequencing based on RAS pathway driver mutation variant allele fraction, copy number variation, and histology estimation.

Driver mutation	Variant allele fraction	Tumor purity (by Driver mutation)	Tumor purity (by Copy number)	Tumor purity (Histology estimation)	RAS Copy number and Zygosity
KRAS c.38 G > A (p.G13D)	12%	24%	31%	25%	Two copy heterozygous
KRAS c.35 G > T (p.G12V)	14%	28%	33%	20%	Two copy heterozygous
NRAS c.35 G > A (p.G12D)	17%	34%	47%	30%	Two copy heterozygous
KRAS c.35 G > C (p.G12A)	18%	36%	27%	50%	Two copy heterozygous
KRAS c.35 G > T (p.G12V)	18%	36%	42%	30%	Two copy heterozygous
KRAS c.35 G > C (p.G12A)	21%	42%	34%	40%	Two copy heterozygous
KRAS c.35 G > A (p.G12D)	25%	50%	54%	60%	Two copy heterozygous
KRAS c.35 G > T (p.G12V)	25%	50%	59%	30%	Two copy heterozygous
BRAF c.1781A > G (p D594G)	26%	52%	49%	70%	Two copy heterozygous
KRAS c.35 G > A (p.G12D)	33%	66%	56%	30%	Two copy heterozygous
KRAS c.35 G > A (p.G12D)	35%	70%	61%	30%	Two copy heterozygous
KRAS c.38 G > A (p.G13D)	82%	82%	90%	40%	Two copy with loss of heterozygosity

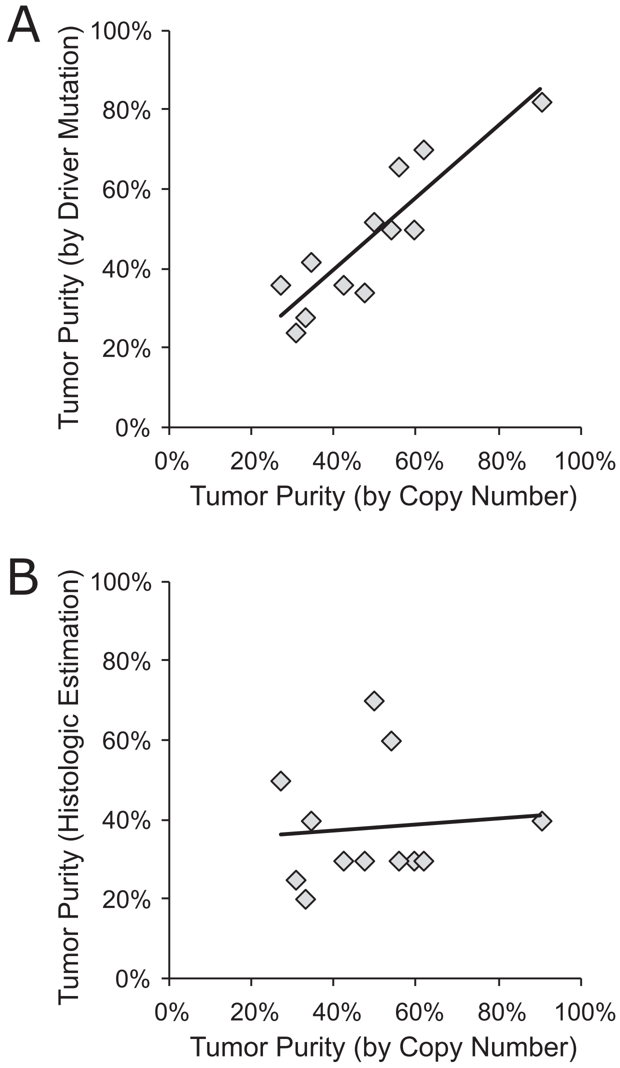


Fig. 2 Copy number-based tumor purity calculation compared to driver mutation allele fraction. A Calculated tumor purity in colorectal cancers based on copy number changes (*X*-axis) versus RAS driver mutation allele fraction (*Y*-axis) shows linear correlation ($R^2 = 0.79$). **B** Calculated tumor purity (*X*-axis) versus histologic estimation (*Y*-axis) shows poor correlation ($R^2 = 0.01$).

