Germline Variants in Cancer Genes from Young Breast Cancer Mexican Patients

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Abstract: Breast cancer (BC) is one of the most frequent cancer types in women worldwide. About 7% is diagnosed in young women (YBC) less than 40 years old. In Mexico, however, YBC reaches 15% suggesting a higher genetic susceptibility. There have been some reports of germline variants in BC and YBC worldwide, such as BRCA1, BRCA2, ATM, CHEK2, PALB2, and POLQ, but also others not commonly reported in YBC. We show further supporting and controversial evidence for some of these genes. We conclude that exome sequencing combined with robust annotation tools and further analysis, can identify more genes and more patients affected by germline mutations in cancer.

Keywords: breast cancer; variant annotation; variant pathogenicity; cancer predisposition; bioinformatic pipeline; cancer genes
1. Introduction

Breast cancer (BC) is one of the most frequent types of cancer among women; 2.3 million cases were reported in 2020, causing more than 680,000 deaths globally [1]. The BC incidence increases with age. The majority of the diagnosed patients are 40 years and older, reaching 89%; 4% between 39 and 35, 5% less than 35, and 1.75% in women under 30 (https://gco.iarc.fr/ (accessed on 1 December 2021), [1]). In Mexico, BC is the first cause of death among women diagnosed with a malignant neoplasia [1], with an estimate of 26% of cancer-related deaths. Notably, the incidence of breast cancer in young women (YBC) in Mexico reached 15% in 2020, which is considerably higher than worldwide [1,2].

It has been observed that clinical outcomes and tumor biology in young patients are different from older women [3,4]. Tumors in young women are more likely to be of higher histological grade and are usually classified as estrogen receptor (ER) and progesterone negative receptors [5]. These patients typically present local recurrences to be diagnosed at an advanced stage and have inferior 5-year survival compared to the older premenopausal counterparts [5]. Moreover, YBC tends to present a more aggressive subtype such as triple-negative or HER2+ [6].

In some populations, up to 24% of the hereditary BC is linked to germline mutations in BRCA1/2 specific genes [7], and the prevalence may reach 14% in patients not showing familial history [7]. Mutations in these genes have a lifetime risk of developing BC up to 65% [8]. Other genes with germline mutations had been reported to confer moderate cancer risk, such as ATM, CHEK2, and PALB2, which are also associated with other cancers [8–10].

In addition to BRCA1, BRCA2, ATM, CHEK2, and PALB2, germline mutations in Latin America have been reported for CDH1, NBN, NF1, TP53, MLH1, BRIP1, MSH2, MSH6, and PMS2 in populations from Chile, Brazil, Colombia, and México [11]. In Mexico, a study in hereditary breast and ovarian cancer, which used a panel of 143 genes, found 21 germline mutations in BRCA1 and BRCA2, while another 19 genes showed 1 or 2 mutations, including the FANC(I/B/C/L/M) gene family accounting for 6 mutations [12]. Importantly, the above study found only 15% of patients (46 of 300) showing a germline mutation in at least one of the 143 panel genes. The low detection rate highlights the need to interrogate more genes in the population known to carry familial susceptibility.

The sequencing of gene panels has been a cost-efficient tool to determine the prevalence of specific genes among cancer populations [13–16]. However, more genes need to be studied to determine pathogenicity in most patients, for example, by whole exome sequencing. One issue is that pathogenicity is challenging to assess in not well-known or reported genes without further functional assays. Fortunately, extensive sequencing efforts, such as the 1000 genomes project [17] and the gnomAD (accessed on 1 December 2021) [18], provide databases of human variation among a relatively healthy population that help remove many common variants. In addition, other databases, such as ClinVar (accessed on 1 December 2021), help to mark pathogenic variants [19]. Still, massive sequencing generates several unseen variants increasing the need to explore methodologies to identify possible causal variants. In this context, Pediatric Cancer Variant Pathogenicity Information Exchange (PeCanPIE, accessed on 1 December 2021) is a web-based tool that integrates many sources of information supported by the guidelines from the American College of Medical Genetics and Genomics (ACMG) that are useful to identify interesting candidate variants quickly [20]. PeCanPIE uses a variant categorization for putative pathogenicity based on a “medal ceremony” concept of four levels. The Gold category is assigned for highly likely pathogenic truncating or splicing variants, whose genes are already found in pathogenic databases and whose variants are rare among healthy populations. Silver variants are in-frame, indels, and truncations in non-cancer genes or predicted to be damaging and matching pathogenic databases. Bronze variants are those whose effects are predicted to be tolerated. An unknown label is assigned otherwise.

