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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.

Introduction

Breast cancer is a leading cause of cancer death in women worldwide, in fact accounting for approximately one-quarter of all cancers in female worldwide [1–3]. Female breast growth, puberty, menstrual cycle, pregnancy, lactation, postmenopausal regressions represent so many influences/pressures on the breast during the female life [4]. There is an increasing risk to get a mammary cancer if the early menarche is correlated with nulliparity [5].

Mammary gland tissue is represented by epithelial cells and associated stroma. Epithelial cells are organized as an epithelium arranged in two layers: (*1*) the luminal epithelial layer and (*2*) the basal myoepithelial layer. Mammary gland stroma is represented by different cell types as fibroblasts, adipocytes, endothelial cells, macrophages, mast cells plus nervous terminals and telocytes as new identified cell phenotype as well as so-called extracellular matrix represented by different kind of proteins and the basement membrane surrounding the whole glandular structure, microvasculature and individual adipocytes [6, 7]. Mention must be made that each different stromal cell types secrete instructive signals playing crucial roles in the development and function of the normal epithelium as well as in mammary cancer development [7]. In fact, both cancer cells and cells of the associated tumor stroma produce a large variety of chemokines, which sustain tumor growth as well as tumor cells migration and dissemination to form secondary tumors, the main cause of death [8–10]. Indeed, in almost all mammary tumors analyzed by transmission electron microscopy (TEM), inflammatory cells are detectable inside of tumor stroma. Moreover, a new player in the peritumoral stroma, namely telocyte become in focus to be investigated [11–18]. These are the main reasons we decide to investigate few particular infrastructural aspects of the tumor stroma in invasive mammary carcinoma.

Most of breast cancer types are due to the mammary gland epithelial tissue malignization [19]. Breast cancer is the main cause of cancer death in Romanian women (about 32% of female cancers and 18% of all cancers), registering over one million new cases annually worldwide. Approximately 5–10% of breast and ovarian cancer patients are likely hereditary [20]. Identifying the *BRCA1* and *BRCA2* genes and their default mutations led to a major change of therapy for woman with hereditary predisposition to breast and ovarian cancer [21]. Most hereditary breast and ovarian cancers (HBOCs) are due to germline mutations in *BRCA1* and *BRCA2* genes. *BRCA* mutations lead patients to important decisions and challenging, both in terms of prevention, screening and early detection, risk of surgical resection and pharmacological options, hormonal and menopausal management [22]. *BRCA1*

and *BRCA2* genes are involved in DNA repair process through the production of tumor suppressor proteins. The *BRCA1* gene is located on the long arm of chromosome 17, locus 17q21 and has 24 exons. It interacts with several proteins involved in cellular signaling pathways such as cell cycle progression and genetic regulation of transcription of the DNA damage response. This area serves to maintain genetic integrity. The loss of this function will accumulate genetic defects that can lead to tumor cells. *BRCA1* is a tumor suppressor gene, and as the majority of genes, may cause variations in some disease, or may be associated with an increased risk to the development of these diseases. There are over 500 *BRCA1* variants considered to be causal, but most are very rare [23].

BRCA2 gene is located on the long arm of chromosome 13, locus 13q12.3 and comprises 27 exons. As *BRCA1* gene, *BRCA2* gene is involved in maintaining the genomic stability. Until now was identified over 1600 mutations in the *BRCA1gene* and over 1800 in case of *BRCA2* gene [24, 25]. Same to the *BRCA1* gene, *BRCA2* gene variations may be causal or in combination with other molecular events present increased risk of developing various diseases. In clinical terms, causal variations are usually referred to as "pathogenic". However, there is no 100% certainty about the fact that a person carrying causal variations will develop the disease during his life. It is known hundreds of *BRCA2* gene variations but these are very rare [24]. Researchers have determined that these genes may also be involved in the development of non-hereditary tumors, and these sporadic tumors containing somatic variants in the *BRCA1* and *BRCA2* genes [26]. Patients who have *BRCA* germline mutations have a proven clinically benefit to therapy with PARP inhibitor [poly ADP (adenosine diphosphate) ribose polymerase – the enzyme involved in DNA repair], olaparib [26, 27]. Because there is the possibility that patients with somatic mutations of the *BRCA* genes may benefit from treatment with PARP inhibitor, it is very important to test all possible variants of the *BRCA* within tumors [26]. Next-generation sequencing (NSG) has provided an unprecedented opportunity for analyzing genomic complexity of breast cancer. NSG is based on parallel sequencing, analyzing multiple genes simultaneously, thereby reducing analysis time and low additional costs. By testing multiple genes simultaneously, can identify a large number of mutations whose clinical significance has not yet been proven, which are called variants of uncertain clinical significance (VUS) [28]. NGS methods provided quantitative measurements of thousands of mutations and copy number aberrations in parallel [29].

In a previous paper, Mihalcea *et al.* (2015) [16] studied PIK3CA (phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha) gene mutations and described some ultrastructural aspects of telocytes inside of mammary carcinoma stromal tissue.

In the present study, another few data are emphasized concerning tumor stroma, especially telocytes infrastructure and their relationships in invasive mammary carcinoma. Determination of *BRCA* variants within analyzed samples was done by NGS.

Materials and Methods

Transmission electron microscopic (TEM) investigation

In order to perform TEM investigations, small tissue fragments about 2–3 mm^3 from the breast tumors resulted by surgery for diagnostic and curative therapy from the patients suffering from breast cancer (surgeon got patients, consent according to the *International Ethical Guidelines for Biomedical Research Involving Human Subjects*) were processed following the routine TEM protocol [16, 30, 31]. Samples from eight patients suffering of mammary invasive carcinoma were analyzed by TEM. Semithin sections were stained with 1% toluidine blue for light microscopy. Ultrathin sections of 100 nm were cut using a diamond knife and collected on 200 mesh grids and double counterstained with uranyl acetate and subsequently lead citrate. The grids were examined by a transmission electron microscope JEOL JEM-1400 operated at an acceleration voltage of 80 kV. Several electron microscopic images were digitally colored.

