



**ROMANIAN ACADEMY
INSTITUTE OF BIOLOGY BUCHAREST**

SUMMARY OF THE PhD THESIS

**INFRASTRUCTURAL AND MOLECULAR
INVESTIGATION OF PARTICULAR ASPECTS IN
THE EVOLUTION OF BREAST CANCER**

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CONTENTS

Page

Introduction	3
Purpose, objectives and originality of the thesis	4
Structure of the PhD thesis	4
Materials and methods	4
Results of experimental studies and discussions	5
A. Mutation analysis of <i>BRCA1</i> and <i>BRCA2</i> genes	5
B. Mutation status of the <i>PIK3CA</i> gene	7
C. Mutational Analysis of the <i>TP53</i> gene	9
D. Polymorphic variations of the <i>TP53</i> gene	11
E. Ultrastructural particularities in tumor breast tissue	13
Conclusions	18
Bibliography	20
Summaries - Scientific papers published in the theme of the doctoral thesis	24

INTRODUCTION

One of the main directions currently pursued, both in Romania and around the world, is the attempt to individualize the treatment in order to increase the positive response to therapy. By identifying patients to whom therapy will have the expected results, it is desirable to avoid inadequate treatment or over-treatment and thus to remove the cytotoxic effects of the drugs.

Breast cancer is the cornerstone in personalized oncology therapy. The status of estrogen and progesterone hormone receptors and the status of the HER2 gene have been studied for many years because they are selection factors for patients to perform hormone replacement therapy or anti-HER2 therapy (Senkus et al., 2014). An important way in customizing treatment is the use of high-performance genomic analysis methodologies (such as Next Generation Sequencing / NSG) that can lead to a new characterization of tumor types and the extension of both the number of patients who can benefit from certain forms of therapy and increased response to treatment.

Breast cancer is the type of cancer that affects with the highest frequency, women around the world, this form of neoplasia being the first place in incidence, and the second on the cause of death. In Romania, the incidence of breast cancer has increased in recent years, with the number of cases doubling over the past two decades, with an estimated 51 new cases per 100,000 women and with 19% mortality rate, approximately 24 deaths per 100,000 women.

A less favorable aspect is represented by the fact that more than 50% of tumors are diagnosed late and therefore having a lower response to curative therapy. Among the factors involved in mammary tumor genesis are: gender, age, family history of disease, genetic factors, early menstruation or late menopause, nullity or age at which a pregnancy occurs, breastfeeding, obesity, increased stress, diet with food contaminated with xenobiotic substances, etc.

In terms of the hereditary factor, family susceptibility accounts for about 25% of all breast cancer cases. The genes with the greatest influence on the development and development of breast tumors are BRCA1 and BRCA2, and mutations at their level are responsible for 20% of familial mammary cancers. The TP53, PTEN and STK11 genes are also associated with an increased risk of developing breast cancer, mutations of which are identified in rare neoplastic syndromes of which Li-Fraumeni, Cowden and Peutz-Jegers can be remembered. Also, RAD51C gene mutations were associated with an increased risk for both breast cancer and ovarian caches (Balmana et al., 2014).

Although, most of the treatment schedules in the clinic are performed by immunohistochemistry, however, due to the multitude of cellular and molecular events, it is necessary to study in depth the mutational patterns of genes that are at increased risk for the appearance and development of breast cancer.

In recent years, due to the advancement of detection methods that have become more easily accessible and with shorter investigative times and scientific data in continuous improvement, personalized medicine has thus become a real therapeutic target based on both molecular investigations as well as on the in-depth knowledge of morphostructural aspects through immunohistochemical analyzes. For the same purpose, additional useful information on the infrastructure alterations of both tumor cells and tumor stroma obtained by electronic microscopy analyzes allow the clinician to approach an optimal treatment regimen for each oncological patient.

PURPOSE, OBJECTIVES AND ORIGINALITY OF THE THESIS

The purpose of this PhD thesis is to determine the mutational status of some genes involved in mammary tumorigenesis and to identify the particular features of mammary tumor cells. It is currently well known that mutations at the molecular level are involved in key biological processes that destabilize the body's functioning. In Romania it is necessary to study in depth the genetic spectrum of the oncological patient in order to establish a therapeutic treatment of precision.

The objectives of this study were:

- ✓ Establishing the mutational status of genes involved in breast cancer: PIK3CA oncogene, TP53 tumor suppressor and BRCA1 and BRCA2 tumor suppressor genes;
- ✓ Identification of cellular ultrastructural peculiarities in mammary tumor tissue by means of electronic microscopy in transmission.

The originality of the study consisted in: development and optimization of modern methods of genetic modification investigation: High Resolution Melting Analysis (HRMA), Sanger sequencing and Next Generation Sequencing (NGS); establishing the mutational status of tumor suppressors involved in familial breast cancer such as BRCA1, BRCA2 and TP53 as well as the PIK3CA oncogene which exhibit a high mutational frequency in mammary tumors; analysis of the mammary tumor tissue infrastructure in order to detect particular aspects. This study can contribute to a better understanding of molecular mechanisms, focusing on individualized therapeutic behavior in mammary carcinomas, increasing the response rate to treatment, and thus increasing the patient's life expectancy.

STRUCTURE OF THE DOCTORAL THESIS

The PhD thesis entitled "Infrastructural and molecular investigation of particular aspects in the evolution of breast cancer" contains 209 pages plus 22 pages of ISI articles published in the theme of the thesis and is divided into two parts: "Bibliographic background" and "Experimental part - own research ". The paper is structured in seven chapters and finally the dissemination of figures and tables from the paper and the scientific articles published or communicated both in the country and abroad both in the subject of the doctoral thesis and outside it.

The **bibliographic background** contains 61 pages representing 29.5% and contains the purpose, objectives and originality of the paper, the glossary of terms, generalities of anatomy and structure of the mammary gland, epidemiology and etiology data, as well as therapeutic strategies in tumors of breast.

The **experimental part** is expanded on 146 pages representing 70.5% and consists of the chapters describing the materials and methods used, the results and the discussions based on them, the conclusions obtained from the investigations carried out, the bibliography and the list of figures and tables, as well as the dissemination of the scientific papers.

MATERIALS AND METHODS

The biological material analyzed to determine the genetic risk factors involved in the appearance and development of breast cancers and some infrastructural characteristics consisted of 22 mammary tumor tissue specimens and 22 apparently normal tissue specimens, freshly harvested by surgical resection

from patients undergoing curative treatment at the Institute of Oncology "Prof. Dr. Alex. Trestioreanu ", from Bucharest, between 2012-2015. All biological samples were taken with informed consent of the patients, both in writing and verbally, thus complying with the rules laid down in the international ethics guidelines for biomedical research involving human subjects.

To determine the mutational status of the *PIK3CA* gene, we used three cell lines as controls: MCF-7 which is a mutated line on exon 9 and wild on exons 1 and 20, SKBR3 cell line used as negative control for exon 9 and T47D positive control for exon 20 and negative for exon 1.

For molecular biology investigations, DNA was extracted, purified and quantified from tumor tissue and apparently fresh normal breast tissue, as well as from cell lines taken as control in the study according to the working protocol specifications of the kit manufacturers used.

All methods used for molecular analysis have been optimized.

The molecular status of the *BRCA1* and *BRCA2* genes was determined by Next Generation Sequencing (NGS) using the Personal Genome Machine (PGM, ThermoFisher Scientific).

