Clinical, Genealogical and Molecular Genetic Studies - Among Twins with Familial Breast Cancer

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Abstract
Breast cancer (BC) is one of the most common malignant diseases in which incidence of cancer structure of the female's population ranked first, accounting for 19.6% in Ukraine.

Aim: To analyse the most common Slavic mutations in the genes BRCA1/2 among twin women with BC with family history on this pathology.

Materials and methods: The material of our study were pedigrees and DNA samples from 120 patients diagnosed with BC who were treated in the Lviv Regional State Cancer Diagnostic Center from June 2008 to December 2012. Molecular-genetic method determined the presence of seven mutations in the gene BRCA1 (185delAG, 4153delA, 5382insC, 188delC11, 5396 +1 G > A, 185insA, 5331 G > A) and 3 gene mutations in BRCA2 (6174delT, 6293S > G, 6024delTA) by allele-specific polymerase chain reaction.

Results: From 120 patients diagnosed with BC Slavic mutations in the genes BRCA1/2 were found in 5 patients (4.2%). We study pedigrees in 3 pairs of twins where both sisters had BC and 2 pairs of twins where one of sister had BC. Among 5 pairs of twins (10 women) 8 of them had BC and only 2 were healthy. BC was diagnosed at age 37-45 in six females and only in family at age 47-48 years.

All 8 women with BC of genes BRCA1/2 were tested. The 6 women with BC no 10 Slavic mutations BRCA1/2 were found. Elder sister from DZ twins had ovarian and breast cancer (tested BRCA1 5382insC mutation positive) died aged 50. A rare gene mutation BRCA2 c.6405_6409delCTTAA (p.Asn2135fs) was found in older sister of MZ twins at age 41 with bilateral breast cancer.

Among 3 pairs of twins (2 DZ and 1 MZ) both sisters were affected. In families with two affected twins only II-II degree relatives were BC diagnosed. Among 5 pairs of twins BC was diagnosed in I-III degree relatives in four families. BC affected mothers were recognized in two pairs of DZ twins.

Conclusion: Cancer incidence in twins is not always caused by known mutations. When disease had one of twins, the other female has a more higher risk for BC. It is not always caused by known mutations and can be an important model for the study of genetic aspects of BC.

Keywords: Breast cancer; Mutation BRCA1/2; Twins; Family tree; Lviv region (Ukraine)

Introduction
A frequency of twins is about 1/100 births in white populations [1]. Dizygotic twins (DZ) are the result of a double ovulation followed by the fertilization of each egg by a different sperm. While MZ (monozygotic twins) twinning rates are quite constant across populations, DZ twinning increases with maternal age until about age 40 years, after which the rate declines. The frequency of DZ twinning has increased dramatically in developed countries during the past two decades because of the use of ovulation-inducing drugs [2,3]. Thus, DZ twins are genetically no more similar than other siblings. Because two different sperm cells are required to fertilize the two eggs, it is possible for each DZ twin to have a different father [4,5].

Cancer is the leading cause of death in economically developed countries and the second leading cause of death in developing countries [6]. The burden of cancer is increasing in economically developing countries as a result of population aging and growth as well as, increasingly, an adoption of cancer-associated lifestyle choices including smoking, physical inactivity, and “westernized” diets [6,7]. It is well established that many major types of cancer (e.g., breast, colon, prostate, ovarian) cluster strongly in families [8,9]. This is due both to shared genes and shared environmental factors. About 1 in 8 (12%) women in the US will develop invasive breast cancer during their lifetime [10].

Heyn et al. using whole blood from 15 twin pairs discordant for breast cancer and high-resolution (450K) DNA methylation analysis, we identified 403 differentially methylated CpG sites including known and novel potential breast cancer genes [11].

Breast cancer (BC) is one of the most common malignant diseases in which incidence of cancer structure of the female's population ranked first, accounting for 19.6% in Ukraine. Over the last years more than 15,000 women has BC in Ukraine [12].

In Ukrainian population gene pool component is like in European population, the aim of our work was to analyse the most common mutations in the genes BRCA1/2 among twin women with BC who have burdened family history on this pathology.

Materials
We studied pedigrees and DNA samples 120 patients with family BC.