Next Generation Sequencing – Ion Torrent Technology

We analyzed eight samples of fresh breast tumor tissues, obtained by resection from patients surgically treated in "Prof. Dr. Alexandru Trestioreanu" Institute of Oncology, Bucharest, Romania. Consent according to the *International Ethical Guidelines for Biomedical Research Involving Human Subjects* was obtained by surgeon from patients who participated in the study. Genomic DNA was extracted from fresh tissue using the QIAamp DNA Mini kit (Qiagen). The amount of DNA and its purity were measured by spectrophotometric absorbance reading at both 260 nm and at 280 nm, using NanoDrop ND 1000 instrument (NanoDrop Technologies). Sequencing was performed on the instrument PGM (Personal Genome Machine – Thermo Fisher Scientific).

NGS method

To achieve next-generation sequencing, we used the Ion AmpliSeq™ *BRCA1* and *BRCA2* Panel (Life Technologies) containing 167 primer pairs in three pools. Multiplex polymerase chain reaction (PCR) was performed using 2 ng/μL genomic DNA in a final volume of 6 μL with a premixed primer pool and Ion AmpliSeq™ HiFi master mix (Ion AmpliSeq™ Library Kit 2.0) for 2 minutes at 99°C, followed by 19 cycles at 99°C for 15 seconds and 60°C for 4 minutes, ending with a holding period at 10°C. The PCR amplicons were treated with 2 μL FuPa reagent to partially digest primer sequences and phosphorylate the amplicons at 50°C for 10 minutes, followed by 55°C for 10 minutes, then 60°C for 20 minutes. The amplicons were ligated to adapters with the diluted barcodes of the Ion Xpress™ Barcode Adapters kit (Life Technologies) for 30 minutes at 22°C, then 72°C for 20 minutes. Adaptor ligated amplicon libraries were purified using Agencourt AMPure XP reagents (Beckman Coulter). Next, we quantify the library on instrument Qubit (ThermoFisher Scientific), and than realizing dilutions of 100 pM in TE buffer. After obtaining the library, go to work on the One Touch 2 device. PCR product was carried out using the Ion OneTouch™ System and Ion OneTouch™ 200 Template Kit v2 (Life

Technologies) according to the manufacturer's instructions. Template-positive Ion Sphere™ Particles were then enriched with Dynabeads MyOne™ Streptavidin C1 Beads (Life Technologies) using an Ion OneTouch™ ES system (Life Technologies). Purified Ion Sphere particles were loaded on Ion 316 Chip V2. Sequencing was carried out on a Personal Genome Machine (PGM) sequencer (Ion Torrent™) using the Ion PGM™ Sequencing 200 Kit V2 according to the manufacturer's instructions [20].

Results

Electron microscopic analysis

Specimens from eight patients with invasive breast carcinoma were analyzed by TEM.

In case of invasive growing mammary carcinomas, tumor epithelium histo-architecture is severely altered. Tumor cells have large euchromatic nuclei, desmosomes are almost missing but interdigitating junctions connect cells each other (Figure 1).

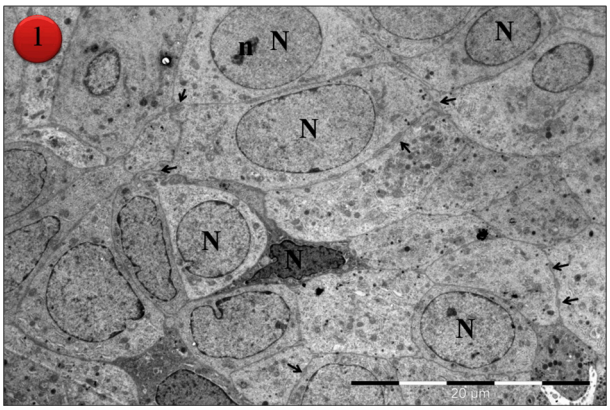

Figure 1 – ***Overview on a tumor mass of invasive mammary carcinoma. A plethora of epithelial tumor cells grown disorganized have large, mostly euchromatic, nucleolated (n) nuclei (N). Desmosomes are almost missing but interdigitating junctions (arrows) connect cells each other.***

In all invasive form of mammary carcinoma, no basal lamina can be detected at the mammary tumor cells – peritumoral stroma interface but some long cell processes termed invadopodia deeply penetrate inside the tumor stroma (Figure 2).

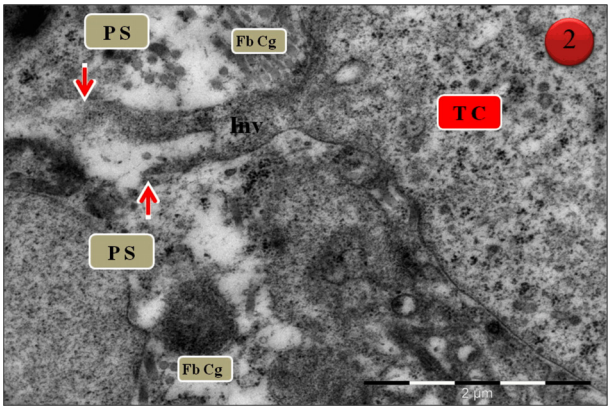

Figure 2 – ***A long invadopodium (Inv) is dichotomized (red arrows) and penetrated deeply into fibrotic peritumoral stroma (PS). No basal lamina can be detected around the mammary tumor cell (TC). Fb Cg: Fibrillar collagen.***

Interestingly, sometimes, promyelocytic leukemia nuclear body can be identified inside of the nucleus belonging to tumor cells described as an amorphous ring, which wraps an electron dense core (Figure 3).

Quite often, tumor cells delivered large shedding vesicles at the tumor – peritumoral stroma interface (Figure 3).