Mutation analysis of the *PIK3CA* oncogene was performed using the High Resolution Melting Analysis (HRMA) technique using the LightCycler480 Real-Time PCR System (Roche) instrument. Subsequent documentation of mutations identified in the *PIK3CA* gene was performed by direct sequencing, the Sanger method, using the 3500 Genetic Analyzer (ThermoFisher Scientific).

Regarding the mutagenic pattern of the *TP53* tumor suppressor, it was performed by direct sequencing, the Sanger method, using the 3500 Genetic Analyzer (ThermoFisher Scientific).

Infrastructural analysis of mammary carcinoma cells and tissues was performed by electron microscopy using the electronic microscope with transmission.

RESULTS AND DISCUSSIONS

A. MUTATIONAL ANALYSIS OF *BRCA1* AND *BRCA2* GENES RESULTS

In this paper we used the next generation sequencing to investigate the coding and non-coding regions of the *BRCA1* and *BRCA2* genes in eight patients diagnosed with sporadic mammary cancers.

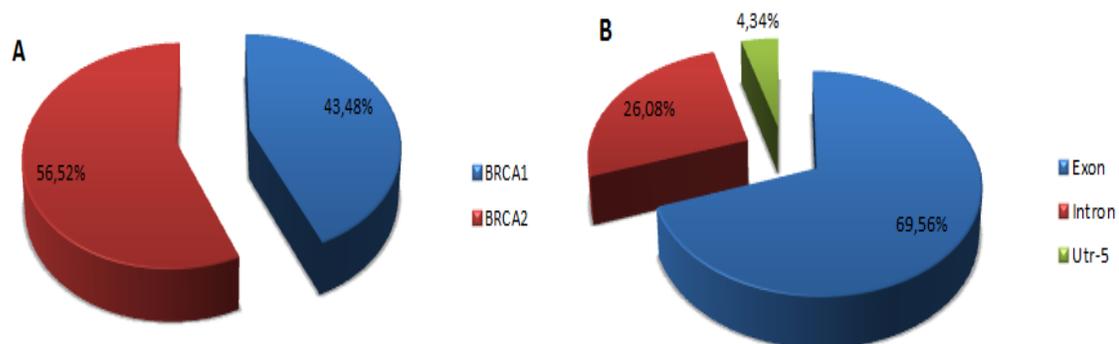


Fig.1. A - The distribution of polymorphisms in the both BRCA genes; B - Exon/Intron distribution of BRCA SNP.

Following sequencing we identified a number of 23 polymorphic variations, namely 43.48% in the *BRCA1* gene and 56.52% in the *BRCA2* gene (**Fig.1, A**). In the literature, three of these SNPs (rs144848, rs1799966 and rs16942) were assessed as having minor risk if taken independently but at increased risk of cancer when their activity is cumulative. A single mutation c. 10067C> G was detected in the *BRCA2* gene at exon 27. Regarding the polymorphism distribution, 69.56% of these were detected in the coding region of the two genes, and 26.08% affected the non-coding region of *BRCA* (**Fig.1, B**) and 4.34% was in the utr-5 region.

DISCUSSIONS

Hereditary breast tumors account for a total of 25% of all cases of mammary carcinomas. The *BRCA1* and *BRCA2* genes are at increased risk for the appearance and development of mammary tumors. *BRCA* mutations are distributed throughout the coding region but with a predominance of these at exon 11 in both genes. Typically, *BRCA* mutations are frameshift mutations or mutations that occur at the splice site resulting in truncated proteins (Mihalcea et al., 2017).

Regarding polymorphic variations, a concordance between *BRCA* mutation carriers and estrogen receptor status associated with breast tumors was observed in the general population. Therefore, polymorphisms associated with cancer risk with negative estrogen receptors (ER-) tend to be associated with mutations in the *BRCA1* gene, whereas tumors displaying positive estrogen receptors (ER +) tend to be associated with mutant carriers in *BRCA2*.

The polymorphic variant rs144848 (N372H) is the only one in the *BRCA2* gene that leads to amino acid modification, having an allele frequency of greater than 10%. There is little data in the literature about the function of this polymorphism, but it is known that the substitution of an asparagine residue that is a neutral amino acid with a residue of a basic amino acid, in this case histidine, affects both the structure and functionality of the *BRCA2* gene modification takes place in a region responsible for histone acetyltransferase P / CAF interaction just before the start of the transcription process of other genes. In the present study, following *BRCA2* gene sequencing, we have identified at the level of exon 9 the polymorphic variation rs144848 (p.Asn372His, c.1114A> C) which is a transversion A → C at position 372 from the protein level. We detected this SNP in 37.5% (3/8) of the mammary tumor specimens analyzed.

Research that has been done on cell lines of lymphocytes B, lymphoblastoid cells and cells extracted from breast tissue, have concluded that the *BRCA1* gene has differentiated allelic expression. The same finding was observed and in some cases associated with the increased risk of breast cancer and ovarian cancer (Chen et al., 2008, Maia et al., 2009, Shen et al., 2011). Available databases revealed that the polymorphic variations of the *BRCA1* gene, including SNP rs16942, were associated with a high expression of the *brca1* protein (Dimas et al., 2009).

Two of the polymorphic variations of the *BRCA1* gene, namely rs16942 (K1183) and rs1799966 (S1613G) having a major allele frequency of more than 10%, are in a strong binding imbalance leading to a homozygous genotype with at least four different alleles. Sequencing identification of all variants that have small individual cancer risks analyzed but which have an increased cumulative effect is complementary to the genome sequence sequencing (Johnson et al., 2007). We identified SNP rs1799966 (pSer1634Gly, c.4900A> G) in exon 16 of the *BRCA1* gene which is a transitions A → G at position 1634 of the protein. The CC homozygous form of

SNP rs1799966 was detected in a single sample of 12.5% (1/8) whereas the heterozygous TC pattern was identified in 50% (4/8) of the investigated samples.

Following gene expression studies performed on murine epithelial mammalian cells, it has been observed that regulating the expression of the *brca2* protein is closely related to the expression of the *brca1* protein and that this regulation process occurs during cell proliferation and differentiation. Regarding the mutagenic spectrum of *BRCA1* and *BRCA2* genes, we can say that it is complex because of the variants that affect both the exon regions and their intronic regions. Among *BRCA* mutations that are thought to have pathogenicity, frameshift (insertions / deletions) mutations and "nonsense" mutations affecting STOP codons, mutations that lead to change in the genomic structure (Tommasi et al., 2012). In the present study we identified a single "nonsense" mutation c.10067C> G (pSer3356Ter) in the *BRCA2* gene at exon 27 and representing a C → G transversion at position 3356 of the protein where a serine residue is replaced with a STOP codon.

B. MUTATION STATUS OF THE *PIK3CA* GENE RESULTS

The sensitivity of the HRM method was tested by making a mixture of DNA extracted from tumor cell lines (T47D and MCF-7) and DNA extracted from normal cell lines in percent of 50%, 25%, 12%, 6%, 3% and 1%. In Exon 9 we were able to detect 3% mutated MCF-7 cells (**Fig. 2**), while for exon 20 the detection limit was 1% of mutated T47D cells from a wild DNA mixture (**Fig. 3**).