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Methods

Genealogy, molecular genetics

The material of our study were DNA samples from 120 patients diagnosed with familial BC who were treated in the Lviv Regional State Cancer Diagnostic Center from June 2008 to December 2012. Each woman signed an informed consent for molecular genetic analysis for determination of mutations in the genes BRCA1/2. Molecular-genetic method determined the presence of seven mutations in the gene BRCA1 (185delAG, 4153delA, 5382InsC, 1886del11, 5396 +1 G > A, 185InsA, 5331 G > A) and 3 gene mutations in BRCA2 (6174delT, 6293S> G, 6024delTA). Determination of mutations studied by allele-specific polymerase chain reaction (PCR), RFLP analysis (restriction fragment length polymorphism).

Genomic DNA (gDNA) was isolated from a total of 120 peripheral blood samples in 120 breast cancer patients with family history. DNA was extracted from peripheral blood lymphocytes using a standard phenol-chloroform extraction [13].

Search for mutations occurred by PCR analysis and melting point temperature Tm obtained amplicon [14]. PCR reaction was performed using reagents firms “Syntol” (Russia), which manufactures reagents with appropriate fluorystsentnymy dyes for analysis of thermal melting point Tm (ie, - dye EvaGreen). PCR reaction was performed in 0.2 ml tubes type of “Eppendorf” (Germany) termotsyklri ABI GeneAmp PCR System 9700 using a graded protocol. Scanning exons 2, 20 genes BRCA1 and BRCA2 gene exon 2 was performed using the method of analysis of the melting point temperature Tm obtained amplicon. The shift of the melting point indicating a change in the nucleotide sequence of amplicon. Point Tm analysis was performed with the aid of ABI 7000 Sequence Detection System (ABI, USA). Reading of fluorescence was performed on the channel SYBR Green dye on each of the intervals. The presence of Tm shift indicating the presence of mutations, mutation identification was carried out by restrict splitting.

The components of the reaction: H2O=14.2 μl, 10x PCR buffer (+ dye EvaGreen) = 2.5 μl, MgCl2 = 2.5 μl, dNTP = 2.5 μl, Taq pol=0.3 μl (20 pmol/μl)

Oligonucleotide primers: Forward=1 μl, Reverse=1 μl, DNA=1 μl

PCR reaction was performed in 0.2 ml tubes type of “Eppendorf” (‘Eppendorf’, Germany) termotsyklri ABI GeneAmp PCR System 9700 using gradient protocol: Amplification program:95°C - 10 minutes, 95°C - 10 sec, 65°C + 55°C 10 sec, 72°C - 10 sec, 95°C - 10 sec, 65°C - 10 sec, 72°C - 30 sec, 72°C - 2 minutes.

Scanning exons 2, 20 genes BRCA1 and BRCA2 gene exon 2 was performed using the method of analysis of the melting point temperature Tm obtained amplicon. The shift of the melting point indicates the change in the nucleotide sequence amplicon, requiring additional analysis identifying mutations.

Point Tm analysis was performed with the aid of ABI 7000 Sequence Detection System (ABI, USA) according to the following programs: 65°C - 2 minutes, 95°C - 5 minutes, 95°C→ 55°C at intervals of 0.5°C/sec. Reading of fluorescence was performed on the channel SYBR Green dye on each of the intervals. The presence of Tm shift indicates the presence of mutations, identification of mutations produced by restrict splitting.

Results

In Lviv region (Ukraine), according to the Statistical Office on January, 2011 population was 2,526,378 persons, including 1,332,405 women (52.7%). Every year 650-700 persons are found to have breast cancer, 99% of whom are women in the Lviv region. Standardized incidence of BC is 50.09 per 100 thousand female populations in Lviv region and slightly below average in Ukraine - 57.53 [15].

The familial aggregation of breast cancer has been recognized for centuries, having been described by physicians. If a woman has one affected first-degree relative, her risk of developing BC doubles. The risk increases further with additional affected relatives, and it increases if those relatives developed cancer at a relatively early age (before 45 years of age) [1,16].

Several genes are now known to predispose women to developing hereditary BC. As found more than 1000 mutations of genes BRCA1/2 [16] in various regions and ethnic groups of the spectrum and frequency of mutations in the genes BRCA1/2 are different [17-19]. Mutations 5382InsC (BRCA1) and 6174delT (BRCA2) were identified in families of patients with BC and ovarian cancer or prostate in Ashkenazi Jews. Mutation 5382InsC is responsible for approximately 2.5% of BC, but under “high risk” (family oncology anamnesis, bilateral BC at a young age), it is found in 10% of patients [18].

From 120 patients diagnosed with BC who were treated in the Lviv Regional State Cancer Diagnostic Center (Ukraine) from June 2008 to December 2012 mutations in the genes BRCA1/2 were found in 5 patients (4.2%).

