Whole genome approach to basal like breast cancer

Aproximación completa del genoma del cáncer de mama basal

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ABSTRACT

Basal like breast cancer (BLBC) composes up to 15% of breast cancer (BC) and is characterized by low or absent expression of estrogen receptor (ER), progesterone receptor (PR), lack of HER2 gene amplification and expression of basal cytokeratins (Cks) 5, 6, 14 and/or 17, epidermal growth factor receptor (EGFR) and/or C-Kit. BLBC constitutes a distinct clinical entity and is associated with poorer clinical outcome (Sørlie et al. 2001). The principal objective of this project is the complete and comparative mapping of genomic abnormalities in a series of BLBC associated or not to *BRCA1* mutations and explores the role of the *BRCA1* pathway in BLBC. For this reason, we are evaluating a 2.1 M feature human exome capture array on 9 frozen samples obtained from the Pathology Department of Centre Jean Perrin. By the time, we identified on one individual (MCD-4) 11,109 variants, of which 7,113 (64%) were described as known single nucleotide polymorphism (SNP) based on the conservative HCDiff algorithm. In regards to the distribution of these variations, chromosomes 10 and 2 were most affected. The novel somatic variations will be confirmed by conventional sequencing. The analyses of the remaining patients are ongoing. Using this next generation methodology, we will contribute to identify new markers or therapeutic targets and help to complete a catalogue of recurrent somatic and inherited variants associated with the development of BLBC.
Keywords: *Breast cancer, Basal like, BRCA1, genomic, mutation, next generation sequencing.*

RESUMEN

El cáncer de mama basal (BLBC) comprende hasta el 15% de los cánceres de mama. El BLBC es caracterizado por la baja o ausencia de la expresión del receptor de estrógreno (ER), receptor de progesterona (PR), ausencia de amplificación del gen HER2 y por la expresión de las citoqueratinas basales (Cks): Cks 5, 6, 14 y/o 17, receptor del factor de crecimiento epidermal (EGFR) y/o C-Kit. BLBC constituye una entidad clínica distinta y presenta un pobre prognóstico clínico (Sørlie et al. 2001). El principal objetivo de este proyecto es el mapeamiento completo y comparativo de las alteraciones genómicas en BLBC asociado o no con mutaciones en el gen BRCA1. Asi también, explorar el rol de la via de señalización de BRCA1 en BLBC. Por esta razón, utilizamos un arreglo de captura del exoma completo de 2.1M en 9 muestras congeladas de mama obtenidas del Departamento de Patología del Centre Jean Perrin-Clermont Ferrand. Actualmente, hemos identificado en un individuo (MCD-4) 11, 109 variantes, de las cuales 7,113 (64%) han sido descritas como polimorfismos (SNP) de acuerdo al algortimo conservativo HCDiff. Con relación a la distribución de estas variantes, los cromosomas 10 y 2 fueron los más afectados. Las nuevas variantes somáticas encontradas serán confirmadas por secuenciación convencional. El análisis de los pacientes remanentes está en avance. Mediante el empleo de esta medotología de nueva generación, nosotros contribuiremos en identificar nuevos marcadores o alvos terapéuticos así como ayudar a completar el catálogo de las variantes somáticas recurrentes y hereditarias asociadas con el desarrollo de BLBC.
Keywords: *Breast cancer, Basal like, BRCA1, genomic, mutation, next generation sequencing.*

INTRODUCCIÓN

Breast cancer (BC) is one of the most common human neoplasms and constitutes a major public health problem, accounting for 22% of all cancers in women worldwide (1).

Basal like breast cancer (BLBC) composes up to 15% of BC and is characterized by low or absent expression of estrogen (ER), progesterone receptor (PR) and lack of HER2 gene amplification (called as triple negative - TN) and expression of basal cytokeratins (Cks) 5, 6, 14 and/or 17, epidermal growth factor receptor (EGFR) and/or C-Kit (2,3). Currently, its identification is based on an immunohistochemistry (IHC) panel: ER/PR/HER2-, Cks 5/6/EGFR+, which is 100% specific and 76% sensitive (2). However, there is not yet a consensus panel established for the identification of BLBC.