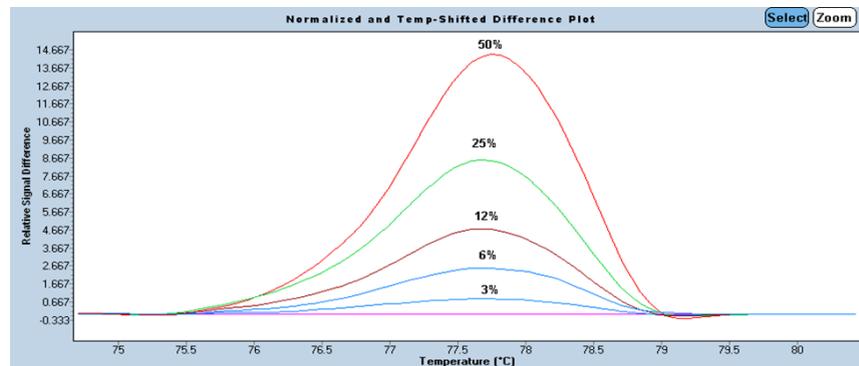


Fig.2. The profile of the sensitivity curves for exon 9 of the *PIK3CA* gene.

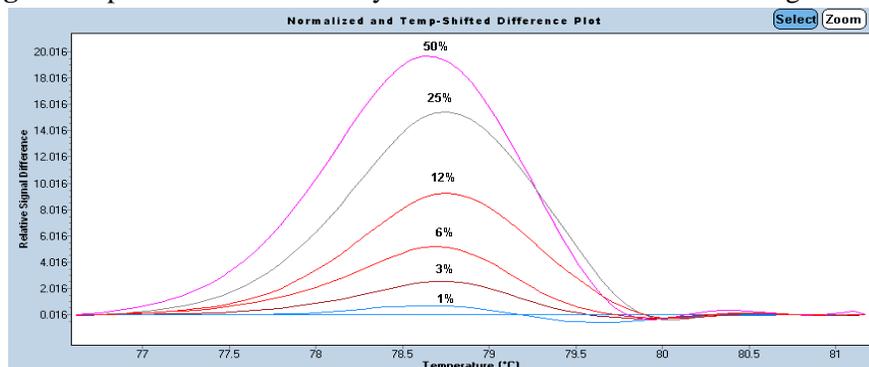


Fig.3. The profile of the sensitivity curves for exon 20 of the *PIK3CA* gene.

The mutation pattern of the *PIK3CA* gene was achieved by analyzing the profiles of the dissociation curves and the melting peaks of exons 1, 9 and 20 using the HRMA method and subsequently documenting them by direct sequencing, the Sanger method.

The mutation rate in the group of 22 mammary tumors analyzed was 36.4% (8/22) of which 13.64% (3/22) were detected in exon 9 of the helical domain and 22.73% (5 / 22) in exon 20 of the kinase domain of the gene (**Fig. 4**). The identified mutations were: Q546 [K, E] and E542 [E, K], which affected the helical domain of *PIK3CA* and H1047 [H, R], which affected the kinase domain of the gene. Exon 1 did not identify mutations.

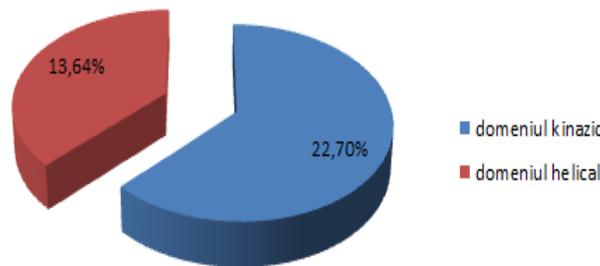


Fig.4. Frequency of mutations in the *PIK3CA* gene.

DISCUSSIONS

The PI3K pathway is one of the most extensively studied signaling pathways due to its oncogenic implications in the vast majority of neoplasias, breast cancer being considered to be strongly influenced by the PI3K pathway. Research has shown that there is at least one molecular mechanism involved in this signaling pathway, one of the most important being mutations in the *PIK3CA* gene. *PIK3CA* mutations were detected for the first time in 2004 following molecular investigations on solid tumors, and since then, numerous studies have been conducted that have highlighted the therapeutic and prognostic implications of mutations of this oncogene (Mukohara, 2015). The oncogene *PIK3CA* is the second most frequently mutated in breast cancer after the *TP53* tumor suppressor gene, the mutations detected at its level having a frequency rate of 20% to 40%. *PIK3CA* mutations represent an early event which has led to the idea that they are involved in initiating the cancer process rather than in invasive propagation. It has been observed that in breast cancer mutations that affect genes other than *PIK3CA* downstream of the tyrosine kinase receptor are rare events compared to other malignancies.

Somatic mutations occurring primarily in the helical and kinase domains, respectively, are three hotspots, namely E542 and E545 in exon 9 and H1047 in the exon 20 of the *PIK3CA* gene. Positions in which these mutations occur have suggested that the mutated protein exhibits intense kinase activity, also having oncogenic properties (Zardavas et al., 2014).

Our study was conducted to determine the frequency of *PIK3CA* mutations in breast tumors, and in this regard 22 pairs of tumoral and apparently normal tissue fragments were collected from patients diagnosed with mammary tumors and operated at the Institute of Oncology Bucharest. As controls in the study we used cell lines MCF-7, SKBR3 and T47D (Mihalcea et al., 2015).

PIK3CA mutations affect three domains of the protein, namely the C2 domain, the helical domain, and the kinase domain respectively. The mutations appearing in the C2 domain are located at the surface of the protein loop increasing the membrane-binding capability of the p110a catalytic subunit. In the helical domain three hotspots (E542, E545, Q546) appear on the external face of the domain, with the possibility of interacting with another protein. Mostly in this area a lysine is inserted, thus concluding that it could even interact with the cell membrane. Following the analysis of exon 9 of the *PIK3CA* gene we identified two mutations: a rare mutation Q546K (c.1636C> A) detected in 9.09% of the samples (2/22) resulting from the substitution of glutamine with lysine at position 546 and E542K mutation (c.1624G> A), also a lysine glycine substitution at position 542. Kinase domain mutations occur adjacent to the protein activation loop, thus influencing both the mobility and the position of this loop (Gymnopolous and colab., 2007).

The H1047R mutation that affects the kinase domain of the *PIK3CA* gene induces cancerogenesis in epithelial cells of lumbar breast cancer, leading to the idea that this mutation is an early event of breast cancer. Tumors with H1047R mutations have variable sensitivity to PI3K inhibition and also have a variable dependence on other oncogenes. In the kinase domain, at the level of *PIK3CA* gene exon 20 we identified the H1047R (c.3140A> G) mutation detected in 22.73% of the samples (5/22) by HRM analysis and subsequently confirmed by direct sequencing, being a substitution at position 1047 of a histidine residue with an arginine residue. Because the frequency of mutations in this oncogene is relatively similar in in situ ductal carcinomas and ductal carcinomas, somatic mutations affecting the *PIK3CA* oncogene can be considered to be early events in mammary carcinogenesis facilitating the transformation of normal breast cells into tumor cells (Miller, 2012).

C. MUTATIONAL ANALYSIS OF THE *TP53* GENE RESULTS

To determine the mutational pattern, DNA samples were amplified with exon-specific primers for exons 4-9 of the *TP53* gene. The purity and concentration of amplicons obtained after the Polymerase Chain Reaction (PCR) was performed by 2% agarose gel electrophoresis (Fig. 5).

