Clinical Oncology: Case Reports

Case Report

A SCITECHNOL JOURNAL

A Case Study of Precision Medicine: Next Generation Sequencing Compared with Oncoscan in a Patient with Breast Cancer in Kentucky

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Abstract

Purpose: Precision medicine of tumors is designed to tailor treatment combinations to individual patients, targeting specific genotypes with corresponding inhibitors. The purpose of this study was to determine whether genotype predicted target gene expression-a critical parameter for treatment-and to test whether alternate modes of gene amplification could affect gene expression.

Patients and methods: The cohort consisted of patients in Kentucky with recurrent breast cancer who were analyzed by next-generation sequencing. Tumors with amplifications in CCND1, AURKA, and MYC were then stained by immunohistochemistry. We subsequently selected a patient with triple-negative breast cancer for analysis by FISH, immunohistochemistry and OncoScan genome-wide copy number array.

Results: The extent of CCND1 staining was highly variable between tumors. In one patient with heterogeneous CCND1 staining, the CCND1 gene was maintained as an extra-chromosomal DNA (ecDNA), which changed with both DNA copy number (increased) and expression (decreased) during metastatic progression. The tumor DNA was then analyzed by OncoScan array, which confirmed an increased CCND1 copy number with treatment and mapped the ecDNA size. Elsewhere in the genome, VAV3 (encoding a RhoA and RhoG guanidine exchange factor) increased in copy number and expression during metastasis. In addition, several actionable genes were detected by OncoScan that were not reported by next-generation sequencing.

Conclusion: NGS reported numerous tumors with CCND1 gene amplification, but the proportion of cells with CCND1 expression was highly variable. Genetic factors, such as mode of amplification, may contribute to expression patterns in tumors.

Keywords

Precision medicine; Oncoscan; Breast cancer

Introduction

The underlying cause of cancer is genetic alterations, which can disturb normal gene expression patterns and/or protein function. The classical approach to identifying genetic changes associated with cancer has focused on individual endpoints and employed targeted therapeutics for a limited number of gene products. There are a number of important success stories with this paradigm, including the targeting of HER2/ERBB2 in breast cancer, and the number of actionable cancer genes is rapidly increasing. The sequencing of the human genome and the advent of inexpensive next-generation sequencing of patient samples has identified a broader array of mutations in cancer and made individualized therapy possible [1,2]. However, the improvement in length of survival from sequencing-based approaches has been modest [3], suggesting that the paradigm that DNA sequence predicts response to an inhibitor can be further refined.

DNA sequence information may be only part of the data set that is required to improve treatment outcomes. In the present study, we compared the results of breast cancer patients that were analyzed by NGS with follow-up studies using immunohistochemistry, FISH and a genome-wide copy number array called OncoScan. In one patient with triple-negative breast cancer, CCND1 [4] (encoding cyclin D1) was amplified, as it is frequently in cancer [5,6]. However, the CCND1 protein was expressed in a highly heterogeneous pattern, and CCND1 was maintained on an extra-chromosomal DNA (ecDNA)-a circular DNA element of approximately 700,000 bp that can replicate separately from chromosomes. CCND1 is one of the genes most frequently maintained as ecDNA [7]. The CCND1 gene is localized to chromosome 11q13.3 adjacent to FGF19, FGF4 and FGF3.

The OncoScan array further characterized the CCND1 ecDNA during disease progression. OncoScan is a whole genome microarray-based copy number array that can be hybridized to DNA from formalin-fixed paraffin-embedded tumor samples. The resulting degraded DNA is detected using molecular inversion probe technology, and copy number variations are reported throughout the genome with 50-100 kb resolution in 900 cancer-associated genes. Together, we report a more comprehensive picture of gene alteration, maintenance, and expression in a patient with triple-negative breast cancer.