Besides usually breast stromal cell types, mainly fibroblasts, adipocytes, other cell types can be detected, as are mast cells and telocytes (Figures 3–6). Sometimes, few telocytes become in close vicinity one to another. Some of them realize so-called a plug and socket synapse (Figure 4). Because their characteristic, cell extensions termed telopodes are extremely thin, the chance to have in the same plane of the ultrathin sections the whole length of the body cell together with telopodes is very rare. Nonetheless, Figure 5 depicts a very long telopode, of cca. 85 µm, embedded into fibrillar collagen of the peritumoral stroma. Except for the cytoplasm of body cell (where the nucleus is located), rich in rough endoplasmic reticulum and Golgi apparatus (not shown here), the rest of cytoplasm appeared prevalently poor in cytomembranes but filled with ribosomes, smooth and poor rough endoplasmic reticulum, some cytoskeletal microfibrils, coated pits and caveolae. It is worthy to mention that, different from the telocytes located in normal stromal tissues, in all our investigated samples of invasive mammary carcinoma, telocytes showed a remarkable paucity in mitochondria (Figures 4–6). Moreover, mention must be made that some telopodes are involved in so-called shedding microvesicles process as is depicted in Figures 4 and 5. Sometimes, two or more telopodial fragments represented by alternating podoms and podomers run together parallel very close but without any direct contact (Figure 6).

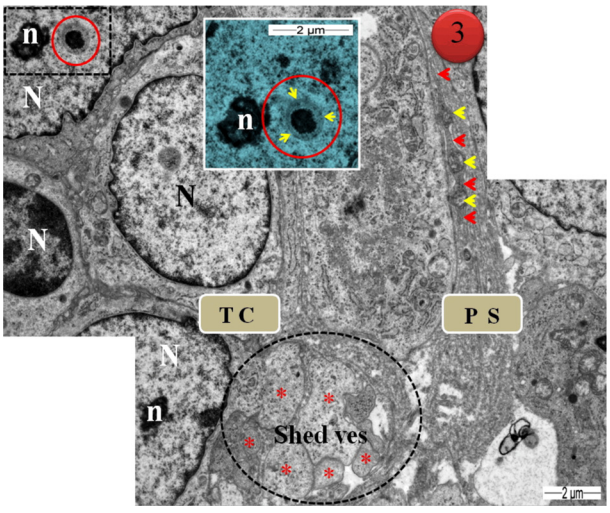

Figure 3 – ***Tumor cells (TC) affronted by peritumoral stroma (PS) have large nucleolated (n) and mostly euchromatic nuclei (N). A promyelocytic body can be seen (encircled area, detailed in inset). An amorphous ring (small yellow arrows) can be identified around an electron dense core. Shedding microvesicles (asterisks) are visible at the tumor cells – peritumoral stroma interface. An alternation of podoms (red head arrows) and podomers (yellow head arrows) suggests a telopodial extension of a stromal telocyte.***

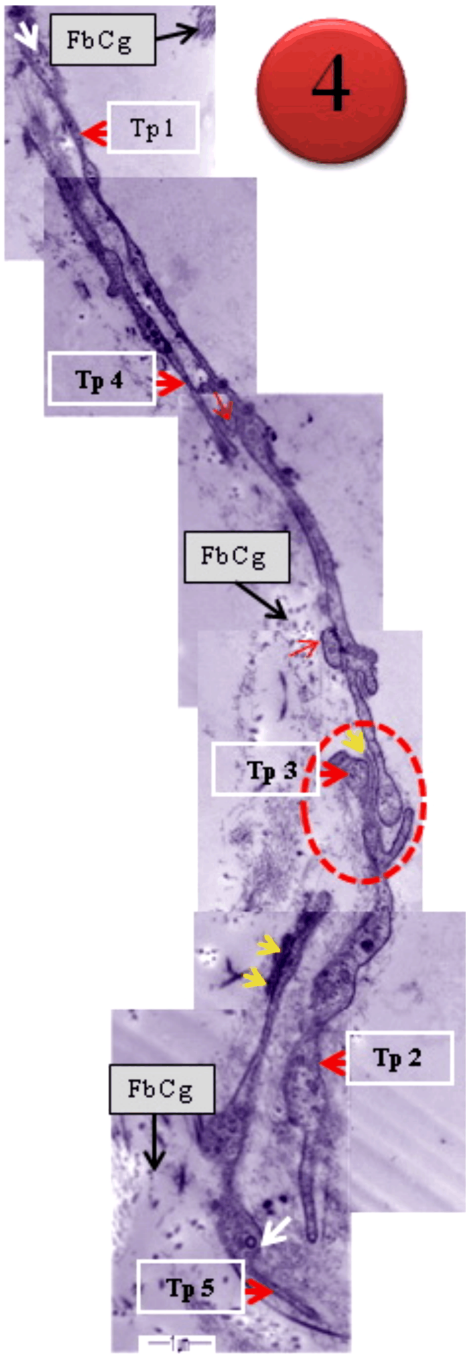

Figure 4 – ***Five telopodes (Tp 1–Tp 5) from a mammary carcinoma peritumoral stroma. Two telopodes are interconnected by a plug and socket synapse (red elliptic area). Short patches of the basal lamina (yellow head arrows) accompany short profiles of telopodes (Tp 1, Tp 3 and Tp 5). Thin red arrows mark extracellular vesicles while white arrows mark caveolae. Fb Cg: Fibrillar collagen.***

Concerning the microvasculature, we observed that the blood vessels relative far from the tumor growing front look quite normal, while those blood vessels close to the tumor exhibited in different degrees some abnormalities. Often, blood capillaries have collapsed lumen (Figure 7) or fibroblasts tend to wrap small blood vessels (Figure 8). Much worst, large edematous space separated endothelial wall from pericytes (Figure 9) to culminate with inter-endothelial gaps formation (Figure 10), and, consequently, blood vessel become dehiscent, a prerequisite for blood cells extravasation, so that, very large fields of extravasated cells were accumulated in front of the growing tumor. Dissolution of long profiles of both endothelial wall and pericyte basement membrane was visible. Moreover, a plethora of Weibel–Palade bodies accumulated inside of the endothelial cells (especially endothelial cells belonging to the capillaries much altered in their histoarchitecture), some of them being very close to the endothelial blebs projected towards the blood vessel lumen (Figure 10).

Molecular investigations by next generation sequencing analysis of *BRCA1* and *BRCA2* genes

In this study, we performed next generation sequencing analysis of *BRCA1* and *BRCA2* genes in eight specimens breast tumor tissue. Target sequencing was performed using Ion Torrent™ PGM System.

Data analysis has not found any mutation with important clinical significance.