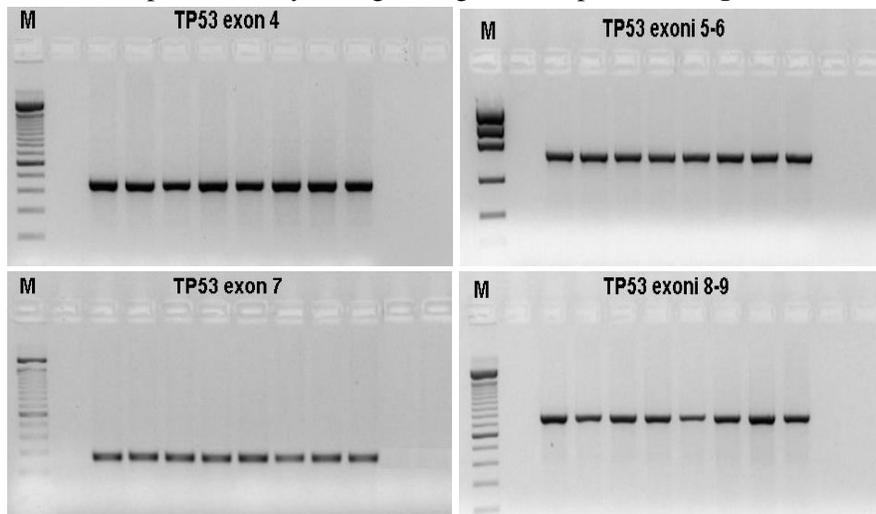


Fig.5. Agarose gel electrophoresis 2%. Determination of amplicon concentration of exons 4-9 of the *TP53* gene using the *Low DNA Mass Lader* molecular marker.

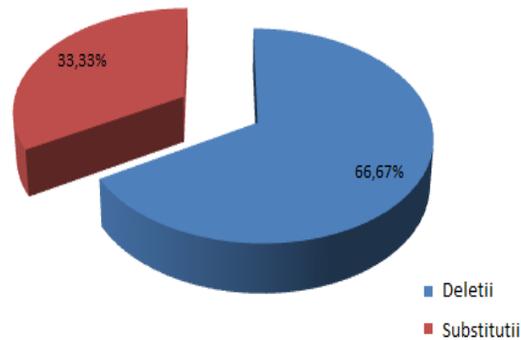


Fig.6. Frequency of mutations in the *TP53* gene.

The *TP53* mutation rate in the investigated group was 27.3%. In the 22 mammary tumor tissue samples, were identified six mutations, of which 2 (33.33%) were substitution mutations (a "missense" mutation R175H, c.524G> A in exon 5 and a "nonsense" mutation pR213 *; c.637C> T in exon 6). Both substitutions were heterozygous. There were also identified 4 (66.67%) deletion mutations (one deletion of two nucleotide bases CC c.213 214del2 in exon 4, a frameshift deletion of a single nitrogen base T p.M133fs * 37, c.398delT in exon 5, a nine-nucleotide AACCGGAGG "inframe" type, p2424-R249 delNRR, c.739 747delAACCGGAGG in exon 7, and a single "frameshift" deletion p.G244 -fs * 3, c.731delG, also in exon 7). A predominance of 66.67% deletion mutations was observed, while only 33.33% represented substitution type mutations (**Fig. 6**).

Following analysis of the distribution of mutations in the *TP53* gene, it was found that 5/6 mutations (83.3%) had the DNA binding domain localization. Of these, 2 (33.3%) were identified in exon 5, 1 (16.6%) was identified in exon 6 and 2 (33.3%) in exon 7 of the gene. Exon 4 also identified 1 (16.6%) mutation.

DISCUSSIONS

The *TP53* tumor suppressor encodes a transcription factor with antiproliferative role that is activated in response to several forms of cellular stress. Somatic mutations occurring in this gene are extreme events that are common in many types of human cancers (20-40% in breast cancer) and hereditary patterns predispose the early appearance of a wide range of neoplasms such as Li-Fraumeni syndrome (LFS) and Li-Fraumeni-like syndrome (LFL). In the *TP53* gene, the majority mutations are "missense" substitutions (75%) followed by "frameshift" insertions and deletions (9%) and last but not least "silent" mutations (Petitejean et al. , 2007). In the present study, six mutations were identified, one missense mutation (16.67%), one nonsense mutation (16.7%) and four deletions (66.67%).

Most *TP53* mutations (90%) are identified in the protein binding domain thereby disrupting its ability to bind the DNA molecule. The mutations appearing here can induce partial or total loss or gain of monomer function, and thus the stability of the *TP53* gene is very important for the proper functioning of the protein. Consistent with the literature, five of the mutations identified in this gene affected the protein binding domain and only one was in another domain (Vegran et al., 2013; Murnyak et al., 2016).

The initiation of the metastasis process has phenotypically similar features to the epithelial-mesenchymal transition process (EMT), including growth of cellular motricity and loss of intercellular adhesion. Data from the literature have shown that the wild-type form of *TP53* inhibits the EMT process

while the mutated form of the tumor suppressor facilitates EMT by its involvement in the proper functioning of the transcriptional regulators of this process, SLUG and TWIST1. Another pathway by which the *TP53* mutant promotes cell proliferation is TAp63 inhibition thus leading to TGFβ-mediated metastasis and stimulation of integrin activity, thus triggering cellular invasiveness. Another effect exerted by *TP53* mutations on tumor progression could be the positive regulation of angiogenesis with the observation that *TP53* wt tumors tend to be less vascularized. Taking into account all of these phenomena, one arrives at the idea that mutations in the *TP53* gene are events that either appear late in the cancer process or may have an important significance in advanced stages of cancer in terms of tumor invasiveness and aggression (Kato et al., 2003).

In human cancers, predominate the missense mutations that affect amino acids 102-292 of the DNA binding domain, the most important hotspots being found at codons R175, Y220, G245, R248, R249, R273 and R282. The classification of mutations in this gene was made basis of its function as a transcriptional factor and two categories can be distinguished: a) contact mutations (R273H and R248W) occurring at the interface between the p53 protein and the DNA molecule, and b) structural mutations (R175H, Y220C, G245S, R248Q, R249S, R282W, etc.) leading to the conformational instability of the p53 protein. Taking into account the R175H mutant allele, an increased rate of spontaneous tumors such as lymphomas and sarcomas was observed. Following the investigation of exon 5 of the *TP53* gene, we identified the heterozygous substitution c.524G> A (R175H), where Arg is replaced by His, CGC> CAG in the hotspot codon 175, this mutation being responsible for modifying the structure of the DNA domain of the protein binding (Xu et al., 2014).

A significant part of the *TP53* mutations are "nonsense" and give birth to truncated and inactive proteins. The most common "nonsense" mutation and even more common than many other "missense" substitutions identified in the *TP53* gene is R213 *. In exon 6 of the *TP53* gene we detected the nonsense heterozygous mutation c.637C> T (R213 *), where Arg is replaced by the stop codon Ter, CGA> TGA in position 213. Repair of such a mutation obviously requires other mechanisms than those for missense mutations.

In the mammary tumor samples analyzed in this thesis we identified 4 deletions: 1) c.213 214del2 which is a double deletion of nucleotide CC which affects codons 71 and 72 of exon 4; 2) c. 398delT (p.M133fs * 37), frameshift deletion of the T nucleotide at position 133 of exon 5; 3) c.739 747del AACCGGAGG (p.2247-R249delNRR) 9-nucleotide & quot; inframe & quot; deletion that affects codons 247, 248 and 249 of exon 7; 4) c.731delG (p.G244-fs * 3), frameshift deletion of nucleotide G at position 244 in exon 7. Except for deletion from exon 4, all other deletions affected the DNA binding domain.

