Rom J Morphol Embryol 2020, 61(2):441–447 ISSN (print) 1220–0522, ISSN (online) 2066–8279 doi: 10.47162/RJME.61.2.13

ORIGINAL PAPER

RJME Romanian Journal of Morphology & Embryology http://www.rjme.ro/

Analysis of *TP53* gene and particular infrastructural alterations in invasive ductal mammary carcinoma

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Abstract

This study was conducted in order to determine the mutational status of *TP53* gene and to determine some particular aspects from ultrastructural level in invasive mammary ductal carcinoma. The cellular signaling pathway involving the *TP53* gene acts in biological deoxyribonucleic acid (DNA) repair processes and cell cycle arrest following a signal transmitted to the p53 protein when posttranslational changes occur in the cell due to stress induced in the cell by both intrinsic and extrinsic factors. Cellular stress activates the transcription factor function of the protein that initiates, as the case may be, either DNA repair or programmed cell death (apoptosis). The *TP53* gene is commonly mutated in many human cancers and also has a highly polymorphic grade. To determine the mutational status of the exons 4–9 of the *TP53* gene, we used extracted DNA from fresh breast tissue, and we analyzed it through direct sequencing. In mammary carcinoma, the mutation frequency of *TP53* is running between 20–40% and, in regards the polymorphism, at least 14 different forms were identified, that are associated with cancer risk. The mutation type distribution showed a predominance of deletions and a reduced frequency of substitutions comparing with *International Agency for Research on Cancer* (IARC) database. Taken in consideration the importance of the tumor associated stroma in tumor development, we have also investigated some particular aspects at the infrastructural level of invasive mammary ductal carcinoma, notably concerning telocytes as tumor stroma interstitial cells by transmission electron microscopy analysis.

Keywords: *TP53* gene, missense mutation, nonsense mutation, polymorphism, exons, telocytes.

Introduction

In order to assure tissue homeostasis, a tightly balance between the rates of cell division and cell death is necessary to be maintained. Conversely, tumors exhibit decreased cell death which leads to an increased cell proliferation. Moreover, genetic changes resulting in loss of programmed cell death (apoptosis) are critical components of tumorigenesis. The *TP53* gene is a tumor suppressor gene considered the guardian of the genome [1] that encodes tumor protein P53 situated in the nuclei [2]. P53 protein plays an essential role in cell cycle regulation, deoxyribonucleic acid (DNA) repair and apoptosis [3]. Regarding p53 protein activity, it may undergo posttranslational changes, associations with other proteins or regulators and even access to chromatin. It has also been noticed that chemotherapy results in the p53 protein being activated, and the transactivated targets and degree of induction varies with cell and tumor type, leading to the alteration of the cell [4]. The *TP53* gene is commonly mutated in many human cancers and also is highly polymorphic. *TP53* mutation leads to transformation of breast cells [5]. In breast cancer, the mutation rate of *TP53* is running between 20–40% and, in regards the polymorphism, at least 14 different forms were identified, that are associated with cancer risk. Studies revealed that the mutations of *TP53* gene may represent a biomarker of chemotherapy response in advanced breast tumors, in particular to Tamoxifen [6]. Over time, the *TP53* tumor suppressor has been extensively studied to determine its role in resistance to chemotherapy for cancer. Although it is well known his involvement in cell cycle arrest and induction of apoptosis, it remains a completely unfulfilled mystery [7]. In patients with early onset cancers, it was found that in a percentage of 7–20% the mutations were *de novo*, which contrasts with familial breast and ovarian cancers, where the somatic mutations are extremely rare. This supports screening for young breast cancer patients for *TP53* mutations, even if they do not have a family history [1]. Single-nucleotide polymorphisms (SNPs) can affect the correct functioning of the *TP53* gene by increasing the risk of developing and progression of breast tumors, also affecting the response to treatment. Currently, over 80 SNPs are known in humans, of which 90% are located in introns, away from splicing sites or in non-coding exons. Despite of this, the role of SNPs in the risk of developing breast tumors remains unclear [2].