The following table notices an only deletion in *BRCA1* gene and 10 single nucleotide polymorphisms (SNPs), three of these are hotspots mutation without clinical significance and seven novel SNPs. Also, in *BRCA2* gene were identified 14 SNPs with overt unknown (Table 1).

In *BRCA1* gene, we identified nine SNPs heterozygous and one SNP homozygous, all pathogenic meaningless and one deletion also unknown. Regarding *BRCA2* gene, we identified 11 SNPs heterozygous and three SNPs homozygous, all of them having a clinical significance unknown. The mutations identified in *BRCA1* gene are: *rs* 16942/COSM148277 – mutation (Figure 11), *rs* 799917/COSM148278 – mutation (Figure 12) and *rs* 1799949/COSM148280 – substitution (Figure 13). Mutation detected in *BRCA2* gene is *rs* 144848/COSM147663 – mutation.

Discussion

A malignant tumor is a complex ecosystem represented by (*1*) genetically altered neoplastic cells and (*2*) the associated tumor stroma represented by (*a*) local connective tissue cells, the microvasculature, terminal nerves as well as transitory cells as imported cells (*e.g.*, mast cells) plus (*b*) extracellular matrix. Cancer develops as a progressive multi-step process, in which involved cells undergo consecutive genetic alterations and in cooperation with stromal cells gradually acquire phenotypic changes so that transformed cells will grow rapidly and uncontrolled to develop a malignant tumor. Mention must be made that there is a body of evidence that alterations in the tumor cells themselves are not sufficient to generate a tumor so that an adequate stromal microenvironment is a stringent condition [2, 8, 31–35]. Long time ago, Ozzello (1971) [36] postulated that, many of the tumors which fulfill the light microscopic criteria of intraductal carcinomas and of lobular carcinomas *in situ*, ultrastructurally are already invasive, even though no stromal invasion can be recognized under light microscope examination. In this context, using TEM investigation, we searched to find some infrastructural abnormalities expressed by mammary tumor cells *per se* and also to detect some ultrastructural particularities of the associated tumor stroma, especially telocytes as newly described stromal cell phenotype [11–17].

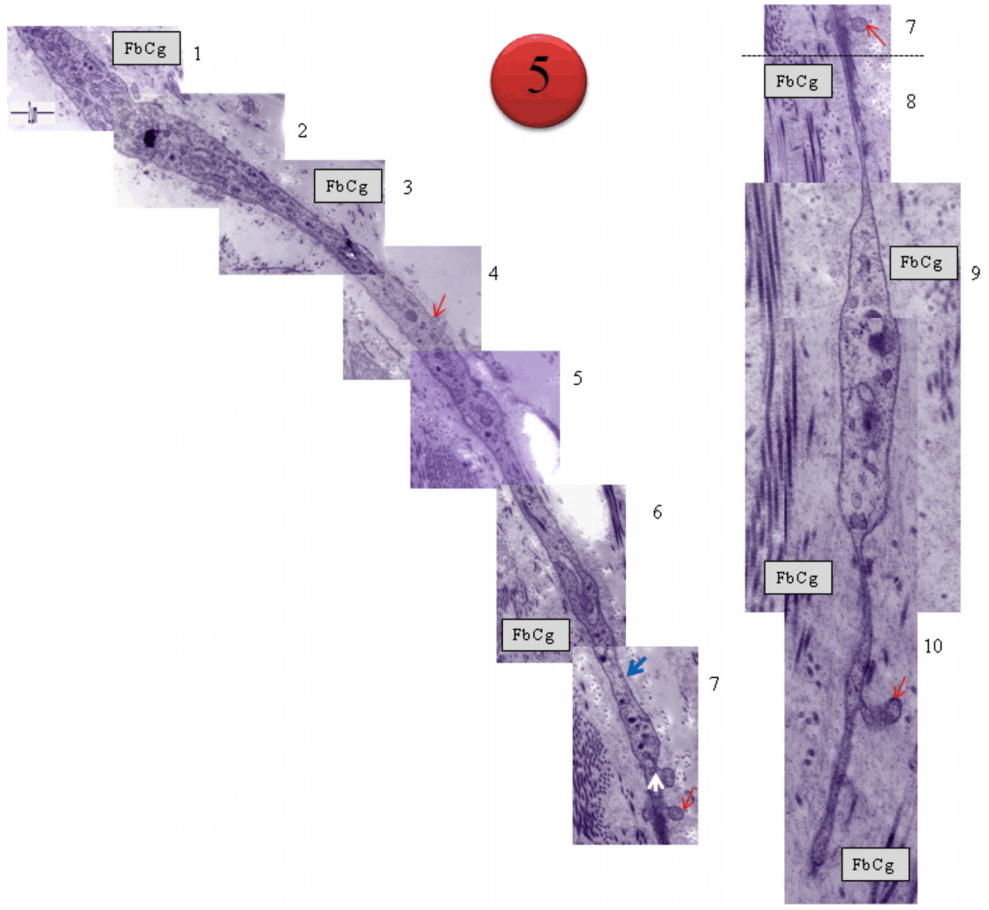

Figure 5 – *A very long telopode (1–10 microphotos) is embedded into fibrillar collagen (Fb Cg) of a peritumoral stroma (invasive mammary carcinoma). Except for a part of the telopodial cytoplasm rich in rough endoplasmic reticulum (1st and 2nd micrographs), the rest is prevalently poor in cytomembranes; nonetheless, ribosomes, smooth and pure rough endoplasmic reticulum and cytoskeletal microfibrils, coated pits (blue arrow) and caveolae (white arrow) can be detected. Red arrows mark shedded extracellular vesicles (ectosomes).*