Frameshift deletions lead to modifying the reading frame of DNA sequence with obtaining of truncated, sometimes short, sometimes extremely long and most likely non-functional proteins. These vary in length from 1 to 37 nucleotides, but the most common are those with the removal of 2-8 nucleotides (Angelopoulou et al., 1998).

D. POLYMORPHIC VARIATIONS OF THE *TP53* GENE RESULTS

Following the direct sequencing of both coding and non-coding regions of the 6 exons investigated in this study, 5 polymorphic variations of a single nucleotide were identified, of which 3 affected the coding region of exons 4 (rs1042522, R72P and rs372397095, P82P) and 6 (rs1800372, R213R), and 2 were identified in intron 6 (rs1625895 and rs17880604) (**Table 1**).

Table 1. Polymorphic variations identified in the *TP53* gene

Patient no.	Exon/Intron	SNP	Genotype	The ancestral allele	The mutated allele	Amino acid
1	Exon 4	rs1042522	16397C>G	C	G	Pro→Arg
2	Exon 4	rs372397095	16428G>A	G	A	Pro→Pro
3	Exon 6	rs1800372	17659A>G	A	G	Arg→Arg
4	Intron 6	rs1625895	17754A>G	A	G	-
5	Intron 6	rs17880604	18225G>C	G	C	-

DISCUSSIONS

The activity of the p53 protein is regulated by several mechanisms that influence both post-transcriptional processes as well as translational and post-translational processes, all in response to a wide range of physical and biological events. Currently, about 100 *TP53* gene polymorphisms are known, some of them varying according to geographical areas, population density and ethnicity (Sagne et al., 2013).

It is well-known that apart from the high frequency of somatic mutations, the *TP53* gene also has a high polymorphic degree. In cancer, in the coding sequences, the mutations of the *TP53* gene, and in particular the substitutions of a single nitrogen base called polymorphisms, can have devastating effects on the proper functioning of the p53 protein. However, it is assumed that only a small percentage of these polymorphisms of the *TP53* gene can have noticeable effects that can be measured (Whibley et al., 2009; Naccarati et al., 2012).

The most studied *TP53* gene polymorphism is rs1042522 (R72P; 16397C> G) identified in exon 4 in a proline-rich region. This polymorphism is a G → C transition of a nucleotide at position 215 of the gene and contains at the level of codon 72 of the TP53 gene either proline (CCC) or arginine (CGC) resulting in cancer predisposition. Some studies have shown that the homozygous genotype of arginine may be an increased risk factor in breast carcinomas and has long been studied in many cancers such as stomach, bladder, breast, colon and lung cancer, a broad distribution based on ethnicity and demographic area. In our study, the polymorphic variation rs1042522 was identified with homozygous GG genotype in 59.09% (13/22) of the samples, and with the heterogeneous CG genotype in 40.9% (9/22) of the samples. Because R72P affects the transactivation domain, it can lead to alteration of the protein expression. It can also influence both transcriptional activity and protein structure due to localization in a hydrophobic zone of the affected codon (Melo et al., 2009).

In exon 4 we identified SNP rs372397085 (P82P, c.246G> A), a synonym polymorphism that represents a G → A substitution at codon 246, CCG → CCA, located in a proline-rich region. There is no literature data on the influence of this polymorphic variation on the risk of breast cancer.

Studies have shown that the cumulative analysis of several tumor suppressors does not indicate a significant percentage of synonymous mutations, but an individual *TP53* gene analysis shows a significantly increased synonymous mutation rate. These are extremely recurrent compared to those found in oncogenes and directly affect nucleotides located at the alternately splice sites, with three such nitrogen bases being affected in *TP53*. SNP rs1800372 (R213R, c.639A> G) is a rare synonymous variation that we have identified in the coding region of exon 5. It is a substitution at position 639 where arginine is also

replaced by arginine, CGA → CGG. It seems that despite the increased rate of these types of mutations, however, they have no clinically significant effect (Supek et al., 2014).

It has been observed that the genetic variations of the *TP53* gene are associated with the occurrence of breast cancer at younger age (Sprague et al., 2007). In our study we identified SNP rs1625895 in both heterozygous (AG) and homozygous (GG) forms, representing an A → G transition in the non-coding region of the gene. Rs1625895 represents the substitution of a single nucleotide at position 61 of the *TP53* gene in intron 6 and although it is still unknown whether it is a regulatory or splice factor and it has been reported that it can affect the level of protein expression but also affect the activity of the protein by modifying its secondary structure (Voropaeva et al., 2015).

Although the rs17880604 (G13964C) polymorphism is one of the most studied intronic SNP in the *TP53* gene but no correlation between it and breast cancer susceptibility has been observed. Some studies have shown that variant 13964G helps maintain the stability of both messenger RNA and stability of p53 protein function. Following the investigation of exons 5-6 of the *TP53* gene we identified the transition G → C at the level of intron 6 in both heterozygous (GC) and homozygous (GG) forms of the SNP rs17880604. Variant 13964C was analyzed in the context of establishing its role in a wide range of human cancers, such as breast cancer, colon and ovarian cancer, but insufficient data were obtained to associate this type of cancer risk with polymorphism (Dehghan et al., 2015).

E. ULTRASTRUCTURAL PARTICULARITIES IN BREAST TUMOR TISSUE RESULTS

Cito-histoarchitecture of invasive ductal breast tumors

Some of the results of the electron microscopic investigations analysis on the identification of particular infrastructural aspects in breast tumors are focused on the invasive ductal carcinoma tumors and have been partially published as two scientific papers in the Romanian Journal of Morphology and Embryology: et al., RJME 56 (4): 1371-1381, 2015 and (2) Mihalcea et al., RJME 58 (2): 445-455, 2017.

In the electronic microscopy analysis of mammary tumor tissues, drastic changes were observed in the histological structure of the mammary gland. In tumor cells from invasive ductal carcinoma, the ratio of nucleus to cytoplasm is increased. Eucromatine predominates in the nucleus of tumor cells. Heterochromatin is visible as a lyser attached to the inner membrane of the nuclear envelope (**Fig.7**). As a rule, nuclei display nucleoli of relatively large size.

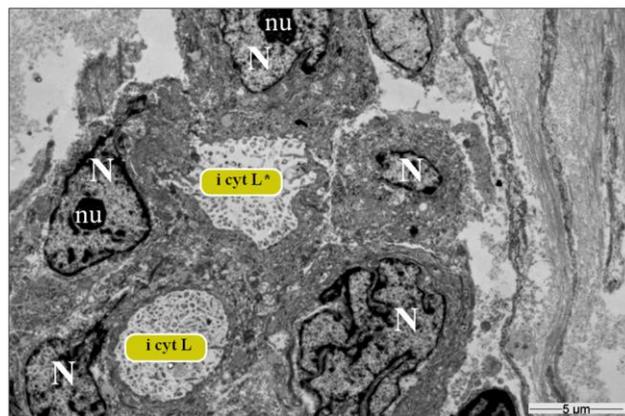


Fig.7 - Neoplastic breast tissue represented by tumor cells with very large nuclei (N) compared to the cytoplasm. The nuclei are almost entirely represented by eucromatine, and some nuclei have large nucleoli

(not). The intracellular lumens (i cyt L) show numerous microvilles. One of the lumens communicates with the extracellular space (i cyt L *). Taking over with the permission of the RJME publisher (Mihalcea et al., 2015).

Cytoskeleton

In the cytoplasm of tumor cells examined, the cytoskeleton is poorly represented. Sometimes cytoskeletal elements with electronmicroscopic appearance of intermediate filaments appear in the form of conglomerates. As a rule, cytoskeleton occurs in the form of finely distributed non-oriented filaments in the cytoplasm (**Fig.8**), probably due to the absence of desmosomal junctions.

