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Genetic alterations in sporadic triple negative breast cancer

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ABSTRACT

Background: Recent studies have aimed to identify gene mutation profiles to explain the cause of TNBC therapy limitations.
Methods: The purpose of our study was to use Next Generation Sequencing (NGS) of 46 genes with a well-defined role in cancer in a cohort of TNBC patients in order to identify novel markers that could lead to the development of strategic, adjuvant, gene-targeted therapies.
Results: A total of 118 gene mutations in 35 genes, 75 mutations in *BRCA1* and 92 mutations in *BRCA2* were identified. The clinical assessment of the identified mutations showed 27 to be possibly damaging and 59 to be damaging. *TP53, KDR, PIK3CA* (rs3729687), *ATM, AKT1* and *KIT* were among the most frequently mutated genes in our TNBC cohort. The SNP *AKT1* (rs3730358) was suggested to modify the risk of breast cancer. SNP *PIK3CA* (rs3729687) is a damaging mutation that we found to be correlated with the prognosis of TNBC. The survival curve analysis showed that the presence of *AKT1, TP53, KDR, KIT, BRCA1* and *BRCA2* mutations is correlated with a poor prognosis.
Conclusion: We show a strong association between TNBC and mutations in *BRCA1/2* genes and the poor outcome of these patients. Moreover, we identified several other unknown mutations putatively associated with the poor prognosis of TNBC tumors. We also discovered novel mutations never before associated with breast cancer that could putatively account for the poor prognosis of the TNBC tumors.

1. Introduction

Breast cancer is among the most common types of cancer worldwide, with an estimated 1.67 million new cases diagnosed every year [1]. The European Cancer Observatory (EUCAN) gives the following estimates for the prevalence of breast cancer: 12.58% for one year, 34.54% for 3 years and 52.88 for 5 years [2]. Although most breast cancers are sporadic, some cases are inherited. This genetic predisposition is often caused by pathogenic mutations in *BRCA1* and *BRCA2* tumor suppressor genes involved in DNA repair; 69% of women with pathogenic mutations in BRCA2 and 72% of women with pathogenic mutations in BRCA1 will develop breast cancer during their lifetime [3].

The triple negative breast cancer (TNBC) subtype comprises

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tumors that lack expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor 2 (HER2). This subtype accounts for approximately 15–20% of diagnosed breast cancers [4]. Each type of cancer displays distinct somatic mutation patterns that can influence tumor development and progression [5]. Moreover, distinct subtypes of breast cancer display different characteristics, behavior, prognosis and responses to therapy.

Previous studies have focused on mutation analysis of genes involved in DNA damage and repair processes; such studies have mainly been restricted to a few genes, like *TP53, BRCA1, BRCA2* and *PIK3CA*, and therefore may miss some genetic events that could be used in diagnosis or treatment of breast cancer. These limitations could be successfully overcome by the completion of the first human genome sequencing and the development of Next Generation Sequencing (NGS) techniques, which allow studies in whole cancer genomes [6,7]. These new technologies are able to generate comprehensive somatic mutation catalogs, which may lead to a greater understanding of the mutational process in the development of cancer [7,8].

Correlations between TNBC and *BRCA* mutations have been previously identified. For example, Reis-Filho et al. [9] observed that 75% of tumors carrying *BRCA1* mutations are also TNBC, and 25% of TNBCs are positive for *BRCA1/2* mutations [10,11].

Several studies have showed the importance of NGS in clinical diagnosis [12–14], while others have described the use of benchtop sequencers for target sequencing in diagnosis (e.g., *BRCA1/2* mutations) [15–17].

TNBC has a poor prognosis and high rates of metastases. Treatment approaches are limited. TNBC patients also have higher relapse rates, suggesting the need for development of novel therapies that would be effective in reducing recurrences. Thus, we molecularly profiled 92 TNBC patients (including NGS of 46 genes and real-time polymerase chain reaction [PCR] genotyping) to identify new direct-target genes for adjuvant treatment.

2. Material and methods

2.1. Patients

The study is a single center retrospective study that included a cohort of 92 patients who were diagnosed with TNBC. The criteria for the inclusion in the study were the diagnosis of triple negative breast cancer, and we included all TNBC consecutive patients. The diagnosis was established using internationally accepted criteria. For all patients, we have 5–6 years of clinical follow-up data. Clinical follow-up was obtained from the Institutional Cancer Registry, Romania, which contains data from the patients' files including patients' data, treatment and survival information. Seventy-one of the patients included in the study were diagnosed with invasive ductal carcinoma. Samples from 30 patients were used for the sequencing study, while for validation experiments we used samples from all 92 patients.