absolute *ERBB2* copy number by NGS and compared this to the absolute copy number assessment by manual counting of FISH specimens. *ERBB2* copy numbers by NGS and FISH were strongly correlated ($R^2 = 0.88$, 95% CI = 0.84–0.98, Fig. 4A). Since clinical guidelines evaluate the ratio of *ERBB2* copy number to a chromosome 17 centromeric probe, we approximated this ratio by assessing the copy number ratio of *ERBB2* to *NF1*, the gene in our NGS panel on chromosome 17q that is closest to the centromere. A comparison of the ratios of *ERBB2* to the chromosome 17 control (*ERBB2* to CEP17 ratio by FISH or *ERBB2* to *NF1* copy number by NGS) showed that the ratios determined by FISH and NGS were linearly correlated ($R^2 = 0.76$, 95% CI = 0.67–0.95, Fig. 4B).

We next adapted the ASCO/CAP algorithm to determine categorical amplification status for our cohort of 19 tumors and found 95% concordance (18 of 19 cases) between NGS and FISH. In the one case of discordance, *ERBB2* amplification was found to be positive by FISH (*ERBB2* to CEP17 ratio 6.7/3.3 = 2.1) but negative by NGS (*ERBB2* to *NF1* ratio 2.0/2.1 = 0.9). Although we cannot completely explain this discordance, this specimen consisted of a breast carcinoma metastasis in a patient with a known prior specimen that was negative for *ERBB2* amplification, and the

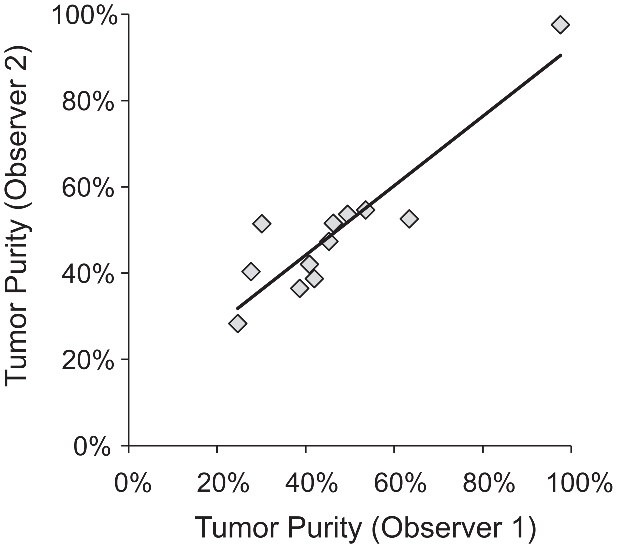


Fig. 3 Interobserver variability between two reviewers was assessed across 12 specimens. The calculated tumor purity between the two observers was linearly correlated ($R^2 = 0.83$).

discordance could be related to tumor heterogeneity. Another possible explanation may be the presence of a hyperdiploid genomic copy number state that could not be deduced from the NGS copy number profile. An additional benefit of *ERBB2* analysis by NGS is the ability to visualize *ERBB2* copy number with respect to the rest of chromosome 17. Examples of *ERBB2* focal amplification, *ERBB2* gain as a part of chromosome 17q arm-level gain, and *ERBB2* gain as a part of complex copy number alterations involving chromosome 17q are shown in Fig. 4C.

Inference of germline alterations using tumor-only sequencing

With an ability to calculate tumor purity, we hypothesized that tumor-only sequencing data could be used to infer the germline status of variants. In most tumor predisposition syndromes, non-neoplastic cells harbor a pathogenic variant in one of two germline alleles, and the wild type allele is lost in neoplastic cells during tumorigenesis, commonly by gene deletion or copy number neutral loss of heterozygosity. The observed variant allele fraction observed in tumor-only sequencing data can be used to infer whether non-neoplastic cells in the specimen harbor the variant.

To test our hypothesis, we evaluated 9 invasive breast carcinomas and 6 ovarian high grade serous carcinomas with that harbored variants in *BRCA1* or *BRCA2* ($n=15$). Eleven of these cases also harbored concurrent sporadic mutations in *TP53*, which were used as additional somatic variants in our analysis. *BRCA1*, *BRCA2* and *TP53* germline status were previously assessed by clinical germline panel testing. In total, the cohort included 26 mutations with known germline status (12 germline, 14 somatic) (Table 2).