Aim

This study was conducted mainly in order to determine the mutational status of *TP53* gene but also to detect some associated particular aspects from ultrastructural level in invasive ductal mammary carcinoma. We focus our electron microscopic investigations on telocytes (a recently described cell phenotype in almost human organs), here as tumor-

associated stroma cell component. Taken in consideration the importance of tumor-associated stroma in tumor development, we have also investigated some particular aspects at the ultrastructural level of breast tumor tissue, notably concerning telocytes as interstitial cells. So far, telocytes role in different diseases (telocytopathies) is poorly known. That was the reason we investigated the distribution and infrastructural aspects of telocytes in the tumor specimens of invasive mammary ductal carcinoma. We present the results on *TP53* 4–9 gene exons by direct sequencing in fresh breast tumor tissue samples, as well as some related peculiar ultrastructural aspects.

Materials and Methods

In our study, we investigated 22 tissue specimens: all were invasive ductal breast carcinomas collected by surgical resection at the Prof. Dr. Alexandru Trestioreanu Institute of Oncology, Bucharest, Romania, between 2012 and 2016 (surgeon got patients' consent).

Genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Venlo, Germany), and DNA concentration and purity were evaluated using the NanoDrop ND 1000 (ThermoFisher Scientific, Waltham, Massachusetts, USA). The samples were amplified by a polymerase chain reaction (PCR) method and sequenced by a Sanger method using 3500 Genetic Analyzer Instrument (ThermoFisher Scientific, Waltham, Massachusetts, USA). Data analysis was done using a specific software Variant Reporter v2 (ThermoFisher Scientific, Waltham, Massachusetts, USA). To determine the mutation 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 PCR was performed by 2% agarose gel electrophoresis. For transmission electron microscopy (TEM) examination, normal breast tissues, as well as small fragments from the invasive ductal breast carcinomas, were processed following regular TEM technique published elsewhere [8, 9]. Briefly, small fragments of mammary carcinomas were prefixed in 4% buffered glutaraldehyde, washed with 0.05 M sodium cacodylate, postfixed in 2.5% osmium tetroxide, washed with 0.05 M sodium cacodylate, infiltrated with propylene oxide, embedded in Glycid ether (Epon 812 equivalent) and polymerized at 60ºC. Ultrathin sections of 70–100 nm were contrasted and examined by a JEOL JEM 1400 transmission electron microscope.

Results

Genetic analysis

Analyzing the mutational pattern of the *TP53* gene, we detected a frequency of *TP53* somatic mutations of 27.3% (6/22), of which 66.67% represented deletions and 33.3% substitutions. Substitution mutations were found in two (33.33%) cases: a missense mutation in exon 5 (*R175H*; *c.524G>A*) (Figure 1) and a nonsense mutation in exon 6 (*p.R213**; *c.637C>T*) (Figure 2). We noticed a predominance of deletions (66.67%) which were located in exon 4 (*c.213_214del2*) (Figure 3), in exon 5 (*c.398delT*) (Figure 4), and in exon 7 (*c.739_747delAACCGGAGG* and *c.731delG*). Of the total mutations detected, 22.73% (5/22) affected the protein in the DNA binding domain, and 4.54% (1/22) was out of this range.

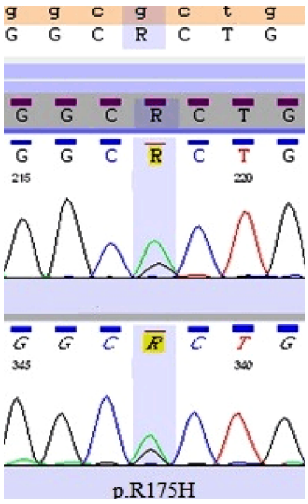

Figure 1 – *Electropherogram of R175H (c.524G>A) missense mutation, TP53 exon 5.*