To determine the possible etiology of BC in young women, in this study, we performed whole exome sequencing followed by the PeCanPie bioinformatic analysis of 115 young patients diagnosed with BC and focused on known 862 cancer genes where pathology
has already been associated with diseases. Briefly, we obtained 49 variants classified as Gold resulting in more than 20 genes that have not been reported previously in LA BC. In addition, 106 variants were classified as Silver.

2. Materials and Methods

2.1. Patients

We included 115 patients from the National Cancer Institute of Mexico (INCAN, Instituto Nacional de Cancerologí) diagnosed at 40 years old or younger with histopathological confirmation of BC. Patients were recruited between September 2015 and December 2017. Medical records and electronic files with detailed clinical and sociodemographic information were obtained from the INCAN. Trained nurses obtained blood samples after patients received genetic counseling and signed the informed consent.

2.2. Ethical Considerations

Regulatory approval was obtained from the INCAN Research and Ethics Committee (Approval ID CEI/1123/16). Genetic counseling and information about potential germline findings were provided to patients in addition to assurance of patient confidentiality and relevant information concerning the project, sample management, and DNA shipment for analysis to the National Cancer Institute (NCI), USA. Based on the recommendations provided by the American College of Medical Genetics, hereditary cancer diagnostic variants in their DNA were reported to the patients.

2.3. Sample and Panel Library Preparation for Sequencing

The DNA was extracted from peripheral leukocytes using the Wizard Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA), following the manufacturer’s instructions. The resulting DNA was purified using Agencourt AMPure XP reagent (Beckman Coulter Inc., Brea, CA, USA) following the manufacturer’s protocol. In addition, an adapter-ligated library was prepared using the KAPA HyperPlus Kit (KAPA Biosystems, Wilmington, MA, USA) with NEXTflex™ DNA Barcoded Adapters (Perkin Elmer Waltham, MA, USA), according to the KAPA-provided protocol.

2.4. Sequencing

The deep whole exome sequencing was performed at the Laboratory of Translational Genomics, Division of Cancer Epidemiology and Genetics (NCI, Rockville, MD, USA). The GRCh37 (hg19) genome assembly was used for genome mapping reference. The DNA sequencing was performed using the Illumina HiSeq 2000 sequencer for 2 × 100-pb paired-end cartridge (Illumina, San Diego, CA, USA). The sequencing included regulatory, splicing, and 3′ and 5′ UTR regions.

2.5. Variant Calling

The reads were aligned using Novoalign software (v3.00.05, Novocraft Technologies Sdn Bhd, Petaling Jaya Selangor, Malaysia). Duplicate reads were removed using MarkDuplicates from Picard Software (v1.126, Broad Institute, Cambridge, MA, USA). For variant calling, the pipeline involved RealignerTargetCreator, IndelReligner, BaseRecalibrator, UnifiedGenotyper, and HaplotyperCaller tools from GATK (v4.1.3.0, Broad Institute, Cambridge, MA, USA). Variants that failed to pass the pipeline control metrics (CScorefilter) had a read depth minor to 10, ABHet (reference to alternate reads ratio) <0.2 or >0.8 were excluded for the analysis. In addition, all variants were filtered using popmaxfreq <0.01. Before variant categorization, variants were filtered to remove those that do not show at least 10 reads in the alternate allele or that the minor allelic fraction was lower than 0.25.

2.6. Variant Categorization

The obtained variant calling files were analyzed using PeCanPIE, which classified the variants into three categories: Gold, Silver, and Bronze [20]. The variants were assigned as
Gold if it is a truncating variant, including splicing, or a loss-of-function associated with a reported disease documented in ClinVar with at least two starts, and at least one of the following databases: IARC: Tp53, ASU: TERT, ARUP: Ret, BIC, PCFP, and COSMIC. The variants assigned as Silver are considered as in-frame indels, truncation events in non-tumor suppressor genes. Variants had to be reported in at least one of the following databases: UMD, LOVD, RB1, or ALSoD [20]. The variant is assigned as Bronze if predicted to be tolerant by in silico algorithms.

