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Genetic alterations of triple negative breast cancer (TNBC) in women from Northeastern Mexico

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Abstract. Triple negative breast cancer (TNBC) is a subtype of breast cancer of heterogeneous nature that is negative for estrogen receptor (ER), progesterone receptor (PR) and growth factor human epidermal 2 (HER2) following immunohistochemical analysis. TNBC is frequently characterized by relapse and reduced survival. To date, there is no targeted therapy for this type of cancer. Chemotherapy, radiotherapy, and surgery remain as the standard treatments options. The lack of a target therapy and the heterogeneity of TNBC highlight the need to seek new therapeutic options. In this study, fresh tissue samples of TNBC were analyzed with a panel of 48 driver genes (212 amplicons) that are likely to be therapeutic targets. We found intron variants, missense, stop gained and splicing variants in *TP53, PIK3CA* and *FLT3* genes. Interestingly, all the analyzed samples had at least two variants in the *TP53* gene, one being a drug response variant, rs1042522, found in 94% of our samples. We also found seven additional variants not previously reported in the *TP53* gene, to the best of our knowledge, with probable deleterious characteristics of the tumor suppressor gene. We found four genetic variants in the *PIK3CA* gene, including two missense variants. The rs2491231 variant in the *FLT3* gene was identified in 84% (16/19) of the samples, which not yet reported for TNBC, to the best of our knowledge. In conclusion, genetic variants in *TP53* were found in all TNBC tumors, with rs1042522 being the most frequent (94% of TNBC biopsies), which had not been previously reported in TNBC. Also, we found two missense variants in the *PIK3CA* gene. These results justify the validation of these genetic variants in a large cohort, as well as the extensive study of their impact on the prognosis and therapy management of TBNC.

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Introduction

Breast cancer (BC) is the most common neoplasm among women worldwide, according to Global cancer statistics (GLOBOCAN) there will be about 2.1 million newly diagnosed female breast cancer cases in 2018, accounting for almost 1 in 4 cancer cases among women (1). In Mexico, BC is the most common cancer in women since 2006 (2). This neoplasm comprises a group of biologically different entities with different pathological and molecular features involved in

their staging and therapeutic management. Based on standard immunohistochemistry tests (IHT), BC is classified into three main groups: 1) luminal: Positive for hormonal receptors; 2) HER2 overexpressed, and 3) Triple-negative BC (TNBC). TNBC is characterized by the absence of hormone receptor expression and lack of HER2 amplification (3-5). Targeted therapies are available for luminal and HER2 amplified categories, but there is no treatment option for TNBC. Therefore, the precise classification in clinically relevant subtypes is of particular importance for therapeutic decision-making. TNBC represents 15-20% of the BC and is more frequent in young women and individuals of African and Hispanic heritage (6). Due to the lack of expression of therapeutic targets, chemotherapy remains a primary treatment option, along with radiotherapy and surgery (7,8). While TNBC patients respond better to chemotherapy than patients with non-TN BC (nTNBC), TNBC patients who do not respond eventually develop the metastatic form of the disease. This form is virtually incurable, so TNBC is characterized by its aggressive clinical course and poor prognosis compared to other BC subtypes (9).

The absence of therapeutic biomarkers of TNBC requires determining the molecular profile of TNBC tumors to propose therapeutic targets. The efforts of the complete genome sequencing have shown that TNBC presents alterations of *TP53* in up to 80% of the cases, followed by a broad set of genes with lower frequencies, such as *PIK3CA* and *RB* (10,11).

Next generation sequencing (NGS) is a powerful method that allows visualizing the genomic landscape of tumors and revealing tumor heterogeneity through the detection of genetic variants that occur in a low percentage (12). NGS has been used to sequence genes linked to cancer (11,13,14) to discover mutations that can modulate the repair capacity as well as the response to chemotherapy (15). Besides, the increased heterogeneity correlates with poor patient outcomes to treatment (16,17).

In the present study, we characterize the genetic alterations of TNBC in fresh tissue biopsies from TNBC patients from the Northeast of Mexico through NGS, with the aim of identifying alternative driver mutations, including those predictive of sensitivity and/or clinical response to chemotherapy and new molecularly directed drugs.