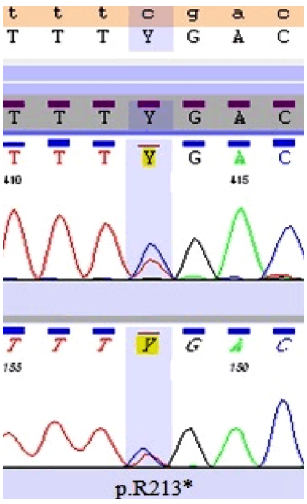

Figure 2 – *Electropherogram of p.R213* (c.637C>T) nonsense mutation, TP53 exon 6.*

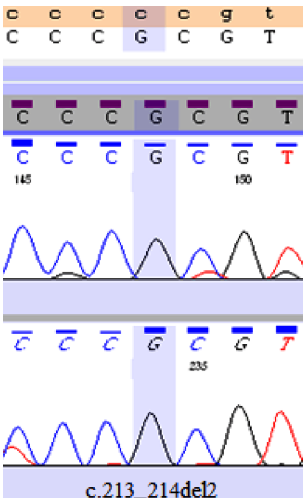

Figure 3 – *Electropherogram of c.213_214del2 deletion, TP53 exon 4.*

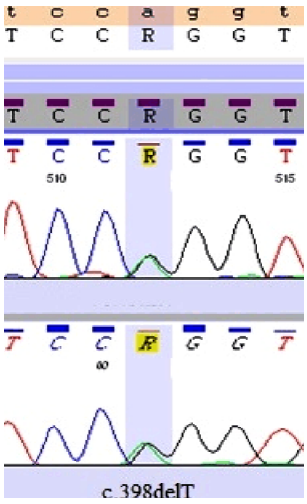

Figure 4 – *Electropherogram of c.398delT deletion, TP53 exon 5.*

Following the direct sequencing of exons 4–9 of both the coding regions and the non-coding regions of the *TP53* gene, we identified five mononucleotide polymorphic variations. Three of them were located in the coding regions of the *rs1042522* gene – *R72P* homozygous mutant *GG*, *TP53* exon 4 (Figure 5), of the *rs1042522* gene – *R72P* heterozygous mutant *CG*, *TP53* exon 4 (Figure 6), *rs372397095* – *P82P* in exon 4 and *rs1800372* – *R213R* in exon 6 (Figure 7) and two in the intron 6 (*rs1625895* and *rs17880604*). Mutant allele corresponding to *rs1042522*, *rs1625895* and *rs17880604* were detected in all samples, either in the homozygous or heterozygous form, while the rest of the *TP53* variants were detected in few samples only.

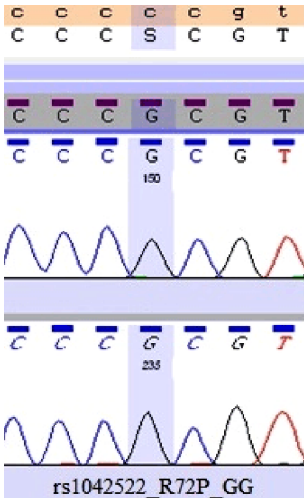

Figure 5 – ***Electropherogram of rs1042522 SNP (R72P, homozygous mutant GG), TP53 exon 4. SNP: Single-nucleotide polymorphism.***

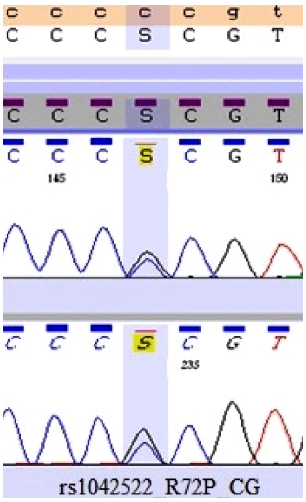

Figure 6 – ***Electropherogram of rs1042522 SNP (R72P, heterozygous mutant CG), TP53 exon 4. SNP: Single-nucleotide polymorphism.***