Materials and Methods

Immunohistochemistry

IHC was performed on 4-micron thick sections cut from formalin fixed paraffin embedded tissue. Heat Induced Epitope Retrieval (HIER) was performed in a Biocare Medical Decloaking chamber using Dako High pH buffer, except for c-myc which used Dako low pH buffer. Staining was carried out at room temperature on a Dako autostainer and visualized with Dako EnVision Flex+ kit or Vector Laboratories Immpress-HRP KIT and 3,3′-diaminobenzidine (DAB) chromogen (Dako, CA) per manufacturer's instructions. Staining protocols for each antibody were as follows: c-myc (Abcam ab32072, 1:50, 1 hour), Envision Flex+with rabbit linker (1 hour each), DAB (10 minutes); Cyclin D1 (Dako IS083, RTU, 20 minutes) Envision Flex+(20 minutes), DAB (10 minutes); Aurka (Company name Catalog#, 1:500, 1 hour), Immpress anti-rabbit-HRP (30 minutes), DAB (5 minutes); FGF19 (R&D MAB969, 1:1000, 1 hour), Envision Flex+with mouse linker (20 minutes each), DAB (1 minute). Slides were lightly counterstained with hematoxylin.

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Received: June 07, 2019 **Accepted:** June 22, 2019 **Published:** July 01, 2019

FISH probes

Cyclin D1 amplification was assessed by FISH on paraffin-embedded tumor sections. Fluorescently labeled DNA probes that bind to CCND1 were purchased from Abbott Molecular and the assay was performed at the University of Kentucky Cytogenetics Clinical Laboratory.

Oncoscan

DNA was extracted from breast tumor specimens using the Qiagen QI Aamp DNA FFPE Tissue Kit by the Markey Tissue Procurement Faculty. DNA purity and concentration was tested with a Thermo Nanodrop Spectrophotometer. 80 ng of genomic DNA was hybridized to a copy number microarray using the OncoScan FFPE Assay Kit (Affymetrix) at the Nationwide Children's Hospital in Columbus, Ohio. Signals were analyzed using ChAS (chromosome analysis suite) software (Affymetrix).

Ethics statement

De-identified breast cancer patient data were obtained after approval from the University of Kentucky Institutional Review Board (UK IRB) and an oversight committee from the Kentucky Cancer Registry. Individual case study information was obtained in the redacted form with approval from the UK IRB.

Results

Next-generation sequencing

We obtained de-identified DNA sequence data from 26 breast cancer patients (Table 1) from the University of Kentucky Hospitals and their affiliates through the Kentucky Cancer Registry (KCR) and the University of Kentucky Institutional Review Board. In the dataset, 58% of the breast cancer patients had gene amplification, often in actionable genes. At UK Hospitals, NGS is generally reserved for patients that are difficult to classify using standard approaches and are not responding to therapy. In this population, 17/26% had TNBC.

Immunohistochemistry

27% (7/26) had amplifications in CCND1, encoding cyclin D1, and we focused initially on this subset of patients. CCND1 amplifications are generally associated with estrogen receptor-positive breast cancer [8-10], and we detected strong, homogeneous CCND1 staining in liver metastasis from an ER-positive patient (Supp. Figure 1A). However, in the present cohort, 5/7 patients with CCND1-amplified tumors were estrogen receptor-negative (Table 1). Among these 5 patients, 2 were HER2-amplified, and CCND1 staining was strong but heterogeneous in breast tumors from these patients (Supp. Figure 1B and 1C). In patients with triple-negative breast cancer, we also detected highly heterogeneous CCND1 staining (Supp. Figure 1E). AURKA was amplified in 6 patients and exhibited a highly heterogeneous staining pattern (Supp. Figure 1D and 1F). Finally, MYC was amplified in 8 patients and exhibited strong, but variable staining in 3 patients with triple-negative breast cancer (Supp. Figure 2).