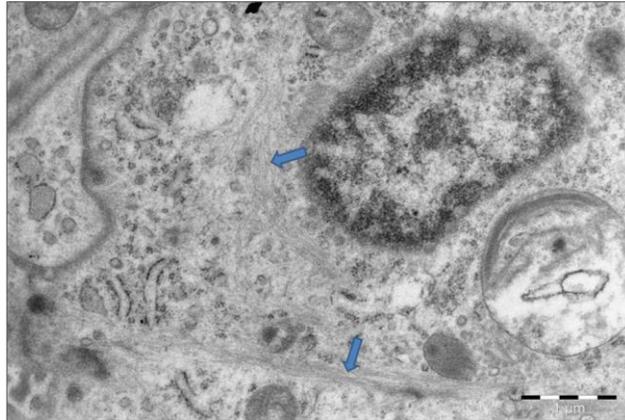


Fig. 8 - Tumor cell sector (invasive ductal breast carcinoma) in which non-oriented distributed cytoskeletal filaments (arrows) are distinguished as desmosomal intercellular junctions are missing.

Intercellular junctions

Similar to other types of epithelial tissues (epidermis, epithelium of the digestive tract, etc.), the epithelium of the unaltered mammary glands is represented by epithelial cells with a distribution characteristic of the histological pattern of breast tissue. This implies that the epithelial cells will have a certain morpho-structural polarity which is mainly due to the existence of specialized junctions that force the cells to remain stable with the adjacent epithelial cells as well as basal membrane-like anchoric structures through their basal pole. Maintaining the polarity of epithelial cells is an essential condition for normal mammary gland physiology.

Analyzes of breast tumor tissues by electron microscopy revealed a gradual alteration of desmosomal junctions, depending on the degree of evolution of mammary tumors, the most dramatic being those recorded in invasive ductal breast tumors. We note here our observation that internalized desmosomes are sometimes detected electronmicroscopically (**Fig.9**).

It should be noted that the aberrant infrastructure aspect of these junctions, which implies an abnormal distribution of molecular components versus the very precise distribution of the various molecules that make up the desmosomes with normal infrastructure (Mirancea et al., 2001; Mirancea and Mirancea, 2010), corroborated by the lack of filament contact cytoscheletics to aberrant desmosomes suggest that in the case of tumor cells the intercellular junctions are precarious. Often, only remnant forms of desmosomes, imperfect forms, are detected.

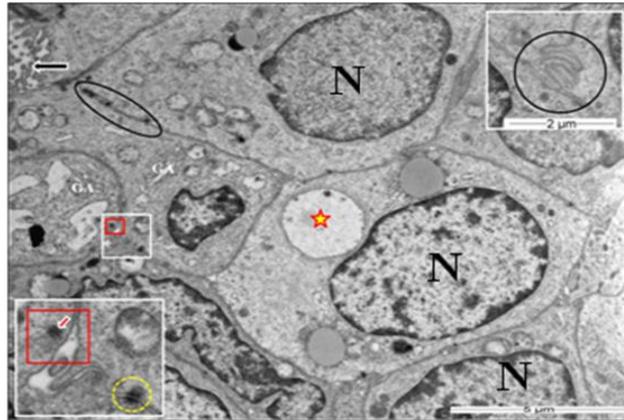


Fig. 9 - Tumor cells without intercellular spaces with large polymorphic nuclei (N) can be observed. Desmosomal junctions are rare (the elliptical area and the yellow circle at the bottom left). Interdigital junctions (in the top right square) are detected. In the red bowl square an internalized desmosom, detailed in the lower left part of the insert (arrow), can be detected. GA - Golgi Apparatus. Taking over with the permission of the RJME publisher (Mihalcea et al., 2015).

Tumor-stromal peritumoral interface

Junction of basal membrane - epithelial cells

The basal membrane, also called the basal lamina (questionable terminology), is an anhist structure that is made up of specific molecules secreted, mainly by both the epithelial cells and the adjacent connective cells. In the case of highly differentiated epithelials such as the epidermis, an unbroken basal membrane of uniform thickness is inserted at the epithelium-connective tissue interface (mesenchymal origin). Such a basal membrane, in the electronmicroscopic examination, distinctly presents a more electronodensive component known as the dense lamina and a weaker electronodensity component called lamina lucida. The application of the microelectronics microscopic investigation method allows for a particularly fine analysis of the spatial distribution of the different molecules that make up the basal membrane (Mirancea et al., 2001).

Epithelial cells of the normal mammary gland have a polarized morphology. Their basal pole is accompanied by a distinct basal membrane. Most often, at the adjacent stromal tumor cell interface, the basal membrane is absent, or small paths from this infrastructure can sometimes be identified.

Invasiveness. Invadopods. Disseminated membrane vesicles (Shedding membranes vesicles)

Tumor invadopods penetrate deep into the peritumoral stroma (**Fig.10**). Shedding membrane vesicles can be released via the invadopod. Close examination of the interface between the tumor cells and the adjacent stroma reveals various aspects of the biogenesis and microevrease release of malignant cells.

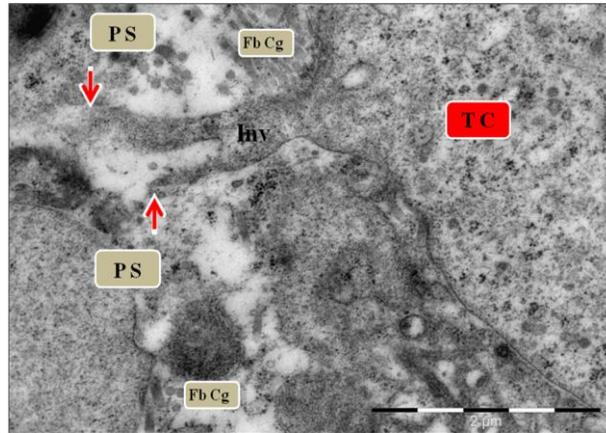


Fig. 10 - An invadopodium (Inv) is dichotomised (red arrows) and penetrates strongly into the peritumoral (PS) fibrous layer. No basal lamina is distinguished around the tumor cell (TC). Fb Cg: fibrillar collagen. Taking over with the permission of the RJME publisher (Mihalcea et al., 2015).

Microvasculature

In most cases, capillaries with collapsed lumen can be identified as a result of burrowing the nucleus and cytoplasm of the endocytes into the capillary lumen that it occupies almost completely, the lumen being detectable in the form of a narrow slit. The endothelial wall of the blood microvasculature is represented by endothelial cells with large nuclei oriented to the capillary lumen. Endothelial cells are interconnected by tight junctions. Often the hazards are absent. In addition to the usual basal membrane surrounding the endothelial wall, we can see redundant basal membranes and a lot of amorphous material. It should be noted that telocytes also occur in the proximity of the capillary vessels but without contact with pericardium or with endothelial cells (**Fig.11**), which obviously contrasts with the situation in the normal mammary gland. An interesting aspect is the phenomenon of danger displacement through the abnormal distribution of redundant basal membranes or the infiltration of an amorphous material between the perils and the capillary wall.