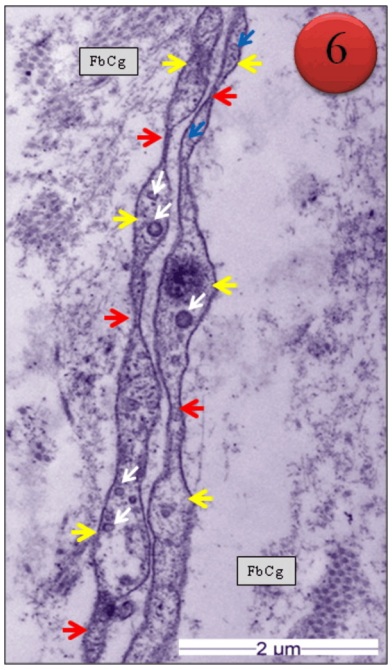

Figure 6 – *Two telopodial fragments represented by alternating podoms (yellow arrows) and podomers (red arrows) run together parallel very close but without any direct contact. Coated pits (blue arrows) and caveolae (white arrows) can be detected. Fb Cg: Fibrillar collagen.*

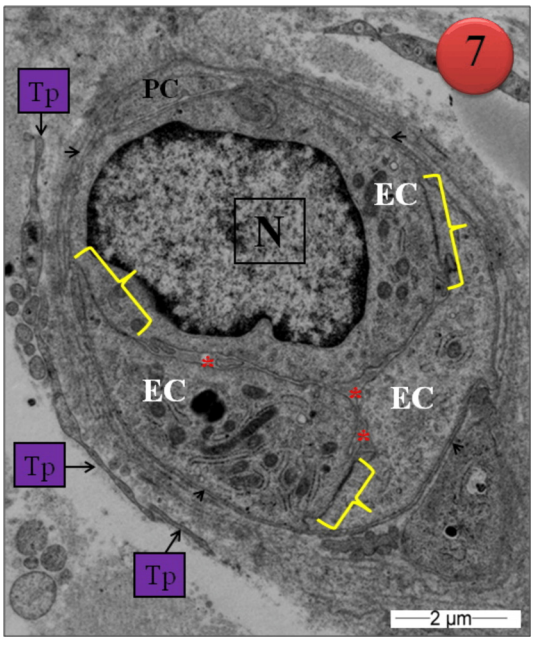

Figure 7 – *A collapsed capillary: a virtual lumen can be seen (asterisks). Endothelial cells (EC) are strongly connected by inter-endothelial junctions (accolades); one of them has a prominent nucleus (N). A continuous basal lamina (head arrows) wrapped the capillary wall. Few telopodes (Tp) follow partially the capillary contour. PC: Pericyte.*

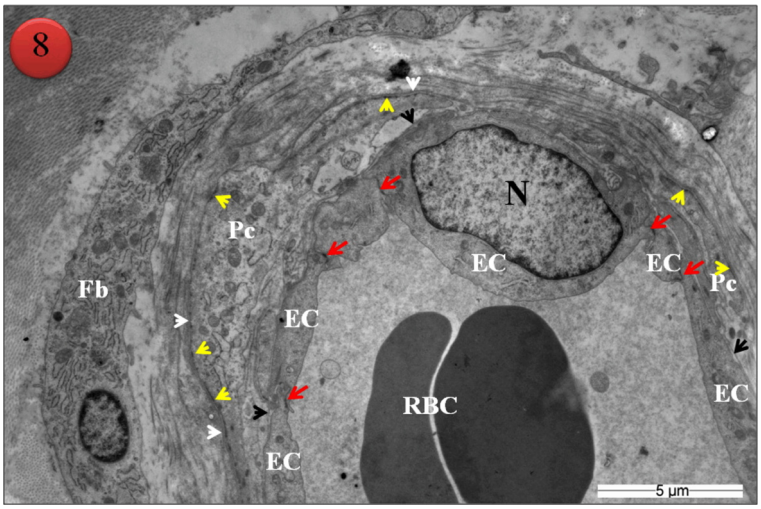

Figure 8 – *A normal blood microvessel inside of the tumor stroma somehow far from the tumor growth front exhibits a continuous basal lamina (black head arrows). Moreover, pericytes (Pc) with characteristic subplasmalemmal densities (yellow head arrows) and associated basal lamina (white head arrows) wrapped endothelial wall. A large blood vessel lumen with red blood cells (RBC) can be seen. N: Nucleus; EC: Endothelial cells; Fb: Fibroblast; Red arrows: Inter-endothelial junctions. L Bl Vs: Lumen of blood vessel.*

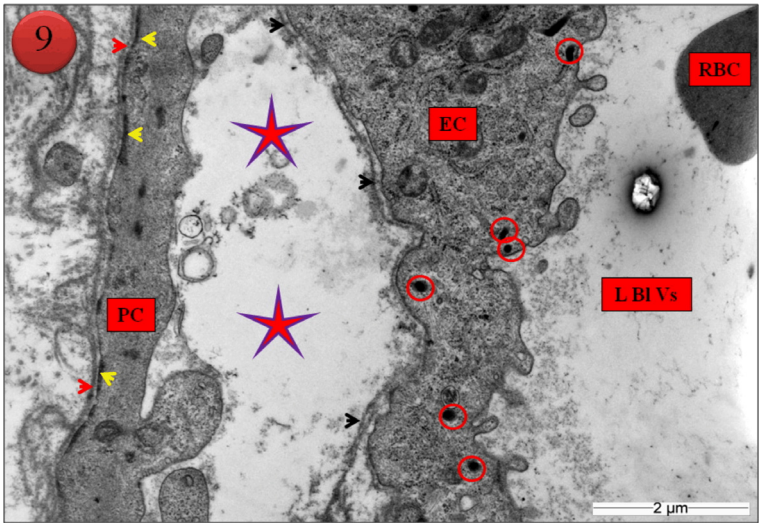

Figure 9 – *An abnormal blood vessel with a very large edematous space (stars) between endothelial cell (EC) and pericyte (PC). Both endothelial wall basal lamina (black head arrows) and pericytic basal lamina (red head arrows) are well preserved. Yellow head arrows mark the pericytes subplasmalemmal densities. Weibel–Palade bodies are visible in endothelial cells (encircled areas). Inside of the blood lumen, a red blood cell (RBC) is visible.*

