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Original Articles

Novel cancer gene variants and gene fusions of triple-negative breast cancers (TNBCs) reveal their molecular diversity conserved in the patient-derived xenograft (PDX) model

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ABSTRACT

Despite the improved 5-year survival rate of breast cancer, triple-negative breast cancer (TNBC) remains a challenge due to lack of effective targeted therapy and higher recurrence and metastasis than other subtypes. To identify novel druggable targets and to understand its unique biology, we tried to implement 24 patient-derived xenografts (PDXs) of TNBC. The overall success rate of PDX implantation was 45%, much higher than estrogen receptor (ER)-positive cases. Immunohistochemical analysis revealed conserved ER/PR/Her2 negativity (with two exceptions) between the original and PDX tumors. Genomic analysis of 10 primary tumor-PDX pairs with Ion AmpliSeq CCP revealed high degree of variant conservation (85.0%–96.9%) between primary and PDXs. Further analysis showed 44 rare variants with a predicted high impact in 36 genes including *Trp53*, *Pten*, *Notch1*, and *Col1a1*. Among them, we confirmed frequent *Notch1* variant. Furthermore, RNA-seq analysis of 24 PDXs revealed 594 gene fusions, of which 163 were in-frame, including *AZGP1-GJC3* and *NF1-AARSD1*. Finally, western blot analysis of oncogenic signaling proteins supporting molecular diversity of TNBC PDXs. Overall, our report provides a molecular basis for the usefulness of the TNBC PDX model in preclinical study.

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1. Introduction

Among the preclinical models developed for testing cancer therapeutics, the patient-derived xenograft (PDX hereafter) model has been drawing increasing attention [1]. This is primarily due to its intrinsic advantages over other models, e.g., retention of a primary tumor's characteristics and relatively good reproduction of diversity of primary tumors [2,3]. Indeed, recent reports demonstrated that the PDX model faithfully reflects chemotherapeutic responses of the patients from whom the tumor was derived [4,5].

Moreover, a PDX tumor can be stored for a later xenograft, overcoming the limitations of research on rare tumors. By far, several excellent studies exemplify the use of the PDX model in preclinical trials [6,7]. In addition, the PDX model can be useful for biomarker discovery [3], tumor microenvironment profiling [8–11] and cancer stem cell biology [2].

Breast cancer is the second most common cancer among women worldwide. In developed countries, the 5-year survival rate is close to 90% [12]. The high survival rate is due to the early detection of cancer by ultrasonography or mammography and availability of multiple targeted therapeutic agents for hormone or growth receptors, such as letrozole or trastuzumab. Nevertheless, there is no effective targeted therapy for TNBC, therefore, more research is

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needed to understand and design new therapy for this subtype of breast cancer [13]. Indeed, recent reports focused new therapeutics for TNBC using PDX models [14,15]. Other reports, however, showed that TNBCs themselves are diverse [16]; accordingly, additional molecular characterization of TNBCs is urgently needed. Furthermore, the sub-classification of TNBCs by molecular profiling [17] or by the response to therapeutic agents will ultimately help researchers to design a strategy to treat this cancer.

Toward this goal, we generated PDXs from TNBC and performed a cancer panel analysis and high-throughput RNA sequencing (RNA-seq) as well as immunohistochemical (IHC) analysis. Unlike ER-positive breast tumors, which showed a poor graft success rate even with estrogen medication, triple-negative breast tumors manifested a remarkable success rate (~45%) for the initial graft. In this article, we describe molecular characteristics of the successful TNBC PDXs in comparison with their original tumors and demonstrate the potential of this model as a preclinical tool for personalized treatment.

2. Materials and methods

2.1. Creation and storage of TNBC PDXs

The animal care protocol for this study was approved by the Institutional Animal Care and Use Committee (IACUC) of the Laboratory of Animal Research at the Asan Medical Center, Seoul, Korea. Five-week-old female NOD/SCID mice were subjected to tumor engraftment and were grown at a specific pathogen-free facility. The surgical specimens were obtained with permission from the institutional review board (IRB) of the Asan Medical Center (No. 2014–0800). Fresh tumor tissues were obtained from breast cancer patients (who underwent surgical resection) and were immediately placed in the Roswell Park Memorial Institute (RPMI) medium (with 10% of FBS and 1% of a penicillin/streptomycin solution) at 4 °C in the refrigerator. Soon after this, the samples were sliced into several 2–3 mm^3 fragments and implanted into the inguinal mammary fat pad of mice. Namely, after cutting the fat pad slightly and making a pouch, we then placed tumor fragments there. This step was necessary to prevent the tumor fragment from coming out. During the time of moving to animal facility, the tube containing the tissue was carried on ice for its freshness. For a tumor implant, animals were anesthetized with 15 mg/kg Zoletil® (Virbac, USA) and 2.5 mg/kg Rompun® (Bayer Korea, Korea) by intraperitoneal injection. Following the implantation, the mice were monitored twice a week for at least 12 months. Once the xenograft tumor had attained a size of 500 mm^2, the tumor was excised, and the mice were euthanized following the protocol of the Laboratory of Animal Research at the Asan Medical Center. The part of the tumor that had been excised from the mouse was then engrafted into another BALB/c nude mouse for expansion, while the residual part of the tumor was placed in a freezing medium supplemented with dimethyl sulfoxide and placed in a deep freezer.