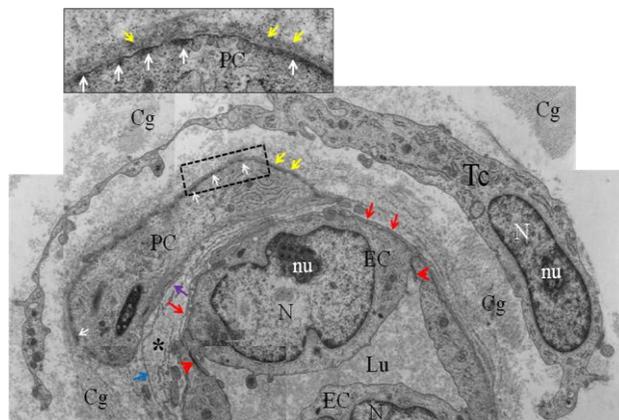


Fig. 11 - Endothelial cells (EC) have large nuclei (N). A pericardium (PC) partially detached from the endothelial wall by redundant basal membranes (red, pink and blue arrows) and an increased amount of amorphous material (asterisk). Dense sub-plaque tiles from the inner face of the pericardial plasmids (white arrows) are visible (see details in the upper bay where the yellow arrows mark the basal membrane associated with the pericardium). Also, a telocit (Tc) is detected in close proximity to a blood capillary,

which does not come into direct contact with endothelial cells or pericardium. N: Nuclei; no: Nucleoli; Lu: Lumen. The red arrow heads mark interendothelial junctions. Cg: Collagen. Taking over with the permission of the RJME publisher (Mihalcea et al., 2015).

Telocytes

Beside to normal stromal cells from the breast like fibroblasts, adipocytes, other types of cells, such as mast cells and telocytes, can be detected. Sometimes, two come in contact one with another by realizing the so-called homocellular synapses.

Since the telopods that representing the extensions characteristic of the telocytes are extremely thin, the chance of having the entire length of the telocitar body together with the telopods is very rare. With the exception of cell cytoplasm (where the nucleus is located), rich in the rugged endoplasmic reticulum and in the Golgi apparatus (not shown here), the rest of the cytoplasm has a low cytomegalovirus content, but with many ribosomes, the endoplasmic reticulum both smooth and smooth, microfibrils belonging to the cytoskeleton and calves. It should be noted that besides telocytes located in normal stromal tissues, in all invasive breast carcinoma specimens investigated by us, telocytes had a remarkable limitation of the number of mitochondria. In addition, it should be noted that some telopads are involved in the processes of membrane microvehicle elimination. Often, two or more pieces of the telescopes represented by the alternation between podomi and podomeri go together very close together, without creating any direct contact between them.

DISCUSSIONS

Using transmission electron microscopy, were examined invasive tumors of mammary carcinomas and we observed that the neoplastic cells represented by the glandular epithelium exhibited a high nucleotide / cytoplasmic ratio. The nuclei are polymorphic. In addition, there is a high frequency of atypical mitosis. Often, 1 or 2 centrioles, sometimes even 3 centrioles in a malignant cell, have been detected in tumor cells, suggesting the process of multiplying tumor cells. Eucromatine is predominant while heterochromatine is mostly attached to the inner membrane of the nuclear envelope. This is a general feature of the nucleoplasm in tumor cells. It is believed that the nuclear structure itself can modulate the cell phenotype and tissue type. Eucromatine is easier to transcribe compared to transcriptionally silent heterochromatin (Bissell et al., 1999; Tamaru, 2010; Mirancea et al., 2010; Mirancea et al., 2013).

Epithelial cells of normal breast are polarized cells. In order to maintain their polarity, these cells must maintain relatively cell-cell and extracellular matrix-cell interactions. Thus, specialized cell junctions are developed and must remain unchanged in their molecular infrastructure and composition. In order to maintain its 3-D structure and communication with neighboring cells in the epithelium, specialized junction adhesion structures for cell-cell interactions and extracellular matrix cell have to be well developed. Intercellular cohesion is supported by the junctional complex, represented in the vast majority of cases of desmosomes. By anchoring the desmosomal cadherins of adjacent cells to the intermediate filaments cytoskeleton, desmosomal junctions contribute to the organization and maintenance of histoarchitecture (Kimura et al., 2007; Stahley et al., 2014). Investigation by electron microscopy of transmission and immunoelectron microscopy of desmosomes has revealed that specific infrastructures

and molecular components are highly localized in the extracellular, transplasmal and intracellular domains (Mirancea et al., 2010; Mirancea et al., 2001).

Through invadopods, tumor cells generate and spread membranous vesicles, including exosomes, within the peritumoral stroma. Among the particularities detected at the infrastructure level are telocytes, which represent a new interstitial / stromal cell phenotype that is considered to have key functions in the cell signaling process, showing a small number of hetero-cellular interactions, suggesting that it could disrupts tissue homeostasis modulation (Mihalcea et al., 2015).

Often, the basal membrane is absent at the stromal tumor peritumoral interface. It is well known that basal membrane integrity is an essential condition of epithelial morphogenesis during embryonic development, which regulates epithelial-mesenchymal interactions (Mirancea et al., 2010).

Extracellular vesicles are nano-membrane infrastructures that range from 30-2,000 nm in diameter and are expelled by several cell types in the extracellular micromedium. Nomenclature, biogenesis and roles are still unclear (Ji et al., 2014). Extracellular vesicles, especially exosomes, carry various molecules such as proteins, lipids, microRNAs, mRNAs and DNA fragments. These act as mediators in intercellular communications by inducing changes in cellular receptors (Ji et al., 2014; Dutta et al., 2014). Also, telocytes can contribute to the elimination of extracellular vesicles (Mirancea et al., 2013; Cretoiu et al., 2014; Fertig et al., 2014).

Telocytes have been described as interstitial cells with the following pattern: the ovoid-shaped cell body in which the nucleus is located has 1-5 (most often two) moniliform-like cell extensions called telopodes (Mirancea, 2016). Cellular extensions are usually very long and very thin. Each telopod has alternatively more swollen zones called podomi and thinner areas called podomeri. The mitochondria are located at the subspecies level. In the telescopes, profiles of the smooth endoplasmic reticulum and caveole (Ca²⁺ + uptake / release units) are visible. This particular subtype of stromal cells has been identified in various normal tissues (Cretoiu et al., 2014, Popescu et al., 2005, Zheng et al., 2014, Rusu et al., 2012). In the normal human mammary gland, telocytes were identified as CD34-positive stromal cells around blood vessels and secretory units (Petre et al., 2016).

CONCLUSIONS

This work has been done to determine the mutational status of genes with an important role in the appearance and development of breast tumors, and moreover the identification of particular aspects of the infrastructure that could add to the establishment of an individualized treatment that improve both the response to treatment and the survival rate of the oncological patient. Mutational profiles of tumor suppressor genes *BRCA1*, *BRCA2*, *TP53* and *PIK3CA* oncogene were determined. Samples were also subjected to electron microscopy investigations that revealed important aspects related to tumor cell ultrastructure. Analyzing the results of molecular biology and electron microscopy in the obtained transmission we can say that:

- Following investigations of mutation profile of the *TP53* gene we detected 27.3% somatic mutations, of which 66.67% were deletions and 33.3% substitutions.
- We identified a single "missense" mutation, p.R175H (4.54%), in exon 5 of the *TP53* gene.
- Also, a single "nonsense" substitution, p.R231 * (4.54%), was detected in the coding region of exon 6 of the *TP53* gene.