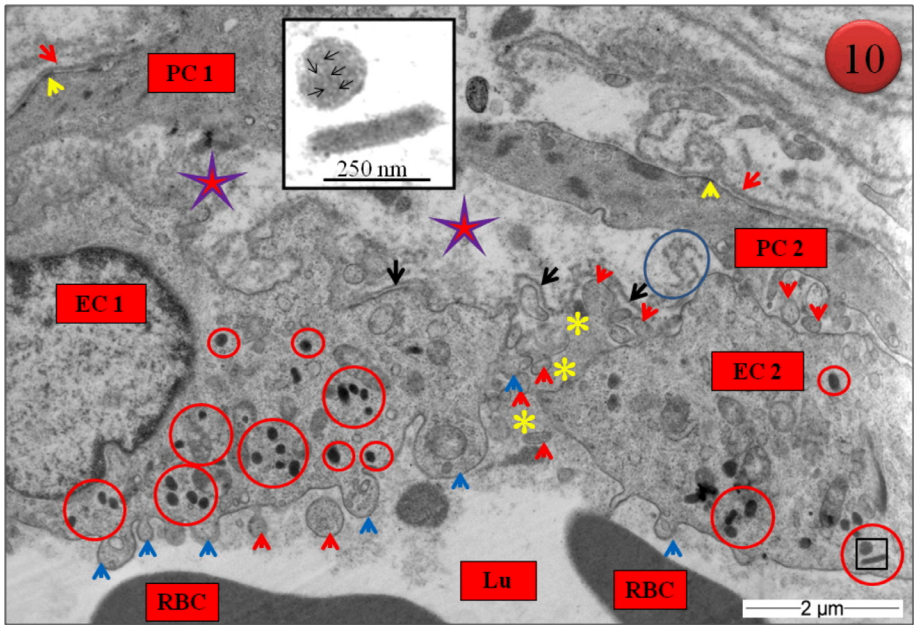

Figure 10 – *An inter-endothelial gap (yellow asterisks) is detectable between two endothelial cells (EC 1 and EC 2). Pericytes (PC 1 and PC 2) are somehow dislocated. Both periendothelial basal lamina (black arrows) and pericyte basal lamina (red arrows) are interrupted. An accumulation of short profiles of basal lamina (blue elliptic area) are detectable. Pericytes exhibit subplasmalemmal densities (yellow head arrows). A plethora of Weibel–Palade bodies (encircled areas) are visible inside of the endothelial cells. Numerous blebs (blue head arrows) are visible towards luminal surface (Lu) as well as towards inter-endothelial space, while some blebs seem to be already detached (red head arrows). RBC: Red blood cells. In inset: Detail from black framed area shows two Weibel–Palade bodies, one cross with electron dense tubules (thin black arrows) and another one longitudinally sectioned.*

Table 1 – ***Genetic and chromosomal changes and their position identified in DNA samples extracted from fresh breast tumor tissue from patients included in the study***

No.	Chromosome	Position	Ref.	Variant	Allele call	Type	Allele source	Allele name	Gene ID
1	chr13	32890572	G	A	Heterozygous	SNP	Novel	---	*BRCA2*
2	chr13	32903685	C	T	Heterozygous	SNP	Novel	---	*BRCA2*
3	chr13	32906729	A	C	Heterozygous	SNP	Hotspot	COSM147663	*BRCA2*
4	chr13	32910328	T	C	Heterozygous	SNP	Novel	---	*BRCA2*
5	chr13	32910430	C	T	Heterozygous	SNP	Novel	---	*BRCA2*
6	chr13	32911888	A	G	Heterozygous	SNP	Novel	---	*BRCA2*
7	chr13	32912299	T	C	Heterozygous	SNP	Novel	---	*BRCA2*
8	chr13	32913055	A	G	Homozygous	SNP	Novel	---	*BRCA2*
9	chr13	32915005	G	C	Homozygous	SNP	Novel	---	*BRCA2*
10	chr13	32929232	A	G	Heterozygous	SNP	Novel	---	*BRCA2*
11	chr13	32929387	T	C	Homozygous	SNP	Novel	---	*BRCA2*
12	chr13	32936646	T	C	Heterozygous	SNP	Novel	---	*BRCA2*
13	chr13	32953388	T	C	Heterozygous	SNP	Novel	---	*BRCA2*
14	chr13	32972717	C	G	Heterozygous	SNP	Novel	---	*BRCA2*
15	chr17	41223094	T	C	Homozygous	SNP	Novel	---	*BRCA1*
16	chr17	41223119	T	C	Heterozygous	SNP	Novel	---	*BRCA1*
17	chr17	41234470	A	G	Heterozygous	SNP	Novel	---	*BRCA1*
18	chr17	41244000	T	C	Heterozygous	SNP	Hotspot	COSM148277	*BRCA1*
19	chr17	41244435	T	C	Heterozygous	SNP	Novel	---	*BRCA1*
20	chr17	41244936	G	A	Heterozygous	SNP	Hotspot	COSM148278	*BRCA1*
21	chr17	41245237	A	G	Heterozygous	SNP	Novel	---	*BRCA1*
22	chr17	41245466	G	A	Heterozygous	SNP	Hotspot	COSM148280	*BRCA1*
23	chr17	41256090	AAAAAAA AGAAAAG	-	Heterozygous	DEL	Novel	---	*BRCA1*
24	chr17	41256878	C	T	Heterozygous	SNP	Novel	---	*BRCA1*
25	chr17	41276247	A	G	Heterozygous	SNP	Novel	---	*BRCA1*

DNA: Deoxyribonucleic acid; chr: Chromosome; A: Adenine; G: Guanine; C: Cytosine; T: Thymine; SNP: Single nucleotide polymorphism; *BRCA*: Breast cancer.