2.2. IHC analysis

Tumors were fixed in 10% formalin for at least 24 h and then embedded in paraffin. Both human and mouse tumor tissues were sectioned at 5 μm thickness and stained with haematoxylin and eosin stain (H&E). IHC analysis was performed to examine the expression of ER, progesterone receptor (PR) and human epidermal growth factor receptor (HER2) in the primary human tumors, in accordance with the protocol of the Department of Diagnostic Pathology at the Asan Medical Center. Formalin-fixed paraffin-embedded tissue slices were stained with an automatic immunohistochemical staining device (BenchMark XT; Ventana Medical Systems, Tucson, AZ). Antibodies to ER (diluted 1:50, NCL-6F11, monoclonal; Novocastra, Newcastle, UK) and to PR (diluted 1:100, 1E2, rabbit monoclonal; Roche, Tucson, USA) were used. IHC analysis of the HER2 protein was performed by means of the anti-HER2 antibody (anti-HER2/neu [4B5] rabbit monoclonal antibody; Ventana Medical Systems), and an UltraView™ universal DAB detection kit (Ventana Medical Systems).

2.3. Comprehensive cancer panel (CCP) analysis

The CCP analysis was carried out according to our previous report [18].

2.4. Confirmation of variants by Sanger sequencing

All the positions of missense variations found in the *Notch1* gene were between 139, 399, 350 and 139, 399, 405 on chromosome 9. Forward and reverse primers were designed to read the sequences of the area. Primer sequences were as follows: (F) 5′-TCC ACC AGT TTG AAT GGT CA-3′ and (R) 5′-AGC TCA TCA

TCT GGG ACA GG-3′. After extraction of DNA from tumor tissue, samples were sequenced by Macrogen Korea (Seoul 08511, Republic of Korea). Sanger sequencing results were analyzed on a chromatogram, and mutations were confirmed.

2.5. RNA isolation and real-time PCR

For each sample, RNA extraction was performed with the TRIzol Reagent (Invitrogen, Carlsbad, CA). Next, 1 μg of total RNA was subjected to cDNA synthesis (PrimeScript RT reagent kit, Cat No. PR037A, Takara). The levels ERBB2 and HEY1 were measured by the Hi-ROX qPCR Green Mix (Ampigene, Cat. No. ENZ-NUC104- 1000), on a LightCycler 480 II (Roche). Before extraction of RNA, MDA-MB-436 cells were treated with 25 μM resveratrol (Sigma-Aldrich, R5010) for 24 h. The primer sequences were as follows; ERBB2: (F) 5′-AGC CGC GAG CAC CCA AGT-3′ and (R).

5′-TTG GTG GGC AGG TAG GTG AGT T-3′, HEY1: (F) 5′-TGG ATC ACC TGA AAA TGC TG-3′ and (R) 5′-CGA AAT CCC AAA CTC CGA TA-3′. Human *RPL13A* served as an internal control gene. Relative quantification was carried out by the $2^{-\Delta\Delta Ct}$ method [19].

2.6. RNA-seq analysis

Except for one sample that failed quality control, 24 PDX samples were subjected to TrueSeq mRNA library construction and RNA-seq on an Illumina HiSeq 4000. Paired-end sequencing with a read length of 101 bp resulted in 60 million reads per sample on average. We employed Bowtie 2 [20] for mapping to the hg19 reference genome. Gene annotation and transcript quantification were performed using RNA-Seq by Expectation-Maximization (RSEM) [21] based on GENCODE v19 [22]. Transcripts per kilobase of a gene per million mapped reads (TPKM) were calculated for normalized quantification of RNA amounts.

2.7. Variant calling

For variant calling from RNA-seq, not exome sequencing, Splice Transcripts Alignment to a Reference (STAR) [23] has been reported to have greater sensitivity than other aligners including Burrows–Wheeler Alignment (BWA). To carry out sample-wise splicing junction analysis, we performed the STAR 2-pass alignment steps. Variant calling and filtering were done using Genome Analysis Toolkit (GATK) [24]. We employed SplitNCigarReads to split reads into exon segments and to hard-clip any sequences overhanging with the intronic regions. We filtered clusters of at