- Of the total mutations detected, 22.73% (5/22) affected the protein at the DNA binding domain, and 4.54% (1/22) was outside this range.
- Following the direct sequencing of exons 4-9 of both the coding regions and the non-coding regions of the *TP53* gene we identified 5 polymorphic mononucleotide variations.
- Three of the identified SNPs were located in the coding regions of exon 4 (rs1042522 / R72P and rs372397095 / P82P) and exon 5 (rs1800372 / R213R).
- In the non-coding region of intron 6 we detected 2 SNPs: rs1625895 and rs17880604.
- Following mutational analysis of exons 9 and 20 of the *PIK3CA* gene we identified 36.4% (8/22) mutations.
- In the helical domain (exon 9) we identified 13.64% (3/22) mutations, these being p.Q546K (c.1636C> A) and p.E542K (c.1624G> A).
- In the kinase domain, the mutation rate was 22.73% (5/22), and the mutation identified was p.H1047R (c.3140A> G).
- The results obtained with regard to the frequency of mutations in the *PIK3CA* gene and the higher weight of mutations in exon 20 are consistent with previously published in literature on breast cancer.
- Following the molecular investigations of the *BRCA1* and *BRCA2* genes, we identified 12.5% (1/8) "nonsense" mutations in the *BRCA2* gene exon 27.
- Also, following sequencing we detected 23 single nucleotide variations, of which 43.48% (10/23) affected the *BRCA1* gene, while 56.52% (13/23) were identified in the *BRCA2* gene.
- 73.91% (17/23) represent exonic polymorphic variations and 26.09% (6/23) are intronic polymorphisms.
- Regarding the polymorphisms detected in the coding region of *BRCA* genes, 87.5% (14/16) represented transitions, of which 50% were A↔G transitions and 50% T↔C transitions.
- Transversions were detected only in the exonic region in two cases (12.5%, 2/16), namely rs144848 representing a A → C transversion and rs206076, a G → C transversion.
- All six polymorphic variants identified in the non-coding region of the genes represented transitions of which 5 were C↔T transitions and 1 was A → G transition.
- In the untranslated area Utr-5 was detected the variation rs1799943 which is represented by a G → A transition.

In the study group of patients, three of them presented two mutations that affected different genes as follows:

- In the patient corresponding to the 1T sample, we identified the mutation c.213 214del2 which represents a deletion in the *TP53* gene exon 4 of two CC nucleotide bases, which leads to modification of the DNA reading frame by obtaining a truncated protein that will most likely be nonfunctional. The same patient also presented the Q546K mutation in *PIK3CA* gene exon 9 which affects less than 1% of the population, being considered rare and acting in the helical domain of the protein being oriented towards the external face of the domain, thus having the ability to interact with other proteins . Because in this area is usually a lysine residue, it has been hypothesized that it could interact with the cell membrane itself.

- Also, the patient corresponding to the 5T probe presented two mutations in different genes, namely: deletion of nine nucleotide-type cDNAs c.739-747delAACCGGAGG that affect three codons in exon 7 of the *TP53* gene (247, 248 and 249) leading to synthesis a short and non-functional protein. The second mutation identified was H1047R in the *PIK3CA* gene exon 20, which affects the kinase domain of the protein adjacent to the activation loop, and thus influencing the protein mobility. This mutation is also responsible for the malignant transformation of epithelial cells into luminal breast cancers suggesting that it is an early event in breast tumorigenesis.
- The last patient with double mutation corresponds to the 7T probe which presented the H1047R mutation in the kinase domain of the *PIK3CA* gene. As a result of this type of mutation, proteins with intense catalytic activity are obtained and these transmit strong signals at the signaling pathway by stimulating the onset of normal cell transformation into tumor cells. This patient was the only one in the analyzed group showing mutation in the *BRCA2* gene. The nonsense mutation c.10067C> G was detected in exon 27 and consists of replacing the TCA (serine) codon with the TGA codon (STOP codon) at position 3356 of the protein and appears to be responsible for alteration of the genomic structure.

Electron microscopic analysis of the cells and tumor tissues has revealed:

- Transmission electron microscopy investigations revealed that invasive ductal carcinoma intercellular junctions, the desmosomal infrastructure is severely altered, or desmosomes may be totally absent, which leads to profound alteration of mammary gland histoarchitecture.
- Tumor cells generate and disseminate membrane vesicles, including the exosomes that are release inside the peritumoral stroma.
- Telocytes generate and release membrane vesicles in the extracellular matrix of the peritumoral stroma.
- Membrane vesicles released into the stromal tumor that can be disseminated away from the producing cells through their contents can play a modulating role through their paracrine effects.
- Heterocyte contacts of telocytes are reduced, suggesting a possible disruption of tissue homeostasis modulation.
- Membrane vesicles produced by tumor cells per se and by the present of telocytes in the tumor stroma through their contents play a putative role in intercellular signaling.
- Limiting the number or the total absence of heterocellular junctions of telocyte and other cell types from invasive ductal carcinoma suggests a possible disruption of tissue homeostasis modulation.

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SCIENTIFIC ARTICLES PUBLISHED IN THE THEME OF PhD THESIS

✚ Particular molecular and ultrastructural aspects in invasive mammary carcinoma

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Abstract

Electron microscopic investigations of invasive mammary carcinoma tumors revealed that intercellular junctions, namely desmosomes are severely altered; some desmosomes became internalized. Tumor cells, especially by their invadopodia, generate and disseminate membrane vesicles, including exosomes, inside of peritumoral stroma. Telocytes, a new described interstitial/stromal cell phenotype, considered to play important roles in cell signaling, exhibited a reduced number of hetero-cellular contacts, which suggests a possible perturbation of tissular homeostasis modulation. Signaling PIK3/Akt pathway plays an important role both in carcinogenesis and in proliferation, differentiation, and cell survival. Alteration of this pathway has been observed in many human cancers, often involving an increase in the activity of PIK3CA, p110 α catalytic subunit of PI3K. Our study confirms the high prevalence of PIK3CA mutations in breast cancer. In accordance with the results of the largest previous studies, 87.5% of mutations detected by DNA direct sequencing were hot spot mutations, most of them located in the kinase domain. High percentage of mutations detected by high-resolution melting makes the assay an attractive choice for mutation scanning, especially, in samples with low percentage of tumor cell.

Keywords: invasive mammary carcinoma, shedding membrane vesicles, telocytes, mutations in exons 9 and 20 of PIK3CA gene.

✚ Molecular analysis of BRCA1 and BRCA2 genes by next generation sequencing and ultrastructural aspects of breast tumor tissue

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Abstract

In this paper, we focus our interest on the dynamics alterations of the tumor–stroma interface at the ultrastructural level and to detect BRCA1 and BRCA2 mutations using next generation sequencing (NGS) of breast tumor tissue. Electron microscopic investigation revealed some peculiar infrastructural

alterations of the tumor cells per se as well as of the tumor–stroma interface: invadopodia, shedding microvesicles, altered morphology and reduced number of telocytes, different abnormalities of the microvasculature. Tumor suppressor genes BRCA1 and BRCA2 are the genes with most hereditary predisposition to breast and ovarian cancer. An early identification of mutation within these genes is essential for determining classification and therapeutic approach to patients. Genetic tests used to determine mutations in BRCA1 and BRCA2 genes are laborious analysis methods which include, among others, NGS. We analyzed a total of eight samples, in which genomic DNA was amplified using Ion AmpliSeq panel BRCA1 and BRCA2. DNA libraries were created, amplified and sequenced with Ion Torrent Personal Genome Machine. The bio-information data obtained allow us to detect all known pathogenic mutation and uncertain polymorphisms.

Keywords: invasive mammary carcinoma, telocytes, BRCA1 and BRCA2, next generation sequencing.