We describe 3 pairs of twins where both sisters had BC and 2 pairs of twins where one of sister had BC. In 4 cases it were DZ twins, and in one - MZ twins. We study familial oncology anamnesis to the I-III degree relatives. Every one pare of twins had one or more cases of BC and another cancer in family tree. Familial aggregation of BC and another oncological disease has been recognized among these families (Table 2).

Case 1

In the younger sister K. of DZ twins’ left breast cancer (Figure 1) diagnosed in 2009 at age 45, T2N1M0, stage II B, histology- infiltrative ductal carcinoma. ER (-), PR (-), HER2neu (+++). Surgery – left radical mastectomy for Madden. Patient had received adjuvant chemotherapy. She is BRCA1/2 negative on 10 Slavic mutations. Menses are normal from 13 years, in 28 days continues 3 days. She has two healthy sons.

At the age of 42 years their mother died from the same disease. On the father’s line grandmother died at age of 70 years from cancer of visceral organs (table 2).

Elder sister of DZ twins is healthy. She is BRCA1/2 negative on 10 Slavic mutations too.
HER2neu (++), histology - infiltrative ductal carcinoma. Diagnosed in April 2010 at age 40, T1N0M0, stage I. ER (++), PR (+++), aunt (mother’s sister), aged 43 years. From throat cancer died her uncle pregnancy in 2000.

She has healthy DZ twins (daughter and son). She had ectopic mutation. Menses are normal from 14 years, in 28 days continues 4 days. She is unmarried.

HER2neu (-). Surgery – mastectomy. Menses are normal from 16 years, cancer T2N1M0, stage II, diagnosed in 2001 at age 37. ER (+), PR (+), radical mastectomy for Madden. She is diagnosed in 2009, aged 42 years, T2N1M0, stage II B. Surgery – right sectoral resection of left breast. She is healthy.

In the younger sister of twins right BC (Figure 2) was diagnosed in 2009, aged 42 years, T2N1M0, stage II B. Surgery – right radical mastectomy for Madden. She is BRCA1/2 negative on 10 Slavic mutation. Menses are normal from 14 years, in 28 days continues 4 days. She has healthy DZ twins (daughter and son). She had ectopic pregnancy in 2000.

From the same disease at age of 39 years died their mother and her aunt (mother's sister), aged 43 years. From throat cancer died her uncle (mother’s brother) at age of 45 years (Table 2).

Elder sister of DZ twins at the time of pedigree - healthy.

In the younger sister S. of MZ twins left BC (Figure 3) was diagnosed in 2012 at age 51, T2N1M0, stage II. ER (+), PR (+), at age of 39 years died their mother and her aunt (mother's sister), aged 43 years. From throat cancer died her uncle (mother’s brother) at age of 45 years (Table 2).

Elder sister of DZ twins at the time of pedigree - healthy.

In the younger sister of twins S. of MZ twins left BC (Figure 3) was diagnosed in 2012 at age 51, T2N1M0, stage II. ER (+), PR (+), at age of 39 years died their mother and her aunt (mother's sister), aged 43 years. From throat cancer died her uncle (mother’s brother) at age of 45 years (Table 2).

Elder sister of DZ twins at the time of pedigree - healthy.

In the younger sister S. of MZ twins left BC (Figure 3) was diagnosed in 2012 at age 51, T2N1M0, stage II. ER (+), PR (+), at age of 39 years died their mother and her aunt (mother's sister), aged 43 years. From throat cancer died her uncle (mother’s brother) at age of 45 years (Table 2).

Elder sister of DZ twins at the time of pedigree - healthy.

In the younger sister S. of MZ twins left BC (Figure 3) was diagnosed in 2012 at age 51, T2N1M0, stage II. ER (+), PR (+), at age of 39 years died their mother and her aunt (mother's sister), aged 43 years. From throat cancer died her uncle (mother’s brother) at age of 45 years (Table 2).

Elder sister of DZ twins at the time of pedigree - healthy.