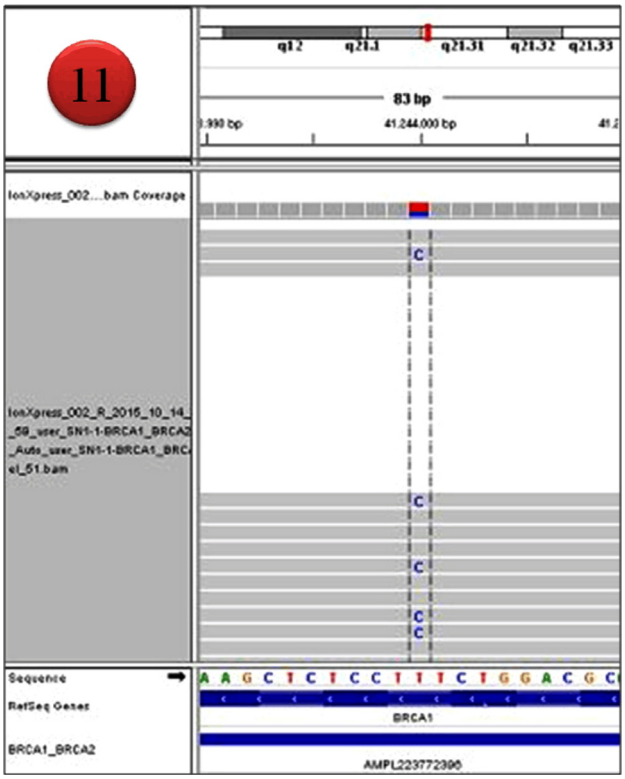

Figure 11 – ***Representative image of chromosome alignment visualized with IVG soft. BRCA1 hotspot mutation [position 41244000 (COSM148277); T>C; heterozygous].***

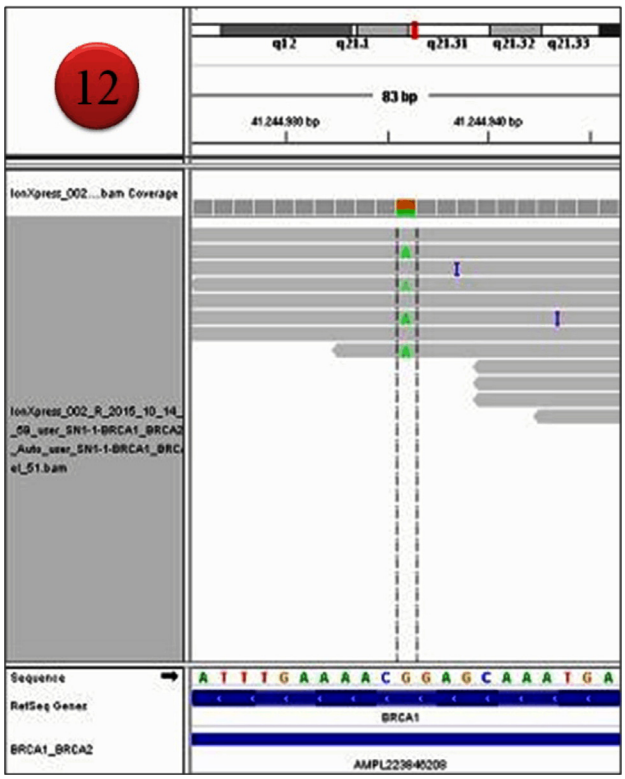

Figure 12 – ***Representative image of chromosome alignment visualized with IVG soft. BRCA1 hotspot mutation [position 41244936 (COSM148278); G>A; heterozygous].***

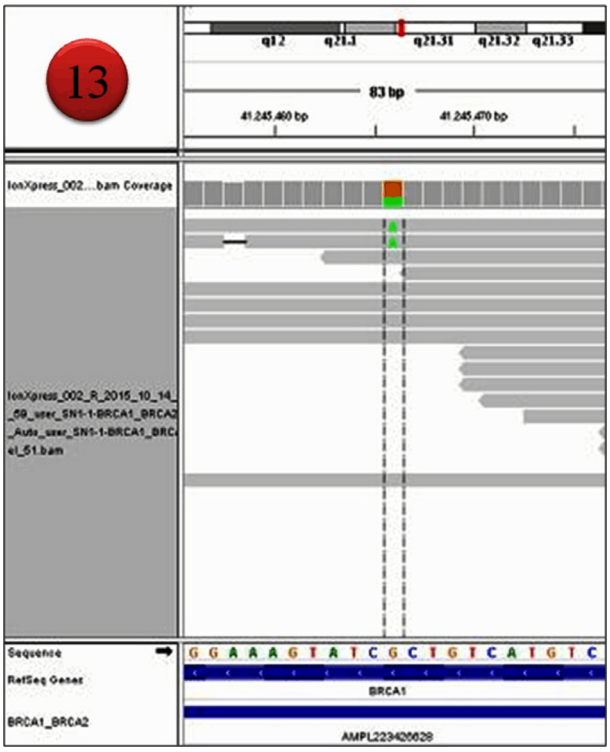

Figure 13 – ***Representative image of chromosome alignment visualized with IVG soft. BRCA1 hotspot mutation [position 41245466 (COSM148280); G>A; heterozygous].***

In all TEM-investigated invasive mammary carcinoma tumor specimens, the normal architecture of the mammary glandular epithelium is abrogated. Desmosomes are almost missing. Mihalcea *et al.* (2015) [16] reported that some desmosomes from malignant cells in invasive mammary carcinoma are internalized, but our extensive TEM analysis showed that interdigitating junctions appeared to connect one cell to another. None the less, mention must be made, such kind of junctions are more weakly compared to desmosomes which contribute to easy cell detachment from the tissue context, a precondition for tumor cell dissemination.

Different from normal epithelial breast cells ultrastructure, in all of our TEM investigated specimens the nuclear infrastructure of malignant breast cells showed that euchromatin is almost prevalently. Nuclear organization itself can modulate cellular and tissue phenotype, knowing heterochromatin remains transcriptionally silent [37]. Moreover, there is a correlation between *BRCA1* tumor suppressor gene and repression of pericentromeric expression, pericentromeric DNA being trancriptionally silent in differentiated cells and epigenetically regulated [38].

Interestingly, in two from eight of our electron microscopic analyzed invasive mammary tumors, promyelocytic leukemia nuclear body (PML-NB) were identified inside of the nucleus belonging to mammary tumor cells. Numerous disease states seem to be related to nuclear body dysfunction [39]. Using an immune electron microscopic study Zhou *et al.* (2002) [40] showed that promyelocytic leukemia gene product was detected as nuclear body shell at the periphery of the PML-NB. Carracedo *et al.* (2012) [41] reported that PML is overexpressed in a subset of breast cancers.