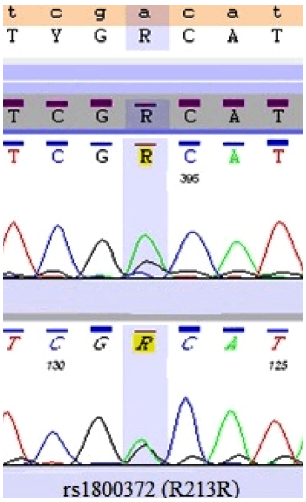

Figure 7 – ***Electropherogram of rs1800372 SNP (R213R, heterozygous mutant AG), TP53 exon 6. SNP: Single-nucleotide polymorphism.***

TEM investigations of tumor samples

In all TEM analyzed invasive ductal mammary carcinomas, frequently epithelial tumor cells exhibited conspicuous intracellular lumina with a plethora of microvillous extensions (Figure 8).

Concerning the fine analysis of the tumor–stroma interface, in this report we focused our observations mainly to the ability of (*i*) tumor cells themselves and (*ii*) telocytes (a recently described cell phenotype) present also inside of the peritumoral stroma to produce small lipoprotein sacks termed extracellular vesicles. In all investigated cases, a plethora of membrane vesicles delivered by tumor cells themselves were detected at the tumor–stroma interface, as is depicted in Figure 9, including the inset. Stromal telocytes with their very long cell extensions termed telopodes are mainly detected closed to the blood vessels and their associated pericytes. To some extent of their profiles, telopodes become each other in very narrow contact (Figure 10) establishing so-called homocellular junctions or with different other cell types, such as tumor cells, fibroblasts, etc., as is visible in Figure 11 (termed heterocellular junctions). Figures 11 and 12 illustrate extracellular vesicles produced by peritumoral telocytes. Figure 12 exhibits extracellular vesicles in different moments of their deliverance from a telopode of a telocyte. Inside of the telopode, membrane vesicles involved in cargo molecules trafficking in different stages of their movement from one plasma membrane surface to another one can be identified. Moreover, clathrin-coated vesicle can be detected inside of the telopodial cytoplasm (Figure 13).

Discussions

The mutational profile of *TP53* gene

Most *TP53* mutations (90%) are identified in the protein-binding domain, thereby disrupting its ability to bind the DNA molecule. The mutations appearing here may 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 [10, 11].

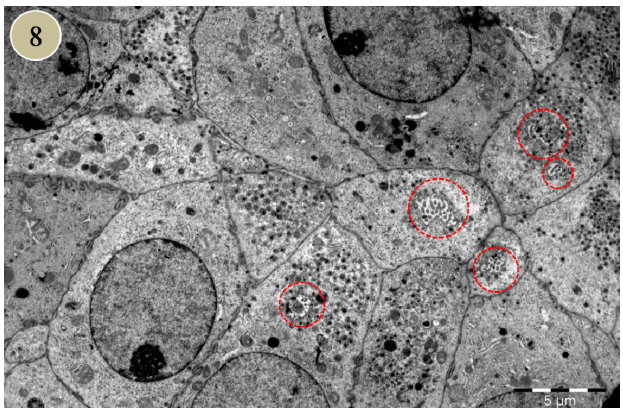

Figure 8 – ***From a field of tumor cells with euchromatic nuclei, many cells exhibited intracellular canaliculi with microvilli (encircled areas).***

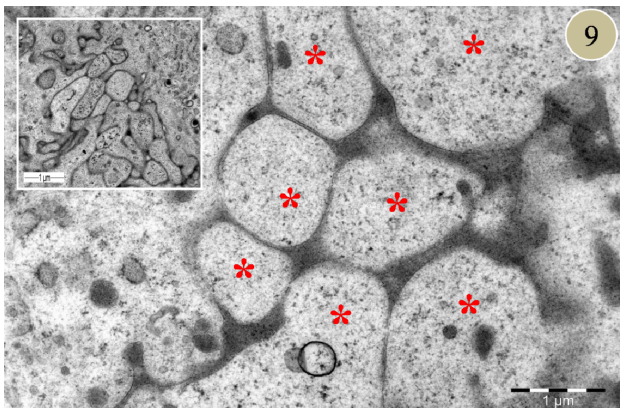

Figure 9 – ***Membrane vesicles (asterisks) of different sizes were detected at the tumor–peritumoral stroma interface. In inset: a TEM overview showing a plethora of membrane vesicles delivered by tumor cells. TEM: Transmission electron microscopy.***