For PeCanPIE, we used the union of their internal set of cancer predisposition genes and the Cancer Gene Census v94 from COSMIC [21] containing 712 and 723 genes, respectively, generating a list of 862 genes.

Only gold and silver variants were considered and studied separately for further analysis and filtering. The variants with fewer than 20 total reads or variant allele frequency (VAF) less than 0.25 were discarded for further analysis.

2.7. Statistical Analysis

We performed a descriptive analysis to calculate central tendency and dispersion measures for quantitative variables and absolute/relative frequencies for categorical variables. We also constructed logistic regression models to analyze the predictors of Gold variants. The final model included age < 30 years at diagnosis, family history of cancer, immunohistochemical subtype, advanced stage (IIB–IV), and continuous BMI. To evaluate disease-free and overall survival, we performed a Kaplan–Meier analysis, stratified by Gold variant, and considered time (in months) from diagnosis to the date of first recurrence or death. To evaluate differences by mutational status, we performed a Log-rank test. For all tests, we assumed a p-value < 0.05 to determine statistical significance. We used STATA 14® software to perform all statistical analysis tests.

3. Results

3.1. Clinical Population

A summary of our cohort characteristics, including 115 patients, is shown in Table 1 (raw data in Table S1). The median age was 33.9 years at diagnosis, and 18.3% were younger than 30. Patients diagnosed at advanced clinical stages (IIB–IV) corresponded to 65% of the cohort. Ductal histology (85%) and Luminal-B (50%) subtype were the most common in this group. A family history of cancer was present in 32.2% of these young women. All participants were premenopausal. The mean age at menarche was 12.34 years, 28.7% of participants were nulliparous, and the average number of children among parous was 2.1. Breastfeeding was practiced in 68.7% of parous women. Overweight and obesity was a condition present in 61.7% of the participants.

3.2. Variant Categorization

From the 1,189,705 raw variants, quality filtering generated 350,546 variants used for PeCanPIE annotation. PeCanPIE detected 6496 variants within the 862 cancer genes selected. After further filtering (for the number of reads described in the Methods section), 49 were categorized as Gold and 106 as Silver, corresponding to 40 and 87 genes, respectively (Figure 1). Only 39 patients (34%) showed high confidence Gold variants, while 74 (64%) showed Silver variants. Overall, 88 patients showed Gold or Silver variants.

3.3. High Confident Germline Variants in Cancer Genes

The criteria for Gold variants involve known pathogenic variants, a strong alteration variant (nonsense, frameshift, truncation) in a known pathogenic gene, and low allele frequency in public non-cancer databases [20]. From the 52 Gold variants obtained before manual filtering, two were removed (chr19, positions 34945343 and 34945354, UBA2 gene) because six patients showed alternative and varied genotypes at those positions, always with fewer reads in the alternate genotype suggesting mapping and sequencing artifacts. Additionally, one variant in (chr16, position 72991715, ZFHX3 gene) was also removed.
because it was present in all patients suggesting a common variant. Thus, 49 variants involving 40 genes and 39 patients were finally designated as \textit{Gold} (Figure 2 and Table 2). All variants were heterozygous. Except for one variant present in two patients (\textit{RBM8A}), all \textit{Gold} variants were observed only in one patient. Of these variants, we noted 20 splicing, 17 frameshift, eight nonsense, three missense, and one 5'-UTR. Most patients showed only one \textit{Gold} variant, but 11 patients of 39 (28\%) showed two. The well-known \textit{BRCA2} gene was the most recurrently altered, observed in five patients, showing four frameshifts and one missense. The following most frequent alterations were observed in \textit{CHEK2}, \textit{PALB2}, \textit{POLQ}, \textit{DDX3X}, and \textit{FLG} affecting two patients. From these genes observed in two or more patients, the variants observed in \textit{BRCA2}, \textit{PALB2}, and \textit{CHEK2} were also found in ClinVar with documented association to BC (Table S2). However, variants in \textit{POLQ}, \textit{DDX3X}, \textit{FLG}, and \textit{RBM8A} are barely reported in BC and will be further described.