Table 2: Results of mutations of genes BRCA1/2 and oncology pathology in pedigree among relatives in twins with breast cancer.

| Ca-Se (Fami-Ly) | Twins | Breast Cancer in Sister | Age | 10 Slavic Mutations of Genes BRCA1/2 | Mutations Of Genes BRCA1/2 Diagnosed in Another Country | Breast Cancer in Another Relatives | Age | Mother/ Father Line | Other Oncology Pathology | Mother’s Line | Father’s Line |
|-----------------|-------|-------------------------|-----|--------------------------------------|----------------------------------------------------------|-----------------------------------|-----|---------------------|------------------------|--------------|---------------|
| 1.K             | DZ    | younger 45              | negative | -                                   | mother 42                                                 | mother visceral cancer            | Grand mother | -                   |                        |              |               |
| 2.F             | DZ    | younger 42              | negative | -                                   | mother, aunt 39                                          | mother’s line pharyngeal cancer   | uncle        | -                   |                        |              |               |
| 3.V             | MZ    | both 40/41              | negative in younger sister | BRCA2 c.6405_6409delCTTAA (p.Asn2135fs) in elder sister | cousin, grandmother 30/60                          | father’s line mother’s line gastric, pancreas | -            | grandmother grandfather |                        |              |               |
| 4.L             | DZ    | both 37/43              | negative in both sisters | -                                   | -                                                       | -                                  | cancer uterus visceral cancer      | mother father    |                       |              |               |
| 5.S             | DZ    | both 47/48              | negative in younger sister | BRCA1 5382InsC in elder sister | grandmother 55                                          | mother’s line                      | -            | -                   |                        |              |               |

Case 2

In the younger sister F. of DZ twins right BC (Figure 2) was diagnosed in 2009, aged 42 years, T2N1M0, stage II B. Surgery – right radical mastectomy for Madden. She is BRCA1/2 negative on 10 Slavic mutation. Menses are normal from 14 years, in 28 days continues 4 days. She has healthy DZ twins (daughter and son). She had ectopic pregnancy in 2000.

From the same disease at age of 39 years died their mother and her aunt (mother's sister), aged 43 years. From throat cancer died her uncle (mother’s brother) at age of 45 years (Table 2).

Elder sister of DZ twins at the time of pedigree - healthy.

Case 3

In the younger sister S. of MZ twins left BC (Figure 3) was diagnosed in April 2010 at age 40, T1N0M0, stage I. ER (+), PR (+), HER2neu (+), histology - infiltrative ductal carcinoma. Surgery – sectoral resection of left breast. She is BRCA1/2 negative of 10 Slavic mutations. Menses are normal from 13 years, in 28 days continues 3 days. She is unmarried.

Elder sister of MZ twins had a mammogram done in September 2010, when she learned of her sister’s illness. Eight months later in January 2011, by bilateral breast cancer was attached older MZ twin sister at age 41. She lived in United Kingdom where BC was diagnosed. Histology-ductal carcinoma. A rare gene mutation BRCA2 c.6405_6409delCTTAA (p.Asn2135fs) was found. She has small daughter – aged 3 years.

Proband and her sister have cancer burdened family history. Their grandmother on mother's line died at age of 60 from breast cancer. BC was diagnosed in cousin on the father's line at the age of 30 years and in period of pedigree construction she has 45 years and feels satisfactorily.

Grandmother of father’s died at age 88 of stomach cancer, and grandfather died at the age of 72 years from pancreatic cancer (table 2). Elder sister of twins is healthy.

Case 4

The younger sister L. of DZ twins (Figure 4) with right breast cancer T2N1M0, stage II, diagnosed in 2001 at age 37. ER (+), PR (+), HER2neu (-). Surgery – mastectomy. Menses are normal from 16 years, in 30 days continues 5 days. Medical history - goiter, grade II, chronic cholecystitis. She has one healthy daughter.

After six years in 2007 her DZ twins sister at age of 43 was diagnosed the same disease. ER (+), PR (+), HER2neu (-), histology - infiltrative ductal carcinoma. Surgery – mastectomy. Menses are normal from 15 years, in 28 days continues 3 days. She has two healthy sons.

At the age of 65 years their mother died from the cancer of uterus, and grandfather on the mother's line died at the age of 75 years from cancer (Table 2).

Both sisters are BRCA1/2 negative on 10 Slavic mutations.

Case 5

In the younger sister Sh. of DZ twins left breast cancer (Figure 5) diagnosed in 2012 at age 51, T2N1M0, stage II. ER (+), PR (+), HER2neu (+). Surgery – radical mastectomy. She is BRCA1/2 negative on 10 Slavic mutations.

Menses is normal from 14 years (28 days continues 3 days) to 41. She is postmenopausal woman since 2002. Medical history- nodular uterine fibroma since 2011. She has healthy son.