Materials and methods

Patients and tissue sample. The protocol was approved by the Ethics and Research Committee of the School of Medicine (Universidad Autonoma de Nuevo Leon), with the registered number BI11-005. Each participant was asked to sign an informed consent. Demographic information and personal data were obtained from the medical records. Tissue samples were obtained from patients under clinical and radiological suspicion of locally advanced BC (Tumor size > 2 cm, palpable ipsilateral lymph nodes, and ulceration) (18) of University Hospital "Dr. José Eleuterio Gonzalez" between 2011 and 2014. Core biopsies were obtained using a 12 Fr gauge (Bard®). Each patient underwent histopathological diagnosis by immunohistochemistry (ER, PR, HER2 and Ki67 status).

DNA isolation. Genomic DNA was obtained from biopsies with the DNeasy Blood and Tissue kit (Qiagen, Inc., Valencia, CA, USA) following the manufacturer's instructions (www.qiagen.com). The tissues were lysed by incubation with proteinase K at 56˚C until the tissues were completely lysed, followed by purification and elution on a centrifugation column. The DNA was initially quantified in the Nanodrop 8000 spectrophotometer (ratio 260/280> 1.8; Thermo Fisher Scientific, Inc., Waltham, MA, USA). A subsequent quantification was done using Quant-iT Picogreen (Thermo Fisher Scientific, Inc.) following the instructions from the manufacturer. DNA concentration was then adjusted to 50 μg/ml.

Sequencing. Libraries were constructed using the TruSeq Amplicon Cancer Panel (FC-130-1008) (19) a kit available on https://www.illumina.com/ that has been designed to cover mutational hotspot of 48 genes associated with cancer that can generate data for treatment with drugs approved by the US Food and Drug Administration. 250 ng of DNA was mixed with the pool of oligonucleotides containing all the primers to generate 212 amplicons (~35 kilobases) from hotspot regions of 48 genes. Libraries were amplified in the Eppendorf EP Master Faster City thermal cycler (Eppendorf, Hamburg, Germany). The quality of the libraries were evaluated in Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA). Each library was standardized according to the manufacturer's instructions. Finally, libraries were adjusted at a concentration of 12 pM. Library pools were loaded into a MiSeq Reagent Kit v3 cartridge (Illumina, Inc., San Diego, CA, USA), and each library pool was sequenced on an Illumina MiSeq instrument using a 150 paired-end design.

Data analysis. The Human Genome build 19 construct (hg19) was used as the reference genome. Alignment and the variant calling were performed with the MiSeq Reporter TruSeq Amplicon (Illumina, Inc.). Variants were identified using Variant Interpreter (Illumina Inc.). Reading quality Q>90 and reading depth>60 were used. Variants with an allelic frequency less than 5% were discarded. The clinical significance of the variants was determined using the ClinVar tool. Also, the tool Polymorphism Phenotyping v2 (Polyphen-2) was applied to predict the possible impact of an amino acid substitution on the function of the proteins.

Results

Patients. A total of 29 frozen tissue biopsies classified as TNBC were collected. However, there were 19 tissue samples available for sequencing. The average age of these 19 women was 51 years, with a BMI average of 27.5. All participants were free of metastases at the time of participating in the study. The main clinical information of the patients is shown in Table I.

TNBC sequencing. We found 65 variants in 25 of the 48 genes analyzed. Of these genes, *TP53, PIK3CA* and *FLT3* genes presented nonsense, missense, stop-gained variants, or variants in the splicing region with pathogenic significance. In the rest of the genes, intronic and variants that have been classified

Table I. Clinical characteristics of women participating in this study. The average age at diagnosis was 52 years, with an age range of 41-71 years. The average Body Mass Index was 27.31. No patient presented metastasis at the time of the study. The participating women were in clinical stages II and III.