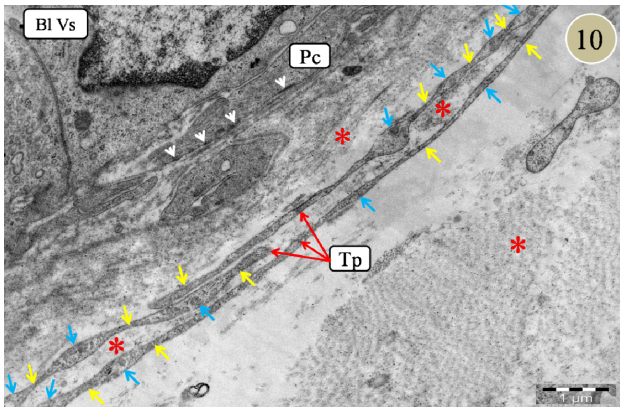

Figure 10 – ***Near a blood vessel (Bl Vs) associated with pericytes (Pc; white head arrows mark the characteristic subplasmalemmal densities) few telopodes (Tp) belonging to different telocytes embedded inside of tumor stroma (asterisks) can be seen. Blue arrows mark the podoms, while yellow arrows mark the podomers.***

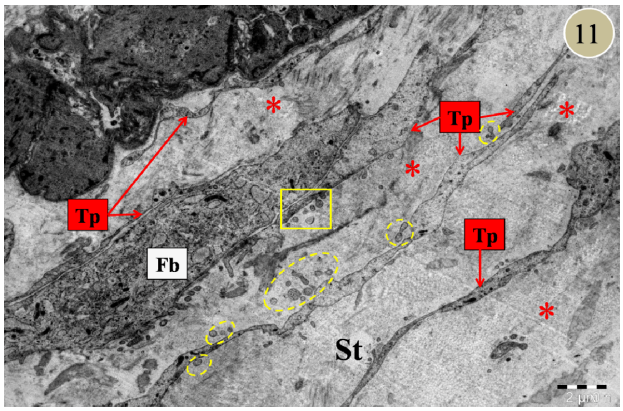

Figure 11 – ***Overview from the tumor–stroma (St) interface in mammary invasive carcinoma. Telopodes (Tp) belonging to different telocytes embedded inside of tumor stroma (asterisks) can be seen. Many extracellular vesicles delivered by Tp (yellow elliptic areas, including framed area detailed in Figure 12) can be detected. Fb: Fibroblast.***

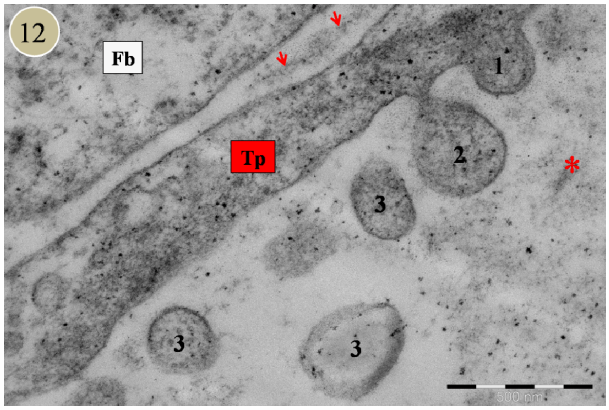

Figure 12 – ***Detail from Figure 11. Extracellular vesicles in different moments of their deliverance from a telopode (Tp) of a telocyte: (1) just budding, (2) still attached to the Tp and (3) already free inside of the tumor stroma. A small patch of basement membrane between the Tp (black arrows) and a fibroblast (Fb) is identified.***