\textbf{Table 1.} Clinical data of 115 BC young patients.

| Clinical Data                  | n  | Frequency (%) |
|-------------------------------|----|---------------|
| Patients                      | 115|               |
| Age                           | 33.9|              |
| Interquartile range           | 31–38|           |
| Age at menarche               | 12.34|             |
| Interquartile range           | 11–13|             |
| Parity                        |     |               |
| Nulliparous                   | 33  | 28.7          |
| 1 child                       | 27  | 23.2          |
| 2 children                    | 32  | 23.5          |
| >3 children                   | 23  | 20.0          |
| Breastfeeding                 |     |               |
| Luminal B (Her2-positive)     | 19  | 13.2          |
| Luminal B (Her2-negative)     | 31  | 28.3          |
| Her2-positive (non-luminal)   | 8   | 6.6           |
| Triple Negative               | 30  | 28.3          |
| Histology                     |     |               |
| Ductal                        | 98  | 85.2          |
| Lobular                       | 6   | 5.2           |
| Mixed                         | 9   | 7.8           |
| Others                        | 2   | 1.7           |
| Clinical Stage                |     |               |
| I                             | 15  | 13.0          |
| II                            | 45  | 39.1          |
| III                           | 49  | 42.6          |
| IV                            | 6   | 5.2           |
| Consumption of Hormonal...    |     |               |
| Yes                           | 65  | 56.55         |
| No                            | 50  | 43.5          |
| First-grade family history of cancer | 90  | 78.3   |
| Yes                           | 25  | 21.7          |
| No                            | 78  | 67.8          |
| Yes                           | 37  | 32.2          |
| Second-grade family history of cancer |     |               |
| Normal (BMI < 25)             | 44  | 38.3          |
| Overweight                    | 45  | 39.1          |
| Obesity (BMI > 30)            | 26  | 22.6          |

\textit{DDX3X}, located in Xp11.4, encodes for an RNA helicase linked to somatic mutations in medulloblastoma [22]. It is also X chromosome inactivated in ovarian cancer [23]. Germline mutations have been reported in female brain development and disability, whose variants were observed on the \textit{Helicase ATP-binding} and the \textit{Helicase C-terminal} domains [24,25].
two observed T→C heterozygous variants in our cohort affect the exon-intron splicing sites located in the Helicase C-terminal region (G539 and S590) responsible for the interaction with the nuclear mRNA export receptor TAP [26]. Moreover, DDX3X plays a role in DNA damage response [27]. There is no evidence in the clinical record for mental disability in these patients. These pieces of evidence suggest that DDX3X is potentially a predisposing gene in young BC patients.

![Diagram](image)

**Figure 1.** The diagram summarizes all the obtained variants with their exclusion criteria. YBC young Mexican breast cancer patients.

*POLQ* encodes a polymerase involved in DNA repair [28,29]. Germline mutations in BC have been reported mainly in non BRCA1/2 carriers [30,31]. In our data, we observed one splicing and one nonsense variant in I2385V and L1430*, respectively. Consistent to the above studies, these *POLQ* positive patients were not carriers of BRCA1/2 or TP53 variants.

*FLG* encodes for filaggrin that aggregates keratin intermediate filaments in the mammalian epidermis. *FLG* germline variants have been recently reported in around 16% of Taiwanese BC patients [28] and 17% of hepatocellular carcinomas from Thailand [29]. In addition, it has been found somatically mutated in 10% of ER + BC patients [30]. We observed two G→A nonsense mutations (R788* and R501*), each one affecting a single patient. One of these patients carrying a *FLG* germline variant had a second primary contralateral breast cancer (K1).
Figure 2. Gold variants detected. The top seven genes show two or more patients affected (black and green), while the rest show one patient only (blue). The 11 patients (in columns) marked with “2” show two Gold variants.

We observed a promoter variant in RBM8A affecting two patients. According to ClinVar (ID 30464), this recessive variant causes a decrease in gene expression [31], which is crucial when combined with another severe variant affecting patients with Radial aplasia-thrombocytopenia syndrome. RBM8A differential expression has been noted in several cancers types but observed more expressed in the tumor than in the normal tissue [32], which seems counterintuitive. The allelic fraction reported in gnomAD is 1.8%, similar to the 1.7% observed in our sample, suggesting that promoter variants may be random. Like reports in ClinVar, it may need to be combined with another unknown alteration. Overall, the evidence is unclear, suggesting that the promoter variant in RBM8A is a variant of unknown significance (VUS).