The calculated tumor purity of breast and ovarian carcinomas in the dataset ranged from 28 to 87%. Overall, the observed variant allele fraction was correlated with calculated tumor purity ($R^2 = 0.53$, 95% CI = 0.47–0.87), and demonstrated close agreement with predicted values for the corresponding germline and somatic status (Fig. 5A, B). Based on comparison of the expected tumor purity and observed variant allele fraction, 26 of 26 (100%) variants were correctly classified as germline or somatic based on tumor-only NGS data (Table 3).

DISCUSSION

The histopathological examination of tumor tissue is a key pre-analytical quality control measure for cancer NGS testing. Inadequate

Table 2. Comparison of absolute ERBB2 copy number estimation by fluorescence in situ hybridization (FISH) and cancer panel next generation sequencing (NGS) in breast cancers.

Tumor purity (NGS)	*ERBB2* copies (FISH)	CEP17 copies (FISH)	*ERBB2* / CEP17 ratio (FISH)	*ERBB2* copies (NGS)	*NF1* copies (NGS)	*ERBB2* / *NF1* ratio (NGS)	*ERBB2* interpretation (FISH)	*ERBB2* interpretation (NGS)
49%	32.90	1.50	21.93	60.87	3.54	17.19	Positive	Positive
59%	29.73	1.63	18.24	43.89	1.93	22.74	Positive	Positive
60%	16.87	1.67	10.10	21.71	2.31	9.40	Positive	Positive
47%	20.10	2.23	9.01	30.18	1.83	16.49	Positive	Positive
53%	29.03	3.33	8.72	29.05	1.40	20.75	Positive	Positive
49%	12.90	1.93	6.68	15.65	2.22	7.05	Positive	Positive
15%	16.30	2.47	6.60	31.43	3.08	10.20	Positive	Positive
64%	13.33	2.87	4.65	8.80	2.03	4.33	Positive	Positive
47%	13.83	4.23	3.27	15.78	5.98	2.64	Positive	Positive
72%	5.68	1.74	3.26	8.73	2.08	4.20	Positive	Positive
40%	6.90	2.80	2.46	14.98	3.75	3.99	Positive	Positive
69%	6.70	3.23	2.07	2.00	2.14	0.93	Positive	Negative
33%	4.50	2.37	1.90	4.75	3.99	1.19	Negative	Negative
52%	2.40	1.90	1.26	2.54	2.05	1.24	Negative	Negative
80%	1.80	1.43	1.26	1.12	1.07	1.05	Negative	Negative
81%	3.03	2.60	1.17	1.36	2.35	0.58	Negative	Negative
69%	2.03	1.77	1.15	2.23	1.94	1.15	Negative	Negative
77%	3.07	2.73	1.12	2.91	3.08	0.94	Negative	Negative
46%	2.10	1.90	1.11	2.96	1.94	1.53	Negative	Negative