Clinical characteristics	n=19	Range	Standard deviation
Age at diagnosis, years	52	41-71	8.63
BMI, Kg/m^2	27.31	20.78-37.01	4.27
Menopause status			
Pre	10	53%	
Post	9	47%	
Diabetes mellitus[a]			
Yes	3	16%	
No	16	84%	
Glucose levels, mg/dl	101.8	91-116	7.69
Number of children[b]			
Nulliparous	0	0%	
1 to 2	6	32%	
>3	13	68%	
Smoking			
Yes	1	5%	
No	18	95%	
TNM			
T1	0	0%	
T2	9	47%	
T3	7	37%	
T4	3	16%	
N0	0	0%	
N1	13	68%	
N2	4	21%	
N3	2	11%	
M0	0	0%	
Clinical stage			
I	0	0%	
II	11	58%	
III	8	42%	

[a]Confirmed at BC diagnosis, [b]Number of pregnancies. T, size of primary tumor; N, node status; TNM, tumor-node-metastasis; BMI, Body Mass Index.

as benign were observed. Fig. 1A shows the distribution of each gene variants. All the samples presented between 18 and 26 genetic variants. Fig. 1B shows the distribution of the variants of each gene analyzed, including the non-reported variants found in *ATM, GNA11, GNAQ, NRAS, PIK3CA, PTEN, RB1, SMAD4, SRC* and *TP53*. The highest number of variants were found in the intronic regions (from 10 to 14 per sample) and 49% were in exonic regions (32/65). An average of 5 synonymous variants were identified regarding the type of variant, and 2 to 5 missense variants were found per sample (Fig. 2). *TP53* was mutated in all the samples and presented 15 variants, of which 7 (46%) were missense variants. We found four genetic variants in the *PIK3CA* gene, including two missense variants. *FLT3* presented one variant in the splicing region between exons 14 and 15 (Table II).

TP53 variants. When comparing the variants found in each sample, we observed the exonic variant rs1042522 located in the *TP53* gene in 94% of the TNBC biopsies (18/19). In addition, we found rs1625895, rs34949160, rs1800372, rs5877580, rs121912654, rs28934574 and rs11540652 variants of *TP53*. The first three variants, located in intron 6 of the gene, are classified as benign according to the ClinVar database. The rs5877580, rs121912654, and rs11540652 variants are probably damaging variants based on PolyPhen-2 with score s of 0.993, 0.998 and 0.992 respectively. The rs28934574 is a variant with a possibly damaging score (0.583). We found 7 non-reported exonic variants. Five of these variants are SNVs: p.Arg213Ter, p.His179Gln, p.Arg196Ter and p.Ser269Arg. Three variants correspond to insertion or deletion of a nucleotide: p.Asn268ThrfsTer77, p.Gys135AlafsTer35 and p.Cys275PhefsTer71. All these variants affect part of the DNA binding domain of p53.

FLT3. The rs2491231 variant of the *FLT3* gene, which has not been reported before for TNBC, was found in 84% (16/19) of the samples. This variant is located in the splicing region between exons 14 and 15.

PIK3CA. Two missense variants were observed in *PIK3CA*: The exonic variant rs121913279 of the *PIK3CA* gene was detected in one sample. This variant has been classified as Pathogenic/Likely pathogenic in ClinVar and the exonic variant, p.Glu1012Gln, that has not yet been reported.

Discussion

NGS is a very useful tool in disease characterization of multigenic origin such as cancer, where the accumulation of a series of mutations in several genes is the key to tumor development. The ability of NGS to evaluate the mutational status of a relevant set of oncogenes and tumor suppressor genes in a single test, such as those evaluated in this work could be helpful to identify TNBC mutation drivers to design better diagnostic and therapeutic strategies. TruSeq Amplicon Cancer Panel was validated in 2015 (19), with the purpose of detecting somatic mutations through hundreds of mutational hotspots of essential genes related to cancer, including *PIK3CA, TP53*, and *EGFR*. Mutations in these genes are related to cancer and are involved in many cellular pathways.

Previously, a difference in the pattern of somatic mutations among the intrinsic subtypes of BC has been observed (11). We found variants in 25 genes, of which variants in *TP53*, *PIK3CA* and *FLT3* showed missense and non-sense variants, or variants in the splicing region. Tumors with a triple negative phenotype have a high prevalence of mutations in *TP53* (80%) vs. luminal breast cancer (12%). In this work, we analyze the triple negative phenotype, observing that the gene with a high prevalence of exonic variants was *TP53*, as previously indicated (20,21). Although in 2012 it was reported that most of the *TP53* variants in basal tumors were non-sense and