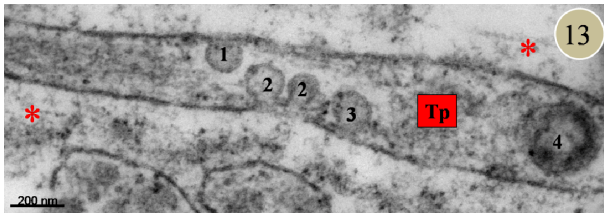

Figure 13 – ***A short segment of a telopode (Tp) embedded into a collagenous matrix (red asterisks) from the tumor stroma. Inside of the Tp, there are membrane vesicles (1–3) in different stages of their movement from one plasma membrane surface to another one. A clathrin-coated vesicle (4) can be detected inside of the telopodial cytoplasm.***

Following the analysis of the distribution of mutations in the *TP53* gene, it was found that 5/6 (83.3%) mutations had the DNA-binding domain localization. Of these, two (33.3%) were identified in exon 5, one (16.6%) was identified in exon 6, and two (33.3%) in exon 7 of the gene. In human cancers, most mutations are of the "missense" type that affect amino acids 102–292 of the DNA-binding domain, the most important hotspots occurring at *R175*, *Y220*, *G245*, *R248*, *R249*, *R273* and *R282* codons. The classification of the mutations in this gene was made on the basis of its function as a transcriptional factor, and two categories can be distinguished: (*i*) contact mutations (*R273H* and *R248W*) occurring at the interface between the p53 protein and the DNA molecule, and (*ii*) structural mutations (*R175H*, *Y220C*, *G245S*, *R248Q*, *R249S*, *R282W*, etc.) leading to the conformational instability of the p53 protein. Considering the *R175H* mutant allele was observed an increased rate of spontaneous tumors, such as lymphomas and sarcomas [12].

Following the investigation of exon 5 of the *TP53* gene, we identified the *c.524G>A* (*R175H*) heterozygous substitution, where Arg is replaced by His, *CGC>CAG* in the hotspot codon 175, this mutation being responsible for modifying the structure of the protein DNA-binding domain. Also, "nonsense" mutations are important because they give rise to truncated and inactive proteins. The most common "nonsense" mutation in the *TP53* gene is *R213**. Repairing such a mutation obviously requires other mechanisms than the "missense" mutations. Amino-glycosides of type G418 and Gentamicin induce DNA sequence reading by *p.R213** mutation, expressing a whole, untreated protein. Despite the fact that treatment with these drugs is limited by their high toxicity, induction of premature reading of stop codons in DNA sequences with "nonsense" mutations is a viable strategy, suggesting the need to study and produce compounds with the same role but with a much more agreeable toxicity to the body [13]. In exon 6 of the *TP53* gene, we detected the *c.637C>T* (*R213**) "nonsense" heterozygous mutation, where Arg is replaced by the stop codon Ter, *CGA>TGA* at position 213. A high frequency in the *TP53* gene is given by hotspot mutations that are often accompanied by indels of type deletions of the secondary allele. Frameshift deletions lead to a modified DNA reading frame, with the obtaining of truncated proteins, sometimes very short, sometimes extremely long and most likely non-functional. They vary in length from 1 to 37 nucleotides, but the most common ones are those with the removal of 2–8 nucleotides [14].

In the investigated mammary tumors group, we identified four deletions: (*i*) *c.213_214del2*, which is a double deletion of nucleotide C that affects codons 71 and 72 of exon 4; (*ii*) *c.398delT* (*p.M133fs*37*), frameshift deletion of the T nucleotide at position 133 of exon 5; (*iii*) *c.739 747delAACCGGAGG* (*p.2247-R249delNRR*) 9-nucleotide type "inframe" deletion that affects codons 247, 248 and 249 of exon 7; (*iv*) *c.731delG* (*p.G244-fs*3*), frameshift deletion of G nucleotide at position 244 in exon 7. Except for deletion from exon 4, all other deletions affected the DNA-binding domain.