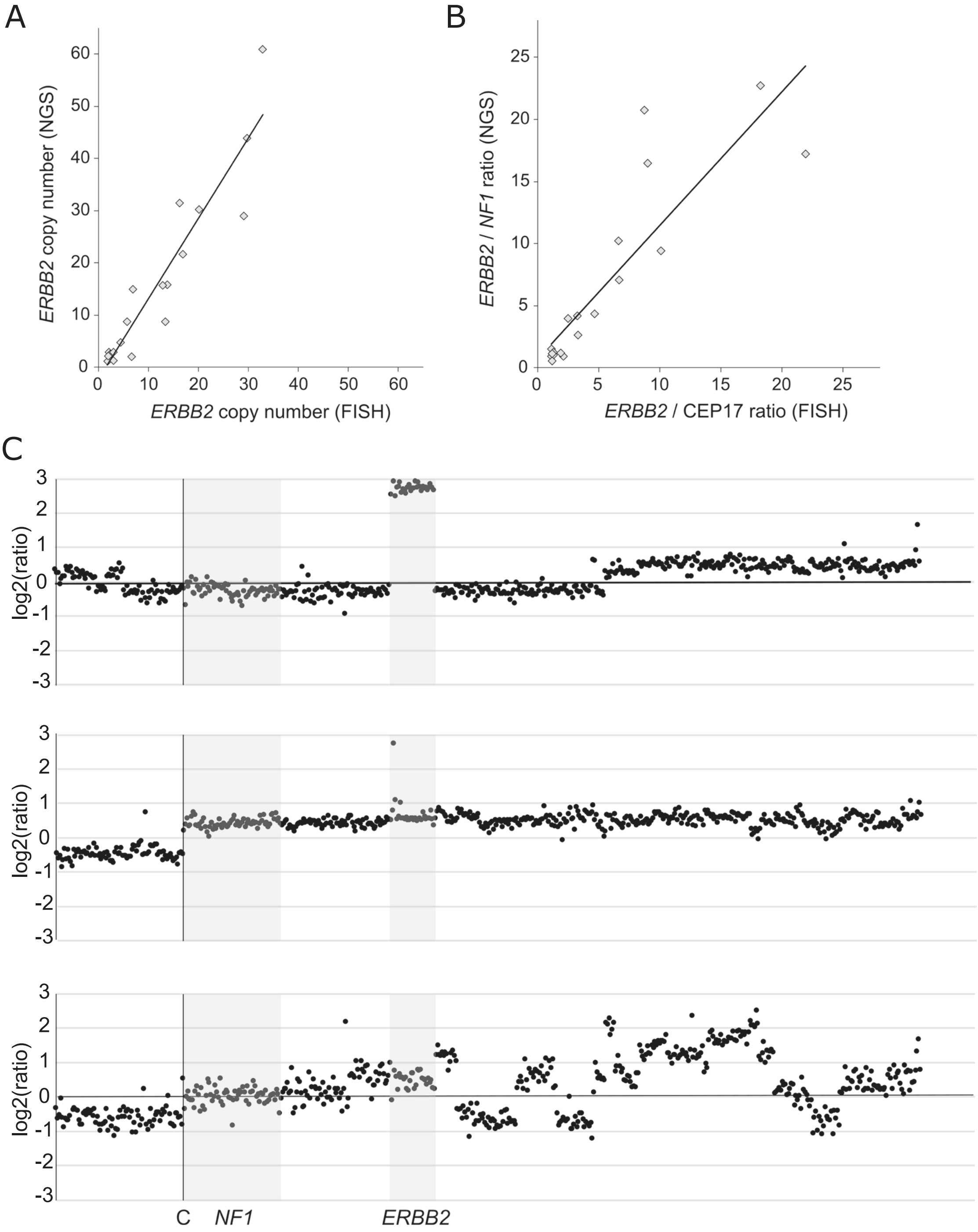
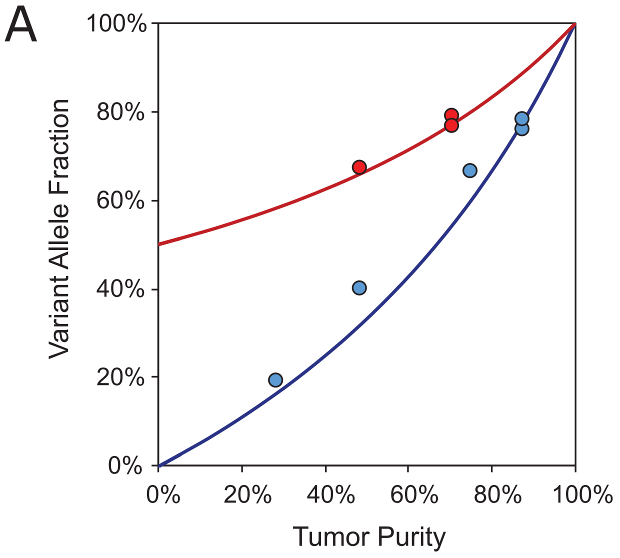


Fig. 4 Copy number estimation of *ERBB2* based on next generation sequencing (NGS) calculated tumor purity. A Absolute *ERBB2* copy number by fluorescence in situ hybridization (FISH) versus NGS (linear regression $R^2 = 0.88$). **B** Comparison of *ERBB2*/CEP17 copy number ratio by FISH versus *ERBB2*/*NF2* copy number ratio by NGS (linear regression $R^2 = 0.76$). **C** NGS data plots of log2(read ratio) for chromosome 17 from three representative invasive breast carcinoma cases; each dot represents read ratio data point from target genes included in the NGS panel; "C" denotes location of centromere and shaded boxes labeled *NF1* and *ERBB2* denote the read ratios corresponding to these target genes. Top: focal *ERBB2* amplification, positive by FISH and NGS. Middle: *ERBB2* gain as part of chromosome 17q gain, negative by FISH and NGS. Bottom: *ERBB2* gain as part of complex chromosome 17q copy number changes, positive by FISH and NGS.