BLBC are associated with high histology and nuclear grade, poor tubule formation, high mitotic and

proliferative indices. BLBC constitutes a distinct clinical entity and is associated with poorer clinical outcome (3,4).

BLBC has been reported to present a greater degree of genetic instability, including highest frequency of DNA losses/gains and less frequently of genomic amplifications than do tumors pertaining to other molecular subgroups (5). The loss of heterozygosity (LOH) and copy neutral changes were shown to be approximately two to three times higher than luminal A or B tumors (4,6).

It is increasingly apparent that BC development not only depends on genetic alterations but also on epigenetic changes involving DNA methylation and histone modifications. Roll et al. showed that BLBC express a hypermethylator phenotype that is characterized by concurrent methylation-dependent silencing of *CEACAM6, CDH1, CST6, ESR1, LCN2* and *SCNN1A* genes. These genes are involved in a wide range of neoplastic processes and their silencing is associated with a poor prognosis (7).

Approximately three quarters of *BRCA1*-mutated BC are BLBC (9,10). The *BRCA1* promoter hypermethylation has been reported in metaplastic BC (a rare type of BLBC) and an over expression of ID4 (a negative regulator of *BRCA1* expression) was shown in sporadic BLBC (7). Thus, the BRCA1 pathway seems to be closely linked to BLBC development, although it is not clear whether *BRCA1* inactivation is the cause or consequence of a BLBC phenotype.

The urgent need for new therapies for BLBC is underscored by the fact that BLBC do not express ER or HER2. Currently, there is not yet a developed targeted therapy to BLBC (3,10).

The principal objective of this study is the complete and comparative mapping of genomic abnormalities in a series of BLBC associated or not to *BRCA1* mutations and explores the role of the *BRCA1* pathway in BLBC.

MATERIAL AND METHODS

Clinical Data. Clinical data from BLBC and TN patients were collected from the Pathology Department. The clinical criteria for the identification of BLBC were based on the morphologic features and IHC panel: ER/PR/HER2-, Cks 5/6/EGFR+ and/or Ck14. About TN tumors, their classification was based on low or absent expression of ER, PR and lack of HER2 gene amplification. The status of *BRCA1* was obtained from Laboratory of Diagnostic Génétique et Moléculaire of Centre Jean Perrin.

Breast Tumors. Frozen samples were obtained from the Pathology Department of Centre Jean Perrin. Frozen samples were cryosectioned in slides and the presence of tumor within each tumor tissue were confirmed by a pathologist by Haematoxylin staining representative tissue sections.

Laser Capture Microdissection (LCM). The specimens were embedded using Tissue-Tek OCT compound (Sakura Fineteck USA, Torrance, CA). The tissue specimens were microdissected into epithelium and stroma using PixCell LIe LCM System (Arcturus). Stained tissue slides were stored in xylene at 4°C for up to 2 hours until ready for LCM. Depending on the specimen size and tumor contents, six to ten 8-µm sections were used in each capture of about 50,000 cancer cells (about 4 mm^2), an amount expected to generate sufficient DNA (750 ng) for the genome profiling.

DNA Extraction. Initially, the genomic DNA was performed using the Arcturus® PicoPure® DNA Extraction Kit protocol (Arcturus, Mountain View, CA), and then cleaned with the QIAamp DNA Micro kit (Qiagen) according to the manufacturer's protocol. But based on the insufficient quantity and poor quality of DNA, we decided to change the protocol of DNA extraction. In all cases, we repeated the LCM procedure as well as DNA extraction according to QIAamp DNA Micro kit (Qiagen) protocol with some modifications. The quantity and quality of DNA was analyzed using PicoGreen® dsDNA quantitation assay (Invitrogen) and Agilent 2100 bioanalyzer, respectively.