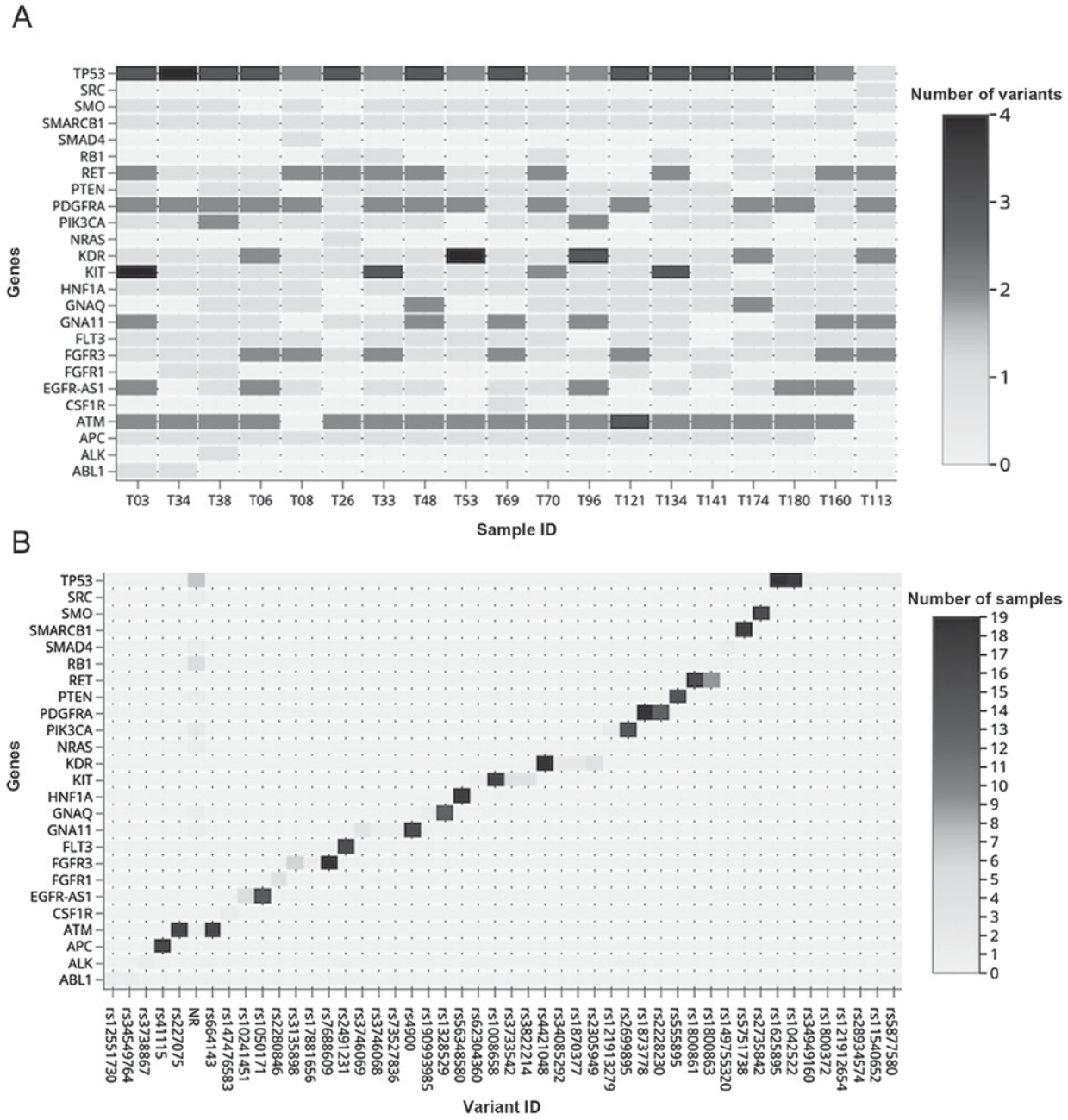

Figure 1. Distribution of the genetic variants by gene and by sample. (A) The graph shows the distribution of the variants of each gene analyzed among the patients. On the x-axis are the sample IDs. On the y-axis is the ID of each gene. On the right side is the gray scale color intensity code that indicates the number of variants found, with light gray=0 variants and darker gray=4 variants. (B) The graph shows the distribution of the variants of each gene analyzed. On the x-axis the IDs (rs) of the variants are shown, NR=Not reported. On the y-axis is the ID of each gene. On the right side is the gray scale color intensity code that indicates the number of samples in which each variant was found, with light gray=0 samples and darker gray=19 samples.