specimens with tumor purity below the molecular limit of detection can be rejected prior to sequencing, saving labor, time and cost. An accurate assessment of tumor purity is useful in downstream analysis and interpretation, including the clinical identification of artifactual or contaminating sequences and the detection of somatic mutations and copy number changes[27]. Despite the importance of histological review, the pathologist's estimation of tumor purity is relatively inaccurate compared to manually counting tumor cells, which may impact the interpretation of molecular test results[28]. Multiple factors can contribute to inaccuracies in tumor purity estimation. Technical factors include changes of tumor purity in deeper levels of the tissue block, particularly in small tissue samples or specimens focally involved by tumor cells. Pathologists may be biased to accept specimens to expand potential treatment options for patients with



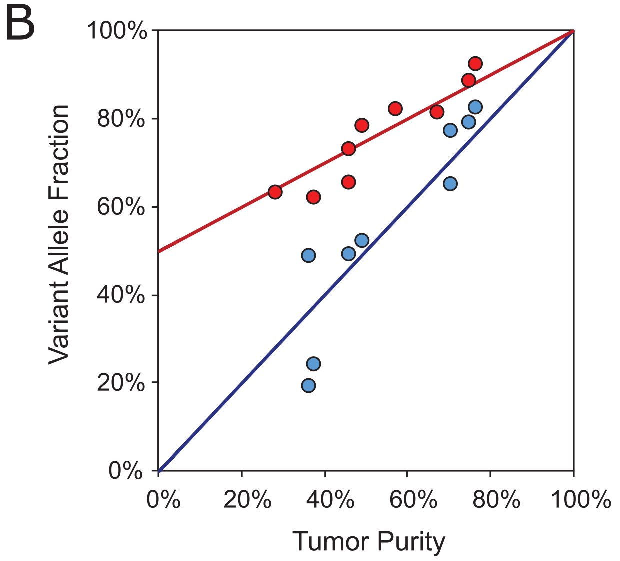


Fig. 5 Inference of germline variants using tumor-only next generation sequencing. A Observed variant allele fraction compared to expected variant allele fraction for known germline and somatic variants with single copy loss. **B** Observed variant allele fraction compared to expected variant allele fraction for known somatic and germline variants with copy number neutral loss of heterozygosity. Observed variant allele fractions (circles) and expected variant allele fractions (lines) are shown in red for somatic mutations and blue for germline mutations.

advanced cancer. Pathologists may also be biased from clinical training to fixate on positive events or diagnostic cells relative to the non-neoplastic background[29,30]. A comparison of pathologist estimation of PD-L1 immunohistochemistry shows relative overestimation of percent of cells with PD-L1 expression compared to automated algorithms[31]. Due to limitations in visual interpretation, calculation of tumor purity based on molecular data may be useful to guide the subsequent clinical interpretation of molecular results.

Establishing the accurate calculation of tumor purity enables us to utilize this value to expand the downstream information yielded in our NGS pipeline. Our first demonstration of this principle is seen with the application of calculated tumor purity to quantitate gene amplification. We describe the reliable quantitation of *ERBB2* copy number in a cohort of breast carcinomas with equivocal (2+) *ERBB2* expression by immunohistochemistry. Previous studies have demonstrated high rates of concordance between NGS and FISH approaches to assessing *ERBB2* amplification across the full spectrum of breast and other carcinomas and within equivocal or clinically challenging subsets[26,32–36]. Compared to prior studies, the current method approximates clinical FISH guidelines with quantitative estimations of absolute *ERBB2* copy number and the ratio of *ERBB2* to the centromeric region of chromosome 17. Future work with larger datasets may enable validation of NGS cut-offs further adjudicate cancers with equivocal findings[33].