More details on the clinical data of the studied population are presented in Table 1.

2.2. DNA extraction

DNA was extracted from formalin-fixed, paraffin-embedded tissues using the PureLink Genomic DNA Mini Kit from Invitrogen with the protocol provided by the manufacturer [18] and using 8 sections of 10 μm for each sample. DNA quantification was performed using NanoDrop (Thermo-Scientific), and the samples showed concentrations between 5.75 and 181.79 ng/μL.

2.3. Next generation sequencing

For the amplicons library, we used 10 ng of DNA from each sample and the Ion Ampliseq cancer panel [19] and Ampliseq Community BRCA 1_BRCA2 primer kit from Life Technologies. The Ion Ampliseq cancer panel contains primer pairs for 190 amplicons of 46 oncogenes and tumor suppressor genes (*AKT1, BRAF, FGFR1, GNAS, IDH1, FGFR2, KRAS, NRAS, PIK3CA, MET, RET, EGFR, JAK2, MPL, PDGFRA, PTEN, TP53, FGFR3, FLT3, KIT, ERBB2, ABL1, HNF1A, HRAS, ATM, RB1, CDH1, SMAD4, STK11, ALK, SRC, SMARCB1, VHL1, CTNNB1, KDR, FBXW7, APC, CSF1R, NMP1, SMO, ERBB4, CDKN2A, NOTCH1, JAK3* and *PTPN11*) and covers 739 COSMIC mutations in 604 loci, providing 97% coverage. The Ion Ampliseq Community *BRCA1_BRCA2* primer kit consists of primer pairs for 167 amplicons to analyze both *BRCA1* and *BRCA2* genes. During the amplicon library preparation, barcode adaptors were ligated to the amplicons, with the help of the Ion Xpress Barcode Adaptors kit (Life Technologies). The libraries were quantified using Ion Library TaqMan Quantification kit (Life Technologies) and the ViiA 7 real-time PCR machine (Life Technologies). All libraries were diluted to a concentration of 20 pM. Template preparation was performed using the Ion One Touch 200 Template kit v2DL (Applied Biosystems) with the upgraded Ion One Touch System (Life Technologies). After the emulsion PCR, the libraries were enriched using the same kit as for the template preparation, together with the Ion One Touch ES apparatus. The enriched libraries were sequenced on 316 chips (4 libraries/chip for cancer panel and 8 libraries/chip for BRCA1/2) using the Ion PGM 200 sequencing kit and the Ion Torrent PGM (Life Technologies).

2.4. Data analysis

For signal processing, base calling and sequence alignment, we used Torrent Suite V4.4 (Life Technologies), and sequences were aligned to the Human Genome Build 19 (hg19). We used the variant Caller 4.4.0.6 plugin for detecting somatic mutations, with Target Regions settings specific for the CP.20131001 AmpliSeq panel. For annotations, we transferred the VCF files generated for each sample to an Ubuntu 10.04 environment and used the command line software ANNOVAR [20], which analyzed our data and annotated each variant with information from several databases, including dbSNP, COSMIC, 1000genomes and refGene, and also attributed a PolyPhen-2 score to the amino acid substitutions that were predicted to be damaging or possibly damaging. For the analysis of the BRCA1/2 sequencing data, we used the Ion Reporter software version 4.6. For both sequencing data analyses, we used the following filters: p value ≤ 0.05, coverage ≥ 500 and frequency ≥ 10.

2.5. Mutation validation

For validation of mutations, we used TaqMan SNP Genotyping assays from Life Technologies; 7 assays were commercial and one assay was custom designed by the manufacturer (Table 2). The assays were used in a real-time PCR reaction together with the TaqMan Genotyping Master Mix (Life Technologies); we used the protocol provided by the manufacturer. Mainly, 2.25 μL of Genotyping Master Mix were mixed with 0.25 μL of 20X SNP assay for each reaction. To this mixture, we added 20 ng of DNA, and the reactions were performed on the real-time PCR machine ViiA 7 (Life Technologies). The analysis was done with the software of the PCR instrument.

2.6. Statistical analyses

All statistical analysis was done in Graph Pad Prism v.6.0. For