frameshift (11), Shah and Weisman *et al* (10,22), independently reported missense mutations in *TP53* on TNBC whereas we found that 7 of 15 variants were missense type. *TP53* is a tumor suppressor gene that encodes for a multifunctional DNA binding protein that regulates the transcription of hundreds of genes related to cell cycle regulation, differentiation, and apoptosis (23-25). The rs1042522 of *TP53* corresponds to an arginine (CGC) by a proline (CCC) change in codon 72 of exon 4. The proteins p53Arg72 and p53Pro72 do not differ in their ability to bind to DNA in a sequence-specific manner, but differ in other ways (26): p53Arg72 protein induces faster apoptosis and suppresses transformation more efficiently than the variant p53Pro72 (27,28). This variant was found in 18 of the 19 analyzed samples, and it is classified in ClinVar as drug response variant. TNBC, unlike the other BC subtypes, responds better to chemotherapy (9). Our results suggest that when p53Arg72 is present, apoptosis is more efficiently activated. However, the association of this *TP53* variant with the risk of various types of cancer, including BC, remains controversial (29,30). The missense *TP53* variants rs121912654 and rs28934574 have been previously associated with hepatocellular carcinoma (31) and osteosarcoma (32), respectively. The rs121912654 located in exon 5 causes the substitution of valine by phenylalanine. The rs28934574 is located in exon 8, and

Table II. Genes with missense, stop gained variants or splicing region variants. TP53, PIK3CA, and FLT3 were the genes that presented variants with transcriptional consequences, in addition to intronic variants. The gene with the highest number of variants was TP53 with 15 variants; PIK3CA presented 4 variants and FLT3 presented a variant.

Gene (variants)	Variant	Samples	Exon	HGVSc/HGVSp	Consequence	dbSNP	ClinVar
TP53 (15)	T>C	19	-	c.672+62A>G	Intron variant	rs1625895	Benign
	G>C	18	004/11	p.Pro72Arg	Missense variant	rs1042522	Drug response
	T>C	1	-	c.672+31A>G	Intron variant	rs34949160	Benign
	T>C	1	006/11	c.639A>G(p.=)	Synonymous variant	rs1800372	Benign
	G>C	1	008/11	p.Arg282Gly	Missense variant	rs28934574	Pathogenic/likely pathogenic
	T>C	1	007/11	p.tyr234cys	Missense variant	rs5877580	[b]
	C>A	1	005/11	p.Val157Phe	Missense variant	rs121912654	[b]
	C>T	1	007/11	p.Arg248Gln	Missense variant	rs11540652	[b]
	A>ACG	1	008/11	p.Cys275PhefsTer71	Frameshift variant, feature elongation	[a]	[b]
	G>A	1	006/11	p.Arg213Ter	Stop gained	[a]	[b]
	A>C	1	005/11	p.His179Gln	Missense variant	[a]	[b]
	G>A	1	006/11	p.Arg196Ter	Stop gained	[a]	[b]
	G>C	1	008/11	p.Ser269Arg	Missense variant	[a]	[b]
	GT>G	1	008/11	p.Asn268ThrfsTer77	Frameshift variant, feature truncation	[a]	[b]
	CA>C	1	005/11	p.Gys135AlafsTer35	Frameshift variant, feature truncation	[a]	[b]
FLT3 (1)	A>G	16	-	-	Splice region variant, intron variant	rs2491231	[b]
PIK3CA (4)	A>G	1	-	c.1252-27A>G	Intron variant	[a]	[b]
	G>C	1	21/21	p.Glu1012Gln	Missense variant	[a]	[b]
	A>G	1	21/21	p.His1047Arg	Missense variant	rs121913279	Pathogenic/likely pathogenic
	C>A	15	-	c.1059+62C>A	Intron variant	rs2699895	[b]

[a]Variants that have not been reported; [b]variants for which there is no information in ClinVar.