A potential limitation of our approach is the use of the chromosome 17q gene *NF1* as a surrogate centromere marker, with a risk that this gene can be affected by chromosomal instability compared to a centromeric control. However, NGS has the advantage of providing copy state information over multiple genes on chromosome 17 that can distinguish focal from broad events, which cannot be determined based on standard FISH approaches[37]. Breast carcinomas exhibiting *ERBB2* absolute copy number gain but without amplification relative to chromosome 17 have been shown to not benefit from targeted therapy[38]. Similar findings can be seen in carcinomas with polysomy 17 or 17q gain[39]. NGS can distinguish between examples of true *ERBB2*-specific amplification, compared with 17q gain or whole chromosome instability (Fig. 4C). Larger cohort studies will be useful to optimize calculation of centromere chromosome 17 controls and to standardize metrics for distinguishing focal *ERBB2* amplification from broader copy number alterations.

A second utility of tumor purity calculation is the ability to successfully infer germline status of variants. Sequencing of paired non-tumor tissue detects pathogenic germline mutations in 3–16% of patients with cancer[3,32,40,41]. Current institutional guidelines including consent protocols for many sequencing platforms do not specifically address germline status, due to the assumption that germline information cannot be ascertained from tumor only sequencing data[42]. However, our findings show that most germline *BRCA1* and *BRCA2* variants can be accurately categorized as germline events from tumor sequencing. While our approach addresses the most common biological scenarios (loss of the wild type allele by deletion or copy number neutral loss of heterozygosity), more complex scenarios, such as greater than two copies of a variant or somatic loss of a germline variant, can be modeled by the same principles. Expected allele fractions for somatic and germline variants at multiple copy number states and tumor purities are provided in Supplemental File 2. A limitation of germline inference is that the expected variant allele fraction of germline versus somatic events converge as tumor purity approaches 100% and may not be informative in specimens with very high tumor purity.

A limitation to this study is an overall high failure rate. 32% of specimens failed due to a flat copy number profiles, indicative of low tumor purity. This high rate of failure in our study is related to the study population of unselected clinical specimens. Compared to research samples used to populate large datasets, clinical specimens in anatomic pathology laboratories have a range of tumor purity estimations depending on tumor biology, growth pattern, and the diagnostic procedure performed. Accurate assessment on low tumor purity specimens remains a clinical challenge in molecular diagnostics. Although not addressed in our current clinical sequencing platform, molecular technologies optimized to detect copy number changes in limited specimens and improved informatics algorithms to reduce artifact in copy number analysis could expand application of quantitative methods in limited clinical specimens.

An additional 17% of specimens failed analysis due to genomic complexity, where a diploid baseline could not be established. This observation reflects the complexity of interpreting cancer genomes, where copy number changes are generated via diverse mechanisms and complex events like genome doubling occur

Table 3. Inference of germline status for pathogenic *BRCA1*, *BRCA2*, and *TP53* variants in breast and ovarian cancers.