From the genes present in only one patient, 12 genes have strong support shown by their reports in ClinVar for pathogenic or likely pathogenic variants in ATM, BLM, BRCA1, CLTCL1, ERCC6, FANCE, G6PC3, MSH6, MUTYH, TP53, and TSC2. Moreover, some of these genes also show germline mutations in BC patients. For example, ERCC6 has also been reported in Brazilian YBC patients [33] and Lebanon familial BC [34]; BLM in Russian YBC patients [35] and USA patients [36]; TSC2 in Italian patients [37]; and ATM is also well known in BC [11].
### Table 2. Gold variants.

| Chr | Position | Ref * | Alt * | Depths | Gene | LA * | Type | AA Chg | Pat | pLI | AF Lat § | ProtSize |
|-----|----------|-------|-------|--------|-------|-------|------|--------|-----|-----|----------|----------|
| 11  | 108175579| G     | A     | 21,19  | ATM   | Splice| E1892E_37| H4    | 0   | 1×10⁻⁴ | 3056     |
| 12  | 91341429 | A     | AG    | 15,25  | BLM   | Frameshift| T1074fs| D8   | 0   | -   | 1417     |
| 17  | 41258472 | C     | A     | 54,35  | BRCA1 | Splice| R71_E4 | G8    | 0   | 3×10⁻⁵ | 1863     |
| 11  | 32914033 | C     | A     | 18,20  | BRCA2 | Frameshift| S1848fs| E0   | 0   | 3×10⁻⁵ | 3418     |
| 11  | 32914122 | AC    | A     | 52,30  | BRCA2 | Frameshift| N1877fs| J2   | 0   | 3×10⁻⁵ | 3418     |
| 17  | 32930635 | C     | CA    | 15,17  | BRCA2 | Frameshift| S2590fs| B2   | 0   | 3×10⁻⁵ | 3418     |
| 17  | 32937507 | A     | G     | 44,30  | BRCA2 | Missense| D272G  | G2   | 0   | 3×10⁻⁵ | 3418     |
| 11  | 32954260 | CG    | C     | 23,17  | BRCA2 | Frameshift| V3079fs| K5   | 2×10⁻⁴ | 3418     |
| 22  | 29091718 | T     | T     | 29,23  | CHEK2 | Frameshift| L413fs | J6   | 0   | -   | 543      |
| 21  | 29115401 | A     | ATGAT| 28,16  | CHEK2 | Frameshift| M222fs| F5   | 0   | 1×10⁻⁴ | 543      |
| 22  | 19222211 | C     | T     | 33,36  | CTCL1 | No Missense| E330K | G5   | 0   | 9×10⁻⁴ | 1640     |
| 17  | 18968190 | T     | A     | 35,26  | COLS1 | No Splice| P869P | J6   | 1   | 9×10⁻⁴ | 1466     |
| 17  | 10192637 | T     | C     | 57,41  | CLXI1 | No Nonsense| Q678* | G2   | 1   | 3×10⁻⁵ | 678      |
| 16  | 50818562 | T     | A     | 30,39  | CYLD   | No Splice| I650_E13| C1   | 1   | 3×10⁻⁵ | 956      |
| 16  | 50754187 | G     | T     | 28,23  | DCC    | No Splice| V621_E11| H2   | 0.99 | 2×10⁻⁴ | 1447     |
| 11  | 41206109 | T     | C     | 46,39  | DDX1X  | No Splice| G539_E15| K3   | 1   | 4×10⁻⁵ | 662      |
| 11  | 41208662 | T     | C     | 44,41  | DDX1X  | No Splice| S590_E16| F3   | 1   | 2×10⁻⁵ | 662      |
| 17  | 45917294 | T     | C     | 21,22  | ERC1C  | No Splice| V235_E7| H0   | 6×10⁻⁵ | 297      |
| 17  | 50680422 | C     | T     | 16,25  | ERC6   | No Splice| R975_E16| G8   | 0   | 3×10⁻⁵ | 1493     |
| 17  | 44228353 | G     | A     | 33,34  | EXT2   | No Nonsense| W535* | A2   | 0   | 1×10⁻⁴ | 718      |
| 17  | 35425330 | C     | T     | 25,34  | FANCE  | No Splice| D286_E3| A0   | 0   | 3×10⁻⁴ | 536      |
| 17  | 18573055 | G     | C     | 34,21  | FAT1   | No Splice| T3356T| B0   | 0   | 4588     |