FISH analysis

To gain further insights into CCND1 expression patterns in the patient cohort, we focused on a patient with stage IIIA triple-negative breast cancer and amplification of CCND1, FGF19, FGF4, and FGF5, which are adjacent and were detected by NGS. FISH staining by a certified clinical geneticist confirmed that CCND1 was amplified (Figure 1A and 1B) and localized CCND1 to multiple puncta consistent with extrachromosomal DNA (ecDNA) elements. The patient was 28 years old at diagnosis and received four cycles of chemotherapy, when she underwent a mastectomy, which is sampled in Figure 1A, 1C, left panels, and 2A. She then received two cycles of paclitaxel with radiation, and four nodules were detected in the chest wall. These tumors are sampled in Figure 1B, 1C, right panels, and 2B. FISH staining detected CCND1 in the mastectomy (Figure 1A, left panel) and chest wall recurrence (Figure 1A, right panel), and the copy number increased from 2.4 ± 1.3 to 5.1 ± 3.0 (Figure 1A and 1B), which was highly significant ($p=1.4X10^{-14}$, t-test, n=100). Notably, increased CCND1 copies were ecDNA (Figure 1A). Approximately half of the breast tumors are positive for ecDNA, and nearly half of tumors that carry CCND1 amplifications maintain the gene as extra-chromosomal DNA [7]. Surprisingly, elevated CCND1 copy number in the chest wall metastasis did not result in increased CCND1 expression. In fact, the opposite was observed (Figure 1C, upper panels). In contrast, FGF19, which is on the same amplified region, increased as expected following treatment (Figure 1C, lower panels).

OncoScan array

Commercial NGS reports a relatively small number of cancer-associated copy number changes, so we analyzed the same TNBC patient using an OncoScan genome-wide copy number array (Figure 2). The results confirmed that CCND1 is amplified in the patient and mapped the amplified region to a 4086 kbp fragment. The fragment increased in intensity by 22% and slightly in size (to 4864 kbp) between the primary tumor and recurrence, consistent with the findings using FISH (Figure 1B and 2C). On chromosome 1p, VAV3 increased from 4.89 to 8.20 copies between the primary and recurrent tumors, suggesting a potential role in the metastatic process. Immunohistochemistry confirmed abundant expression of VAV3 in both the tumor and recurrence. VAV3 is a guanine nucleotide exchange factor for RhoG and RhoA which is described in greater detail in the Discussion section.

Table 1: Next generation DNA sequence data from 26 breast cancer patients.

Patient number	23
Age range	28-64
Appalachian/non-	8/15
Metastatic/non-	14/9
Breast cancer sub-type	
ER+/PR+	3
ER+/HER2amp	3
ER-/HER2amp	1
Unknown/HER2amp	2
Triple-negative	14
Tumor suppressors	
BRCA1/2 mutant	1/2
TP53 mutant	18/23
PTEN mutant	3/23
Actionable alterations	
HER2/neu/ErbB2	6
Cyclin D1-amplified	6
Aurora kinase-amplified	5
VEGFA-amplified	1
Topoisomerase 2A-amplified	1
RARA-amp+rearrangement	1
EGFR-amplified	1
Targets of developing therapeutics	
MYC amplified	7
JAK2-amplified	2
AKT1/2	½