Targeted Sequence Capture. Genomic DNA (approximately 750 ng) was captured on a NimbleGen 2.1M human exome array following the manufacturer's protocols (Roche/NimbleGen) with some modifications. DNA was sheared by thermic shock (95°C) and adaptors were ligated to the resulting fragments. Extracted DNA was amplified by ligation-mediated PCR, purified, and hybridized to the capture array at 42°C using the manufacturer's buffer. The array was washed twice at 47.5°C and 3 more times at room temperature using the manufacturer's buffers. Bound genomic DNA was eluted using 125mM NaOH for 10 minutes at room temperature, purified and amplified by ligation-mediated PCR. The resulting fragments were purified and are sequencing on the Roche genome analyzer, following the manufacturer's protocol. Capture and non-capture amplified samples were subjected to

quantitative PCR to measure the relative fold enrichment of the targeted sequence.

Confirmation by Capillary Sequencing. For follow-up confirmation of identified novel variants, we will also apply capillary sequencing on tumors and blood samples. PCR primers will be designed flanking approximately 200 bp of a given variant and will be sequenced on an ABI 3730 capillary sequencing instrument following standard procedures. Capillary sequence reads will be analyzed using the Sequencer software package.

Data Analysis. The results from next generation sequencing will be analyzed using bioinformatics programs.

RESULTS

Clinical Data and Breast Tumors. Overall, 9 breast tumors were analyzed: 6 fulfilled BLBC criteria (4 of them with *BRCA1* mutation) and 3 fulfilled TN criteria. Table 1 shows the pathological characteristics and status of mutation for each tumor sample.

Table 1. Characterization of 9 breast tumors based on *BRCA1* status and pathological diagnosis.

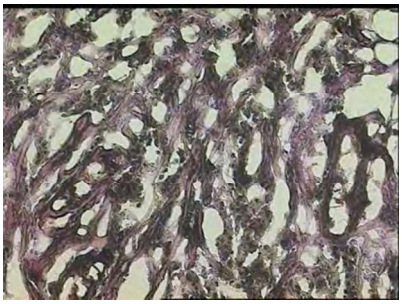
Tumor Identification	Status of *BRCA1* Mutation	Pathological Diagnosis
MCD-1	c.3839_3843del5ins4	Triple Negative
MCD-2	c.4128delC	Basal like
MCD-3	Non	Basal Like
MCD-4	c.3583delC	Triple Negative
MCD-5	c.4251_4252delGT	Basal Like
MCD-6	c.3960delCA	Basal Like
MCD-7	c.1808C>G	Triple Negative
MCD-8	4247delA	Basal Like
MCD-9	Non	Basal Like

MCD: Laser microdissection sample.

LCM and DNA Extraction. The LCM was performed by collaboration with the Faculty of Medicine of University of Auvergne (Clermont-Ferrand) and with the presence of a pathologist of the Department of Pathology of the Centre Jean Perrin. Because our work was the first application of this methodology in the University, we conducted a series of standardizations, such as time of coloring, the number of cell captured and DNA extraction method. With respect to the LCM, we obtained good results in relation to the coloring and number of cells. Figure 1 shows the sample before being microdissected (A), the capture of tumor cells (B) and finally the tissue without cells tumor (C) confirming the performance of this procedure.

Regarding the xtraction of DNA, we used QIAamp DNA Micro kit (Qiagen) protocol with some modifications. The quantification was performed using PicoGreen® dsDNA quantitation assay and we obtained high quantity of DNA (from 750ng to 2ug) that was enough for the next steps. Regarding the quality of DNA, we performed Agilent 2100 Bioanalyzer DNA 7500 Chip (Figure 2).

Targeted Sequence Capture. We performed exome capture and next generation sequencing in 6 out of 9 samples. Figure 3 shows a variant profile per chromosome obtained from sequencing data (MCD4). We identified 11,109 variants, of which 7,113 (64%) were described as known single nucleotide polymorphism (SNP) based on the conservative HCDiff algorithm. In regards to the distribution of these variations, chromosomes 10 and 2 were most affected.