There are many reports which clearly demonstrate that progression of an epithelial lesion to a malignant transformation (carcinoma) is very much dependent on the so called reactive stroma that provide structural and vascular support for tumor growth and, eventually to become invasive and, finally to metastasize [9, 15, 34, 42]. There are many players involved in cell–cell and cell–extracellular matrix communications [34]. Very tightly mechanisms of control, represented by autocrine and paracrine factors, maintain normal local/regional tissue homeostasis. Among different other factors involved in paracrine cell communication, shedding vesicles (formation and delivery of microvesicles inside of the micromedium) by different cells are now considered to play an important role. There are three identified mechanisms by which microvesicles are formed and delivered inside of extracellular matrix (ECM): (*1*) within endosomal multivesicular bodies, which are redirected to the cellular surface (membranes of Golgi-derived vesicles, tubules and granules, *via* exocytotic pathway fuse with plasma membrane being extracellular delivered as exosomes of 30–100 nm in diameter, (*2*) blebbing of the cell plasma membrane termed ectosome (also known a microvesicles) of 100–1000 nm in diameter delivery into micromedium and (*3*) breakdown of dying cells/undergoing programmed cell death into apoptotic bodies of 50 nm up to 4000 nm in diameter [2, 17, 18, 35, 43, 44]. Indeed, by their content (receptors, proteins, lipids, microRNAs, mRNA, DNA fragments) such kind of infrastructures might act as vehicles reaching other cells inducing phenotypic changes in recipient cells [17, 18, 45]. Like any other normal or malignant cell types [2, 3, 14, 15], mammary tumor epithelial cells delivered a plethora of extracellular vesicles [15]; see also in this study emphasized by Figure 3. Moreover, telocytes are involved in delivering shedding vesicles inside of tumor stroma as is depicted in Figures 4 and 5. Telocytes have been described in the interstitium of many organs: heart, skin, skeletal muscle, liver, including mammary gland [46]. There are many reports which emphasize that telocytes play a major role as integrators of many intercellular functions [17, 47, 48], regeneration and repair processes [49], including tumor development [15, 16, 50, 51].

Now is well stated that telocytes have the capability to generate and deliver exososmes, ectosomes as well as multivesicular cargos and consequently may be involved in horizontal transfer of information [17, 18, 52]. Díaz-Flores *et al.* (2014) [53] reported that telocytes are the principal non-macrophage cells with phagocytic-like properties. The old hypothesis concerning horizontal transmission of malignancy *via* transfection-like uptake of tumor-derived nucleic acids by susceptible cells becomes in focus [54].

In normal telocytes, podoms harbor mitochondria. Different from the normal situation, in almost podoms belonging to telocytes from peritumoral stroma in invasive mammary carcinoma we investigated, the mitochondria are almost missing. Is hard to have a pertinent comment related to this particular aspect but mention must be made that, obviously, like other peritumoral cell types, telocytes attenuate apoptosis, might contribute cancer cells adaptive to tumor microenvironment including hypoxia [50].

The gradual alterations of microvasculature from the normal aspect to the worst aspect, when capillaries from the immediate vicinity of the tumor growth front become dehiscent and the blood cells are extravasated, we may speculate to be related with gradiental concentration of delivered growth factors by tumor cells *per se*, able to make the stromal cells their accomplice to sustain their uncontrolled proliferation. Concerning the increased number of the Weibel–Palade bodies (WPBs), especially inside of the endothelial cells belonging to the capillaries much altered in their histoarchitecture, we may correlate this aspect with the fact that WPBs contain a supply of mediators that could be deployed in response to signaling molecules or mechanical stress, allowing vascular endothelial cells to influence hemostasis, inflammation, angiogenesis, and vascular tone [55]. All these mentioned putative capabilities/roles of WPBs might be involved in/correlated with/invasive tumor growth inside of the surrounding stroma.

In order to make an accurately differential diagnosis of breast lesions, besides currently histopathological observations, TEM investigations and immunohisto-chemistry analysis, a new trend is to perform genetic analysis.

The full sequencing of the entire genome has led to the identification of several rare and non-recurrent mutations in individuals with the same disease diagnosis. Detection of mutations in different genes or cellular signaling pathways may reveal heterogeneous genetic diseases like cancer. An incomplete documentation of these mutations may have an impact concerning the sensitivity of these methods [56]. Hereditary factor in breast carcinoma accounts for about 25% of all cases of breast cancer. *BRCA1* and *BRCA2* genes have a high penetrance and increase the breast cancer risk. *BRCA1* and *BRCA2* mutations explain about 20% of familial cases of breast cancer [57]. Mutations are distributed throughout the coding region and flanking by intronic sequences, with a high concentration in exon 11 of the two genes. Most mutations are frameshifts or splice site alterations which leading to truncated proteins [58].

In a recent published paper, we investigated PIK3CA mutations in breast cancer Mihalcea *et al.* (2015) [16].

In this study, we analyzed a total of eight breast tumor tissue specimens by next-generation sequencing using Ion Torrent Personal Genome Machine (PGM). In total, four BRCA variants were identified, three of them are BRCA1 mutation and have been reported: c.3548A>G, c.2612C>T and c.2082C>T. *BRCA2* mutation also has been reported: *rs* 144848. All four variants *BRCA* show independently a minor risk but cumulative the risk is significantly increased for breast cancer.

These results confirm that the next generation sequencing is very efficient and fast, with affordable prices and could be the suitable sequencing method for developing countries.

Conclusions

Our TEM investigations showed that different from the normal tissues or other tumor cell type, in almost podoms belonging to telocytes from peritumoral stroma in invasive mammary carcinoma, we investigated the mitochondria are almost missing. In this study, we analyzed samples of fresh breast tumor tissue through new generation sequencing method. In *BRCA1* gene, we identified nine SNPs heterozygous and one SNP homozygous, all pathogenic meaningless and one deletion also unknown. Regarding *BRCA2* gene, we identified 11 SNPs heterozygous and three SNPs homozygous, all of them having a clinical significance unknown. The variants with no clear clinical significance may present a diagnostic challenge and the interpretation of results when we make a new sequencing. Using the new generation sequencing is suitable for germline mutations screening, both in terms of costs and reducing work time.

Conflict of interests

The authors declare no conflict of interests.

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