Note: Numbers in parentheses represent the total length. * Represent whether the gene has been reported in Latin-American BC patients in the Urbina-Lara et al. analysis [11]. § Allele frequency in Latino population from GnomAD website (https://gnomad.broadinstitute.org/), accessed on 1 December 2021. Variants in GnomAD slightly different to those found are explicitly indicated or marked with * * . A total of 50 variants is shown, 49 unique (RBMSA is present in two patients). Genomic positions correspond to hg19. Ref and Alt refer to reference and alternate alleles respectively. 

The remaining Gold variants genes are, by definition in PeCanPIE, associated with diseases in databases; inquiringly, they do not show clear evidence of pathogenicity, specifically in ClinVar. All these variants carry protein truncations in genes where the loss of...
function (LoF) mutations is a known mechanism of disease (BLM, COL3A1, CUX1, CYLD, ERCC1, EXT2, FAT1, FLCN, NFkBIE, NSD1, PBRM1, PMS1, PRDM9, PTCH2, RAD51C, RPS7, USP6, WRN, and ZFHX3). Many of these genes already show evidence of variants in BC in other populations such as BLM in Slavic [38] and Brazilians [39]; ERCC1 in Brazilians [40]; PMS1 in men [41]; WRN in Latins [42], or in other cancers such as CYLD in head and neck [43]; EXT2 in osteochondromas and chondrosarcomas [44,45]; FAT1 in retinoblastomas [46]; NSD1 and PBRM1 in renal cell carcinoma [47,48]; PRDM9 in acute lymphoblastic leukemia [49]; PTCH2 in rhabdomyosarcoma [50]; RAD51C also in breast cancer [51]; RPS7 in hypocellular bone marrow failure [52]; SPEN, USP6, and ZFHX3 in pancreatic adenosquamous carcinoma [53]; TICAM1 in thyroid cancer [54]; ZFHX3 also in endometrial cancer [55]; and DCC in gallbladder cancer [56].

We also noted seven genes marked with “caution” in PeCanPIE showing the truncation close to the C-terminal (CUX1, FLCN, FLG, MSH6, NSD1, PRDM9, and USP6), questioning its functional effects in the cancer context. To provide additional support for these variants, we considered the probability of LoF intolerance (pLI) provided by gnomAD [18]. Natural selection purifies highly deleterious variants, therefore, genes showing fewer than expected LoF variants in a large healthy population are seen as highly LoF intolerant, proposing association to disease when observed in an individual. Thus, pLI close to 0.9 and up to 1 are a strong indicator of LoF intolerance as recommended by gnomAD. We noted pLI = 1 in NSD1, strongly suggesting some contribution to disease consistent with previous evidence [47]. We also noted pLI = 1 for CUX1, but the stop codon gain is shown at the last protein amino acid (Q678), marked in gnomAD as ‘low confidence loss of function’. In between, we observed pLI = 0.79 in FLCN and pLI = 0.77 for NFkBIE. Contrary, we noted pLI = 0 for PRDM9 and USP6; thus, although categorized as Gold, these variants are less likely to be pathogenic. We also noted pLI > 0.9 for COL3A1, DCC, DDX3X, PBRM1, RPS7, SPEN, TSC2, ZFHX3, and ZMYM3, in which a considerable proportion of the protein is altered by a frameshift, splice, or nonsense variant. We noted that one of the patients carrying TP53 and NFkBIE presented a second primary glioblastoma (I9).

We observed that the variant allelic fraction in Latino populations, is in general, low (Table 2), validating the PeCanPie filtering. Nevertheless, few variants in the order or few per a thousand (10^−3) could indicate a random finding due to our sample size.

We explored associations between patients carrying Gold variants and those not, along with clinical co-variables. The adjusted model showed an association of Gold variants with first- and second-family history of cancer (OR 3.21; CI 95% 1.15–8.95) and age < 30 years (OR 3.74; CI 95% 1.20–11.70). None of the tumor subtypes was associated with carrying a Golden variant.