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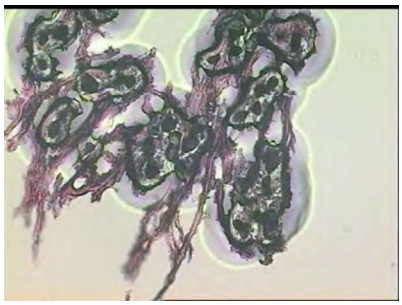

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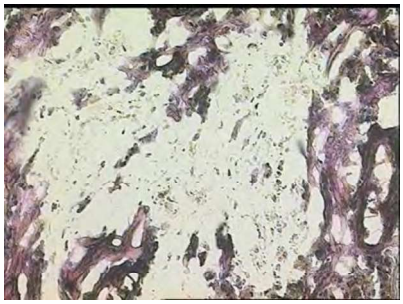

C

Figure 1. Microdissection of breast cancer cells by laser capture microdissection (LCM) in MCD-6 sample. (A) One tissue section was prestained with H&E for histologic diagnosis and for orientation during dissection; (B) The cell nests were punched out and transferred to a cap-tube for DNA extraction and (C) LCM was carried out using tissue sections.

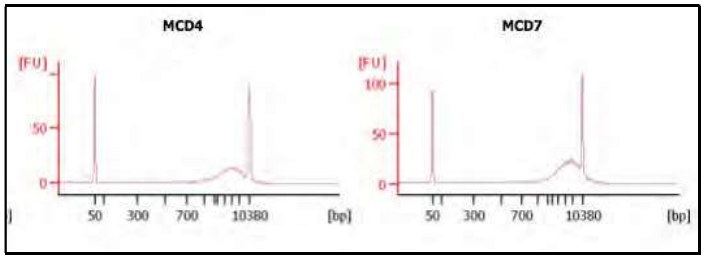

Figure 2. A representative MCD sample quality assessment results. After DNA extraction by QIAamp DNA Micro kit (Qiagen), all MCD samples showed a good quality and quantity of DNA by Agilent 2100 Bioanalyzer DNA 7500 Chip.

DISCUSSION

Our goal was to evaluate the genomic profile of BLBC with or without *BRCA1* mutation using NimbleGen 2.1 Array Human Exome technology

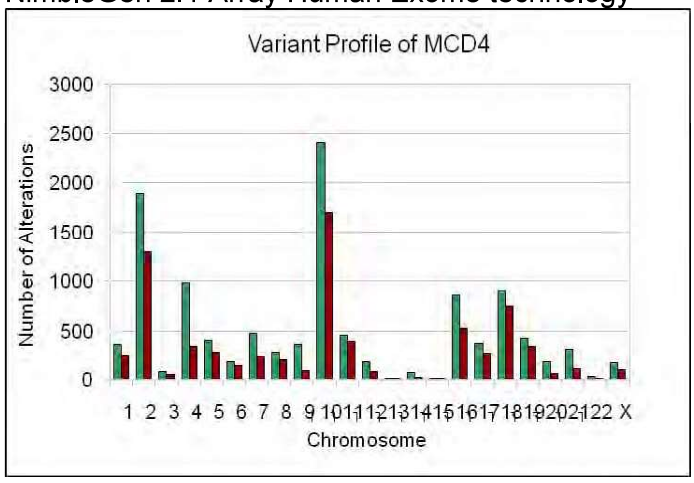

Figure 3. Variant profile for sample MCD4 based on number of alterations per chromosome. Green lines described unknown variants and red lines described known SNPs.

This new technology enables to capture of nearly all regions of the human genome that code for proteins (~180.000 human coding exons and ~550miRNA exons) on a single array. Based on it, we expect to have a unique portrait of all genetic abnormalities in BLBC. This portrait may indicate genes implicated in initiation of BLBC and may allow the discovery of key genes in the tumorigenesis of BLBC appointing new therapeutic targets.

Respect to *BRCA1* tumors, our results should help us to address our hypothesis about *BRCA1* inactivation and its role as a cause or a consequence in a BLBC phenotype.

Based on the complexity of our research and the sophisticated and new methodologies used in this project, we almost completed the experimental part of this project and are starting data analysis. Our approach has not been previously described and is scientifically interesting for use in medical practice into identification of BLBC and personalized treatment.

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