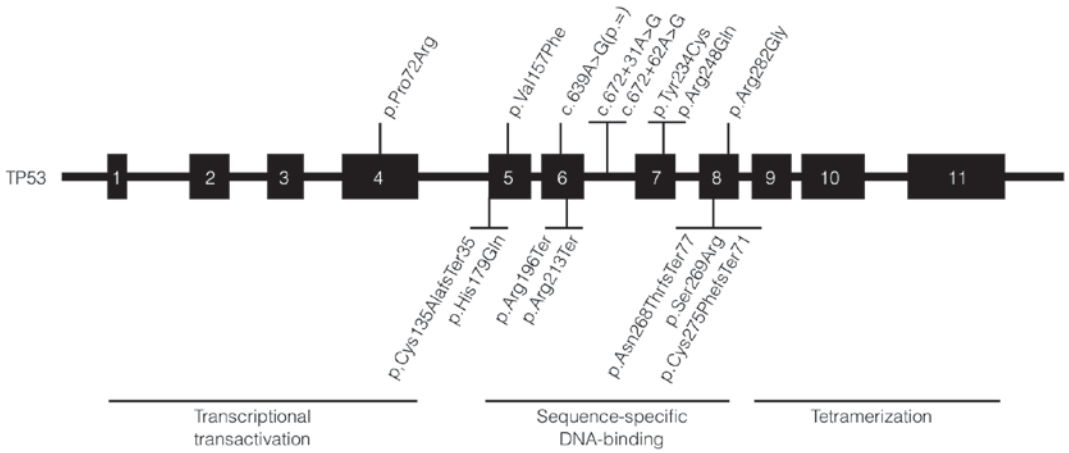

Figure 2. Map of *TP53* and the domains that transcribe each region with the location of the variants reported (upwards) and not reported (downwards).

the final product is a substitution of tryptophan by arginine. The rs28934574 variant meets the criteria published in 2013 by the American College of Medical Genetics (ACMG) (33) as a variant that is recommended to inform the patient.

We found seven unreported variants in *TP53* (p.Arg213Ter, p.His179Gln, p.Arg196Ter, p.Ser269Arg, p.Asn268ThrfsTer77, p.Gys135AlafsTer35 and p.Cys275PhefsTer71) in exons 5, 6, 7 and 8, which affect the

DNA binding domain of the protein. These results match with those reported in 2014 by Silwal-Pandit *et al* (34). Their results conclude that more than 80% of mutations in BC are grouped in exons 5-8 (the rest was eliminated). They observed that the p.Arg213Ter variant was located in a hotspot area of basal tumors. We found this variant in 1 of 9 patients. It would be important to analyze whether these changes affect the function of p53.

PIK3CA codes for the catalytic subunit p110 alpha (p110α) of the phosphatidylinositol 3-kinase enzyme (PI3K) (35). The PI3K signaling pathway is essential for several cellular processes, including cell growth, proliferation, migration, and survival (36). Some works have shown gene amplifications, deletions and, more recently, missense mutations in the *PIK3CA* gene in human cancers, including colon, liver, stomach, brain, lung and breast cancers (37). We found four variants of the *PIK3CA* gene, two of them already identified as rs2699895 and rs121913279. The first is a variant located in the intron 5. The second is a missense variant. The ancestral allele is an adenine changed by guanine, which represents a change of histidine by arginine in position 1047 of the protein. In ClinVar, it is classified as probably pathogenic since it has been associated with breast and colorectal cancer, melanoma and non-small cell lung cancer. We also found two not previously reported variants, c.1252-27A>G, and p.Glu1012Gln. The first one is located in an intronic region and the second one is located in exon 21, therefore a change from glutamic acid to glutamine is observed. In previous works, it has been reported that *PIK3CA* is the second most frequently mutated gene in TNBC (10,38). *PIK3CA* presents at least one variant in 15 of the 19 samples analyzed, and two samples have an exonic gene variant. It has been reported that the frequency of mutations in *PIK3CA* in BC is 10-30% (39), what is similar to our data. However, most of the mutations reported are located in exons 9 and 20 (39). These variants produce a gain of function and transformation capacity in the *PIK3CA* protein, thus it is relevant to investigate the role of the variants that we found in this work. Lips and colleagues reported that mutations in *PIK3CA* are associated with mutations in *BRCA1* (40). In this work, we do not investigate the *BRCA1* gene, so it would be interesting to analyze this theory in these patients.