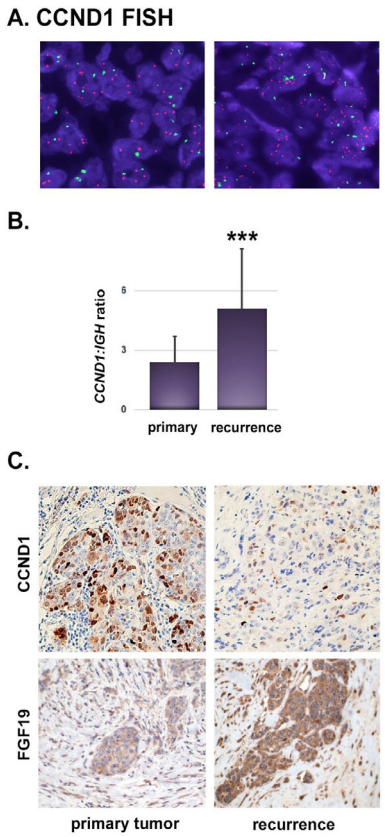

Figure 1: CCND1 extra-chromosomal DNA (ecDNA) in a patient with triple-negative breast cancer. The patient was selected based on FoundationOne sequencing results indicating amplification of CCND1. (A): FISH results showing increased number and heterogeneity of CCND1 (pink) relative to the control IGH (green); (B): Increased CCND1 copy numbers after tumor recurrence based on FISH staining; (C): Immunohistochemistry showing decreased CCND1 expression (top panels) in a breast tumor recurrence from the same patient (top right panel) but increased expression of FGF19 (lower panels), the gene adjacent to CCND1.

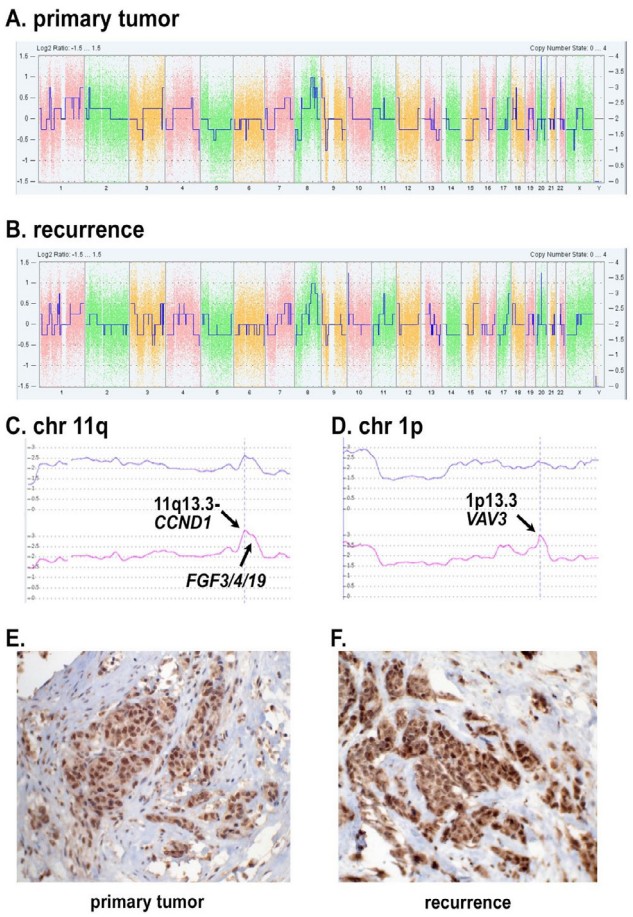

Figure 2: An OncoScan array of the patient from Figure 1. Panel A is from the primary tumor, while panel B is a recurrent metastasis. (C-F) Closer views of chromosome 11q (C) and 1p (D), with the primary tumor (blue line) and metastasis (red line) indicated. (E-F) Immunohistochemistry of VAV3 in the primary (E) and metastatic tumor (F).

Finally, we probed for agreement between the NGS analysis and OncoScan array. The original sequencing analysis of this patient indicated amplification of CCND1 and the linked genes FGF3, FGF4 and FGF19, as well as RAPTOR (regulatory associated protein of mammalian target of rapamycin), BCL2L1 (B cell lymphoma protein 2-like) and others, while CDKN2 (cyclin-dependent kinase inhibitor 2) and FAT1 gene dosage decreased (Table 2). Two pairs of amplified genes detected by NGS were on common amplicons (ASXL1 and BCL2L1 on 20q11.21 and PRKAR1A and RAPTOR on 17q24-25), (Figure 3A and 3B). Among the amplified genes detected by NGS, only CCND1 increased by more than 20% between the primary tumor and recurrent tumor (Table 2). Indeed, ASLX1 decreased by 20%. We then searched for actionable genes that were not reported by NGS but identified by OncoScan, and we detected increased copy numbers of the kinases ALK, PI3KCA and MET (Table 2 and Figure 3C). Immunohistochemistry confirmed the expression of MET in both the primary and metastatic tumor (Figure 3D).