We also observed that an increase in one unit of continuous BMI raises the odds of detecting a Gold variant in young women with breast cancer (OR 1.19; CI 95% 1.07–1.34), suggesting that BMI might be a modifier in women with Gold germline variants, and might reduce the age of breast cancer presentation. This phenomenon has been described previously for BRCA1/2 carriers, but our results suggest BMI could be a modifier for other genes as well [57–59].

We noticed that women with a Gold variant were diagnosed in advanced stages: IIB-IV (OR 3.21; CI 95% 1.21–8.98), suggesting that a Gold variant might increase breast cancer aggressiveness. It has been described that BRCA1/2 carriers have a higher risk of lymph node involvement at diagnosis [60].

The Median follow-up of this cohort was 62 months (48–73). Although not significant at p < 0.05 using a Log-Rank test probably due to the small sample size, we found that disease-free survival could be higher in women without Gold variants (89%) versus women with Gold variants (74%) (Figure 3A) after adjusting for clinical prognostic factors. We observed fewer differences for overall survival (OS), with 91.5% of OS in women without carrying a Gold variant and 86.8% OS in women with a Gold variant (Log-Rank test p = 0.48, Figure 3B). These results are consistent with many previous studies involving hereditary breast cancer and survival [61,62].
Figure 3. Survival analyses of YBC patients carrying Gold variants. (A) Disease free survival. (B) Overall survival.

3.4. Modest Confidence Germline Variants

The criteria for Silver are in-frame indels, truncation events in non-tumor suppressor genes but associated to diseases, variants predicted to be damaging by in silico algorithms, and matches to databases such as ClinVar with fewer than two stars, BRCA Share, ALSoD, LOVD, and a locus-specific database for APC, MSH2, and RB1. In addition, we filtered for the 862 genes associated with cancer. Under these conditions, PeCanPIE detected 1128 Silver variants. To further filter Silver variants to choose those more likely pathogenic, we reasoned that if a variant is the same as a somatic mutation found in a cancer patient, specifically in the tumor biopsy, it could indicate a higher degree of confidence. Thus, to further explore potential pathogenic variants, we only considered Silver variants categorized as Gold or Silver in the PeCanPIE somatic category. This additional category considers somatic databases such as COSMIC and PCGP [63].

Thus, 106 Silver variants were obtained distributed in 87 genes (Table S2). Of 115 patients, 74 (64%) presented one or more silver variants, all heterozygous. Of these, 18 were missense, four splice site, one frameshift, one nonsense, six protein deletions, eight protein insertions, and 78 splice regions. Of these genes, the most frequent were AKAP9 and ATM in four patients, followed by KMT2D, MGA, COL3A1, NCOR2, ERBB2, and MLLT3 in three patients each. We noted the following gene families: BRCA1/2, CDK 4/N2A, ERBB 2/3, ERCC 1/2/3, FANC A/D2/E/M, MRE 11/11A, NOTCH 1/2, and SMARC A4/B1/E1. Interestingly, we detected the same missense mutation in ERBB2 (R896H) in two patients. The gene position has been reported to activate HER2 function (R896C) [64], suggesting that R896H could affect normal function and potentially contribute to tumorigenesis.

4. Discussion

In the current work, we report an exome analysis of 115 young Mexican BC patients using the pipeline PeCanPIE focused on well-defined evidence of pathogenicity following ACMG guidelines. To our knowledge, this is the first effort for Mexican patients covering the germline whole exome. Previous efforts in Mexico and Latin America have focused on gene panels from 20 to 140 genes irrespective of the age of diagnosis [12,65]. Similar approaches showed a prevalence of 10.2% of pathogenic variants in BC in the USA [66]. Nevertheless, age at diagnosis is important because it may indicate an accelerated tumorigenesis process supported by recent reports showing an increase in BC incidence in young women [1,2,67–70]. Therefore, we focused on extreme phenotypes patients, where BC was diagnosed at 40 years old or less. We found that 39 patients (34%) showed a likely pathogenic Gold variant in 40 genes. This finding is higher than recent prevalence estimations in Latin American countries (13–25%) [42], which is likely due to our increased analysis in over the more than 800 genes by whole exome, and a higher genetic risk background of the younger population.
Comparing our *Gold* variants results with those of Quezada-Urban et al. in Mexican’s BC, where more than 53% were older than 40 years old and using a panel of 143 genes [12], only six genes were overlapping (*BRCA1/2, ATM, WRN, RAD51C, and CHEK2*). We did not find variants in 15 of the 21 genes reported (*MSR1, ERCC3* [1 Silver], *LIG4, PDE11A, ATR, FANC(I/B/C/L/M), RECQL4, SDHB, MLH1, NBN, and PTEN*), and we found 36 other genes which were not present in the Quezada-Urban et al. study. Some genes, however, showed similar gene families, such as *ERCC1/6* and *FANCE*. We also noted that many genes were not reported in BC for Latin-American countries (Table 2).