Case	Primary site	Tumor purity	Variant	Allele fraction	Gene copy number	Known status	Inferred status
1	Ovary	46%	*BRCA2* c.7068_7069delTC (p.L2357Vfs*2)	73%	2	Germline	Germline
2	Ovary	71%	*BRCA1* c.5266dupC (p.Q1756Pfs*74)	79%	1	Germline	Germline
3	Ovary	49%	*BRCA1* c.3254_3255dupGA (p.L1086Dfs*2)	78%	2	Germline	Germline
4	Ovary	48%	*BRCA1* c.1165delA (p.S389Vfs*5)	67%	1	Germline	Germline
5	Ovary	38%	*BRCA1* c.68_69delAG (p.E23Vfs*17)	62%	2	Germline	Germline
6	Ovary	75%	*BRCA1* c.3627dupA (p.E1210Rfs*9)	89%	2	Germline	Germline
7	Breast	87%	*BRCA2* c.5286_5287delTC (p.S1764Kfs*3)	76%	1	Somatic	Somatic
8	Breast	58%	*BRCA2* c.5857 G > T (p.E1953*)	82%	2	Germline	Germline
9	Breast	71%	*BRCA1* c.1964delA (p.Y655Sfs*46)	77%	1	Germline	Germline
10	Breast	77%	*BRCA1* c.4868 C > G (p.A1623G)	92%	2	Germline	Germline
11	Breast	75%	*BRCA1* c.1687C > T (p.Q563*)	79%	2	Somatic	Somatic
12	Breast	36%	*BRCA1* c.1214 C > G (p.S405*)	19%	2	Somatic	Somatic
13	Breast	46%	*BRCA2* c.8584_8585insT (p.E2863Rfs*6)	65%	2	Germline	Germline
14	Breast	68%	*BRCA2* c.572_573delAT (p.M192Vfs*13)	81%	2	Germline	Germline
15	Breast	28%	*BRCA1* c.3817 C > T (p.Q1273*)	63%	2	Germline	Germline
1	Ovary	46%	*TP53* c.214_216delCCCinsGC (p.P72Afs*51)	49%	2	Somatic	Somatic
2	Ovary	71%	*TP53* c.452 C > G (p.P151R)	65%	2	Somatic	Somatic
3	Ovary	49%	*TP53* c.536 A > G (p.H179R)	52%	2	Somatic	Somatic
4	Ovary	48%	*TP53* c.818 G > A (p.R273H)	40%	1	Somatic	Somatic
5	Ovary	38%	*TP53* c.527 G > T (p.C176F)	24%	2	Somatic	Somatic
6	Ovary	75%	*TP53* c.724 T > A (p.C242S)	66%	1	Somatic	Somatic
7	Breast	87%	*TP53* c.743 G > A (p.R248Q)	78%	1	Somatic	Somatic
9	Breast	71%	*TP53* c.701 A > G (p.Y234C)	77%	2	Somatic	Somatic
10	Breast	77%	*TP53* c.1024 C > T (p.R342*)	82%	2	Somatic	Somatic
12	Breast	36%	*TP53* c.527 G > T (p.C176F)	49%	2	Somatic	Somatic
15	Breast	28%	*TP53* c.1009 C > T (p.R337C)	19%	1	Somatic	Somatic

frequently[43]. While our analysis is based on purely analytical features of NGS data to set baseline copy number states, incorporation of additional biological and clinical information, such as expected copy number changes for tumor type, may provide tumor purity estimations for more samples. Alternatively, assessment by an orthogonal method with absolute copy number quantitation, such as FISH analysis of a limited subset of gene targets, would be expected to provide baseline ploidy for most cancer specimens.

The ability to determine germline status in tumor specimens for a variety of pathogenic variants has broad implications for clinical care. For example, <20% of patients with breast or ovarian cancer meeting eligibility criteria for germline evaluation undergo genetic testing[44]. With appropriate patient consent, germline alterations could effectively be incorporated into analytical pipelines to yield clinically relevant information, while being masked within reporting schemes according to patient preference[45,46].

Tumor-only sequencing uses population databases to filter germline events; however, studies have shown that population databases are insufficient to remove rare private variants from cancer sequencing panels[47]. These considerations are important in the calculation of tumor mutational burden, an emerging biomarker that predicts response to immune checkpoint inhibitor therapy. The consideration of variant allele fraction in the context of tumor purity may assist in filtering incidental germline event and improving accuracy for tumor mutational burden calculation, especially for non-Caucasian individuals who are underrepresented in population databases.

In summary, we have demonstrated that NGS data can be used to quantitate tumor purity, which can be integrated with analytical algorithms to improve quantitative copy number analysis and infer the germline status of variants without paired normal sequencing. Cancer NGS interpretation requires molecular pathologists to integrate complex data and make clinical recommendations. This paper demonstrates that we can derive more clinically useful information from existing sequencing data with little additional cost, which has potential to expand the utility of cancer NGS and better integrate histopathological and molecular data into patient care.

DATA AVAILABILITY

All data used and analyzed in the current study are available in the published paper and Supplemental Files.

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AUTHOR CONTRIBUTIONS

S.E.S. and F.D. performed study design, development of methodology, data collection, writing, and revision of the paper. D.K.M. and P.K.D. performed data collection and revision of the paper. All authors read and approved the final paper.

COMPETING INTERESTS

The authors declare no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This project was approved by the Mass General Brigham Human Research Committee, the institutional review board for Brigham and Women's Hospital, and was conducted in accordance with the Declaration of Helsinki. Informed consent was waived as this research was deemed no more than minimal risk.

ADDITIONAL INFORMATION

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Correspondence and requests for materials should be addressed to Fei Dong.

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