Discussion and Conclusion

Techniques such as Foundation One sequencing are being used to classify cancer patients as candidates for targeted therapies. The genetic information is provided in a facile format, focusing on actionable genes. Precision medicine approaches have improved survival, but only by weeks-months on average [3]. One shortcoming is that many of the point mutations detected by NGS are not well characterized, so increases in gene copy number are frequently used to drive treatment recommendations. However, increased copy number does not necessarily predict high, uniform protein levels, and treatment recommendations are often made without knowing whether the encoded protein is expressed and how it changes with treatment. In the breast cancer patients in this cohort, only one had strong, homogeneous CCND1 expression, even though CCND1 was amplified in each case.

In one patient, CCND1 was maintained as an ecDNA, rather than a homologous staining region. Cancer cells treated with antimetabolites decrease ecDNA levels [11], and one would expect that ecDNA would be reduced by multiple types of chemotherapy. However, the TNBC patient in this study had elevated CCND1 DNA after chemotherapy. This finding is consistent with a widely accepted model for ecDNA generation, in which replication pauses cause DNA fragments to be released from the genome and replicate independently [12]. While the CCND1 copy number increased, the tumor had lower CCND1

Table 2: Amplified genes detected by foundation one sequencing.

Gene location	NGS-amp	OS-amp1	OS-amp2	amp size
ASXL1	20q11.21	1.52	3.67	3.06
BCL2L1	20q11.21	2.79	3.45	3.4
CCND1	11q13.3	1.69	2.54	3.11
CDKN2	9p21.3	0.51	0.95	1.28
DNMT3A	2p23.3	1.51	2.58	2.58
KDM5A	12p13.33	1.54	2.8	2.78
PRKAR1A	17q24.2	1.47	2.47	2.68
RAPTOR	17q25.3	1.68	2.81	2.94
RUNX1T1	8q21.3	2.2	3.29	3.33
FAT1	4q35.2	0.49	0.8	1.18

Table 3: Additional amplified genes detected by OncoScan.

Gene	Primary	Recurr
AKT1	1.64	1.81
ALK	2.33	2.33
BRAF	2.06	2.15
CDK4	1.75	1.83
CDKN2A	0.95	1.28
c-KIT	2.29	2.15
ERBB2	1.54	1.61
ERBB3	1.73	1.8
MAP2K1	2.15	2.44
MDM2	1.8	1.93
MET	2.8	2.61
PIK3CA	2.86	2.64

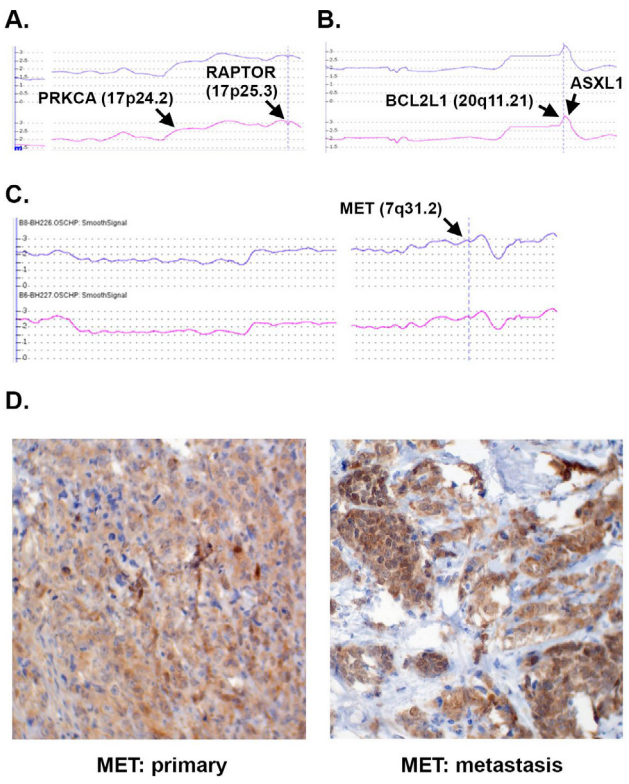

Figure 3: DNA copy number array of selected regions detected as amplified by next generation sequencing. The regions shown are from chromosomes (A): 17p; (B): 20q11 and; (C): 7q31 with actionable amplified genes indicated. The blue and red lines are the same pattern as Figure 2. (D): Immunohistochemistry of the MET tyrosine kinase in the primary and metastatic tumor from Figures 1 and 2.