In our sample, we noted that 58% of the patients were overweight at the time of diagnosis, which is consistent with the 60% reported by Quezada-Urban et al. [12] and other reports in Mexico [2]. Weight loss in the young woman has been associated with lower cancer risk in *BRCA1* carriers [71]. Thus, we explored possible associations of known variants (*Gold*) to BMI. We observed a small but significant increase in BMI among *Gold* variant carriers. These results should be confirmed in larger cohorts.

We noted many *Gold* variants in genes not previously reported in Latin American cohorts but reported in BC in the USA, Europe, Asia, or other cancer types and gene families. For instance, Fanconi anemia and excision repair genes (*FANC and ERCC* genes) have been reported in Latin American BC cases [11]. These findings highlight the use of a broader set of genes combined with powerful analysis tools, to expand the results.

PeCanPIE uses a pipeline considering the observed variant frequency among ‘healthy’ populations deposited in databases such as ExAC, which is primarily based on Caucasian populations [72]. In the *Silver* category, we noted few variants present in many individuals in our cohort even with low allelic fraction in ExAC, confirming that estimations of Latin America variations are needed to identify common variants in this population. Although PeCanPIE was initially conceived for pediatric cancers, they included several gene databases from other cancers reaching 712 genes. To complement this, we added 723 genes from COSMIC v94. Thus, our analysis was limited to the unified 862 genes from these two sources. If we extend the analysis to the whole exome and focus on *Gold* variants, besides the two common variants that would need to be removed, six genes are added (*C8B, DMD, HBB, IRF8, KCNQ1, and MYBPC3*), of which three are splices, two frameshifts, and one nonsense). Nevertheless, these were not considered in our analysis, since we focus on more likely genes with a stronger background in the cancer context.

We focused on *Gold* variants because pathogenicity is theoretically the highest provided by PeCanPIE. Nevertheless, *Silver* variants may also show interesting results, such as that mentioned for HER2 (*ERBB2 R896H*) and other gene families such as *CDK, BRCA1/2, ERBB, ERCC*, and *FANC*. However, more careful revision is needed for *Silver* variants. For example, we noted a missense mutation in five patients in *WRN* (R834C) that has been shown to abolish important WRN function [73]. Although the variant was filtered out because of quality criteria (fewer than 10 reads), this polymorphism is frequent in the Mexican and is also unlikely to be pathogenic [74]. Thus, *Silver* variants should be handled more thoroughly. This evidence also highlights that annotation tools are crucial to facilitate interpretation but that results must be revised, and tools should be continuously updated.

Overall, our study provides candidate pathogenic variants in Mexican YBC, a barely studied population. Some variants need more careful analyses; for example, those regarding splice site variants and those in *RBM8A*. In addition, recent evidence rises questions even for well-known breast cancer genes [75]. Thus, confirmatory information may be needed either by specific experimental assays or analyses of large cohorts to potentially translate our findings into clinical practice.

### 5. Conclusions

We conclude that using whole exome sequencing to analyze an extended set of cancer genes, and a rigorous bioinformatic pipeline that includes PeCanPIE, we were able to identify candidate pathogenic genes for a more extensive set in young, Mexican breast cancer patients.
Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cancers14071647/s1, Table S1: Clinical Data, Table S2: Gold Variants.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Research Ethics Committee of National Cancer Institute of Mexico (INCAN, Instituto Nacional de Cancerología) (protocol code CEI/1123/16 approved in 2016).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study may be available on request from the corresponding author. The data are not publicly available due to embargo period.

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