In Mexico, Vaca-Paniagua *et al* (41) reported *TP53* and *RB1* as the most frequently mutated genes in TNBC by complete exome sequencing. Differences in the results found in our work with respect to *RB1* could be attributed to the design of the study. In the work of Vaca-Paniagua, the whole exome was sequenced in paraffin embedded tissues (n=12), while we sequenced the hotspot regions of the 48 genes in frozen tissue biopsies. Although they obtained a greater coverage of the gene, we have a greater depth of reading. To properly compare both studies not only an intrapopulation analysis is needed, but also an interpopulation comparison is required. Finally, differences also could be explained by the clinical criteria of selection, geographic origin of the patient and his ancestors, analytical methods, sample size, exposure to environmental risk factors and dietary, among others factors.

In addition, we found the rs2491231 variant of *FLT3*, which corresponds to an SNV type change for which there is no evaluation in the ClinVar database. This variant is found in a region of splicing between exons 14 and 15 that is part of the region that codes for the cytoplasmic domain of the protein. The *FLT3* gene encodes a tyrosine kinase receptor class III regulating hematopoiesis. When this receptor is activated, it phosphorylates and activates multiple cytoplasmic effector molecules in pathways involved in apoptosis, proliferation, and differentiation of hematopoietic cells in the bone marrow. The most common reported mutations are located in exons 14 or 20 and result in constitutive activation of this receptor, which are observed in acute myeloid leukemia and acute lymphoblastic leukemia (42,43). It would be essential to evaluate the effect of the *FLT3* variant on TNBC.

It would be interesting to determine the pathogenic significance of the genetic variants reported in this work in TNBC, because these genes are potential therapeutic targets. Currently, new compounds with different specificity and potency are being developed, targeting different components of the PI3K/AKT/mTOR pathway (44), small molecule compounds that specifically target the mutant p53 (45,46) and compounds that inhibit tyrosine kinase enzymes, such as *FLT3* (47). Mutations in DNA are not the only form of gene regulation; it is important to consider some other molecular events, including Copy Number Variation, chromosomal and epigenetic alterations, as well as the role that play micro RNAs (miRNAs) and non-coding RNA (ncRNA) (48).

In the present study, the diagnosis of TNBC was the only criterion for the variant analysis, other factors, such as age, ethnicity as well as the mutational signature will be considered in a future study, including damage to DNA and its repair components (49). Although for this study we do not have healthy tissue and our sample number is small, we can draw some conclusions. First, the mutation spectrum remains diverse even in a carefully selected and untreated group of patients with TNBC. All samples were from the same institution and the laboratory procedures were carefully monitored. Our results strongly suggest that each tumor has its unique molecular composition. However, it is observed that the total of the biopsies studied have at least two variants in the *TP53* gene. The rs1042522 drug responsive variant is the most representative, since it was found in 94% of the samples analyzed. We also found seven previously unreported variants with probable deleterious characteristics of the p53 tumor suppressor protein. The variant rs2491231 of the *FLT3* gene was identified in 84% (16/19) of the samples, which has not been reported before for TNBC.

In conclusion, we found intron, missense, stop gained and splicing variants in *TP53*, *PIK3CA,* and *FLT3* genes. Some of these variants have not been reported. Studies should be carried out to elucidate if they have a role in the development of TNBC y and their possible role as therapeutic targets. It is important to validate the presence of these variants in a large cohort that includes healthy tissue and non TNBC tissue as well as in cell culture to evaluate their impact on diagnosis, prognosis and management of such aggressive TBNC.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

GIUP conducted the experiments, acquired, analyzed and interpreted the data, and drafted the manuscript. SKSF designed the study, conducted the experiments, analyzed and interpreted the data, and critically revised the manuscript. CNSD made substantial contributions to the conception of the study, and drafted and critically revised the manuscript. SCH, GMM, JFGG and JVG as clinicians, selected the patients, performed the biopsies to obtain the tissue samples, collected the clinical information from medical records and contributed to the design of the study. PRF and ARM as geneticists selected the patients and participated in the interpretation of data. JRBW and LEOR assisted in technical support during the experimental work and made substantial contributions in the analysis and intepretation of the data. GSGM, ABQ, OBQ and RGG as pathologists, conducted the histopathological diagnosis of the patients. ROL made substantial contributions to the conception and design of study, and drafted and critically revised the manuscript.

Ethics approval and consent to participate

The protocol and informed consent was approved by the Ethics and Research Committee of the Faculty of Medicine (Universidad Autonoma de Nuevo Leon), with registration number BI11-005.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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