At age of 40 her DZ twins sister was diagnosed ovarian cancer, at 48 aged – breast cancer. From the same disease at the age of 50 years she died in Canada. She was diagnosed mutation of the gene BRCA1 (5382 Ins C). Family doctor of eldest sister proposed our proband to go through molecular-genetic testing for BRCA1/2. Our patient is negative on mutation BRCA1 5382 Ins C and other 9 Slavic BRCA1/2 mutations.

Grandmother of mother’s line had BC at aged 55 (table 2).

In this study, in the pedigrees of all twins cancer affected relatives has been recognized. BC was affected in I-III relatives on both lines (mother's and fathers). Another oncological disease (visceral, pharyngeal, gastric and pancreas cancer) in I-II degree relatives was found.

This study is the first to use 10 Slavic mutations BRCA1/2 in twins with BC in Ukraine. Among 5 pairs of twins (10 women) - 8 of them had breast cancer (Table 1), only 2 were healthy (case 1 and case 2). All 8 women with BC of mutations of genes BRCA1/2 were tested. The 6 women with BC tested in Ukraine no 10 Slavic mutations BRCA1/2 were found. Difference in age at occurrence of BC in twins is low, which may indicate the genetic nature of the disease.

BC was diagnosed at age 37-45 in six females (3 pairs) and only in family S. - at age 47-48 years. In this family (case 5) younger woman was BRCA1/2 negative, but her elder sister who had OC and BC was BRCA1/2 positive. Elder sister from DZ twins that had had BRCA1 5382InsC tested in Canada died aged 50. Difference in age at occurrence of BC in twins is low, which may indicate the genetic nature of the disease.

Only one rare tested mutations of genes BRCA1/2 - BRCA2
In another two families with DZ (case 5) and MZ (case 3) twins BC affected in II degree relatives – grandmother on mother’s line. In cousin of MZ twins BC on father’s side was recognized (case 3) and also another kinds of cancer (gastric, pancreas) was founded on father’s line.

In families with both affected twins (cases 3-5) only II-III degree relatives were cancer affected.

Frequently of Slavic mutations $BRCA1/2$ were diagnosed only in 1 from 8 affected women from twins. It means that another mutation should be found in those families because of family tree with BC. In genetic counseling great attention should be pay to the twins, where one of them has a breast cancer because if a woman has one affected first-degree relative, her risk of developing BC is higher than in population. The risk increases further with additional affected relatives, and it increases if those relatives developed cancer at a relatively early age (before 45 years of age).

Good collected clinical and genealogical analysis are crucial for the purpose of molecular genetic research. Over the past decade, issues of etiology, early detection and prevention of breast cancer is closely associated with genetic counseling, which is aimed at studying the role of genetic susceptibility in the development of cancer pathology, its genetic heterogeneity and research in the field of molecular genetics.

**Conclusion**

From 120 patients diagnosed with BC who were treated in the Lviv Regional State Cancer Diagnostic Center (Ukraine) from June 2008 to December 2012 mutations in the genes $BRCA1/2$ were found in 5 patients (4.2%). We study pedigrees in 3 pairs of twins where both sisters had BC and 2 pairs of twins where one of sister had BC. Among 5 pairs of twins (10 women) 8 of them had BC and only 2 were healthy.

c.6405_6409delCTTAA (p.Asn2135fs) was diagnosed in elder sister with bilateral BC (case 3) in another country.

All women with BC had normal menstrual function. Menstrual function were without complications but stopped early due to surgical and chemotherapy treatment. Reproductive function was not affected.

Kim et al. to date, a meta-analysis on the effects of twin birth on the risk of maternal breast cancer has not been conducted [20]. In our two cases DZ twins (case1 and case 2) women with BC had I degree relatives with the same disease - mother. In the our analyses we found the expected increased risk associated with a relatives with BC on mother’s side opposed father’s line.
All 8 women with BC mutations of genes BRCA1/2 were tested. The 6 women with BC no 10 Slavic mutations BRCA1/2 were found. Elder sister from DZ twins had ovarian and breast cancer (tested BRCA1 5382InsC mutation positive) died aged 50. And a rare gene mutation BRCA2 c.6405_6409delCTTAA (p.Asn2135fs) was found in older sister of MZ twins at age 41 with bilateral breast cancer.

In all families with twins BC was diagnosed in I-III degree relatives. In families with both affected twins only II-III degree relatives BC was diagnosed. In families with BC great attention should be pay to the relatives, even in cases where no common Slavic mutation was found. The risk increases if affected relatives developed cancer at early age (before 45 years of age).

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