expression. In some triple-negative breast cancer cell lines, loss of CCND1 expression increases invasion [13,14], and the recurrent tumor in this study had metastasized to the chest wall. FGF19 was co-amplified with CCND1, but FGF19 expression was induced under the same conditions that repressed CCND1 expression. Discordant results between FISH and immunohistochemistry have been reported for HER2 [15,16], with the $FISH^+$ IHC^- a subset of patients responding poorly to trastuzumab.

OncoScan arrays (1) confirmed our FISH analysis that CCND1 copy number increased following chemotherapy and tumor recurrence (2) confirmed the set of amplified genes detected by NGS, (3) identified actionable genes that were amplified in the tumor but not reported via NGS. The amplified genes detected by OncoScan included MET, which was confirmed by immunohistochemistry. MET amplification and overexpression have been reported previously in breast cancer [17-19], and MET can be inhibited by crizotinib and cabozantinib. MET inhibition is active in vitro for triple-negative breast cancer [20], ideally as combination therapy.

The OncoScan array also extended the analysis to the rest of the genome, identifying novel candidate genes that may contribute to the patient's disease. VAV3 is a guanine nucleotide exchange factor for RHO family GTPases [21], and VAV3 is a prognostic indicator for poor survival in breast [22], colorectal [23], gastric cancer [24,25] and pancreatic cancer [26]. VAV3 is associated with estrogen receptor activation [27] and estrogen receptor inhibitor resistance [28] in breast cancer, but the patient in this study had triple-negative breast cancer. However, VAV3 regulates a transcriptional program in breast cancer that is associated with lung metastasis [29], which may be relevant for metastatic cancer in this case study. Perhaps of greater relevance to this patient, VAV3 is associated with receptor tyrosine kinase signaling through EGFR, PDGFR, Ros, insulin receptor, and IGF1R, and VAV3 activates PI3K, NFB, and others [30,31].

In summary, this case study suggests that multiple modes of DNA copy number validation combined with a simple test of protein expression are useful in breast cancer. Gray et al. came to the same conclusion in a prostate cancer case study [32]. The results also suggest that gene regulation may be aberrant on ecDNAs and that simply understanding DNA copy number for an ecDNA could be misleading in directing therapeutics.

Acknowledgement

The authors are grateful to Madison Sands and Whitney Barbour for contributions in the initial phases of the research and Dr. Jill Kolesar for advice. We are also grateful to Aimee McKinney at Nationwide Children's Hospital (Columbus, Ohio) for performing the OncoScan analysis and to Dr. Sainan Wei of the University of Kentucky Oncogenomics Core Faculty.

Research Support

This research was supported by the Biospecimen Procurement and Translational Pathology and Cancer Research Informatics Shared Resource Facilities of the University of Kentucky Markey Cancer Center (P30CA177558), a Research Incentive Fund from the Department of Pharmacology and Nutritional Sciences, and the Markey Cancer Center Joseph Pulliam, M.D. Memorial Fund.

Author's Contribution

Rolf J. Craven conceived, coordinated and performed the research and wrote the manuscript. Kuey-Chu Chen performed the OncoScan analysis. Steven Schwarze coordinated and analyzed the FISH study. Sydney Lay analyzed the data and prepared in Table 2. Dana Napier directed and performed the immunohistochemistry and extracted DNA for the OncoScan analysis. Rachel Stewart analyzed the immunohistochemistry.

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