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 action of mutations in the *TP53* gene, and in particular the substitutions of a single nucleotide 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 *TP53* polymorphisms can have notable effects that can be measured [15, 16]. The most studied *TP53* polymorphism is *rs1042522* (*R72P*; *16397C>G*) identified in exon 4 in a proline-rich region. This polymorphism is a $G \rightarrow C$ transition at the 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. Structurally, the *TP53* gene which contains proline in codon 72 is different from that it contains arginine, because tumors that containing 72Pro are smaller and develops more easily compared to 72Arg tumors. Apparently, both forms are at increased risk for tumor cell development, but a higher frequency was observed for 72Arg-containing cells. Because *R72P* affects the transactivation domain, it may lead to alteration of the protein expression. It can also influence both the transcriptional activity and the structure of the protein due to the localization in a hydrophobic zone of the affected codon [17]. In our study, the *rs1042522* polymorphic variation was identified with homozygous *GG* genotype in 59.09% (13/22) of the samples, and with the heterozygous *CG* genotype in 40.9% (9/22) of the samples. In exon 4 of *TP53* gene, we identified the *rs372397085* (*P82P*; *c.246G>A*) SNP, a synonym polymorphism that represents a $G \rightarrow A$ substitution at codon 246, $CCG \rightarrow CCA$, which are located in a proline-rich region. There is no evidence in the literature about the influence of this polymorphic variation on the risk of breast cancer.

By analyzing several tumor suppressors, the synonymous polymorphic variations do not appear to have a high incidence, but in the separate investigation of *TP53* gene, this type of polymorphism appears to be quite high and affects directly the nucleotides located in the splicing sites, three such nitrogenous bases seems to be affected in the *TP53* gene. Despite the increased rate of these types of variations, they have no clinically significant effect [18]. In the coding region of exon 5, we detected a rare synonymous variation, *rs1800372* (*R213R*; *c.639A>G*) representing a substitution at position 639 where arginine is also replaced by arginine, $CGA \rightarrow CGG$. Over time, the researchers have analyzed the *TP53* haplotypes of the *rs1042522*, *rs1625895* and *rs17878362* polymorphic variations in relation to the risk of breast carcinoma. Although the results are insufficient, it has been observed that the *rs1625895* (*IVS6+62A>G*) polymorphism is associated with an increased risk due to the minor allele. These three SNPs present a risk associated with the occurrence of invasive breast cancer, but with the observation that patients with heterozygous tumors have a lower risk than patients whose tumors are homozygous, considering the major alleles. It has been observed that at early ages, genetic variations occurring in the *TP53* gene are associated with an increased risk of breast cancer [19].

In our study, we identified *rs1625895* SNP in both, heterozygous (*AG*) and homozygous (*GG*) forms, representing an $A \rightarrow G$ transition at position 61 in the non-coding region of intron 6 of the *TP53* gene. One of the highly studied intronic polymorphisms of this gene is *rs17880604* (*G13964C*). The *13964G* variant of this SNP helps maintain the stability of both messenger ribonucleic acid (mRNA) and p53 protein stability, while *13964C* variant has been analyzed in the context of establishing its role in a wide range of human cancers, such as breast, colon and ovarian cancer, but insufficient data were obtained to associate this type of polymorphism with cancer risk [20].

Following the investigation of exons 5–6 of the *TP53* gene, we detected the $G \rightarrow C$ transition at the level of intron 6 in both heterozygous (*GC*) and homozygous (*GG*) forms of *rs17880604* SNP.

Electron microscopic investigations

Our electron microscopic investigations of the invasive ductal mammary carcinoma tumors showed a lot of conspicuous intracellular lumina. Goldenberg *et al.* (1969) [21] emphasized that such peculiar aspects were detected almost exclusively only in infiltrating human carcinoma.

In this study, we observed that very often, a plethora of shedding membrane vesicles can be counted, delivered especially by the invasive mammary carcinoma tumor cells, at their interface with tumor stroma. Such delivered microvesicles by tumor cells were associated with paracrine signaling [22]. By their delivered exosome-derived microRNAs (miRNAs) from tumor cells to the recipient cells *via* fusion and internalization, they can regulate target gene expression [23, 24]. Inside of recipient cells, the mRNA shuttled by exosomes was shown to be translated into protein [25, 26]. Mention must be made that next-generation sequencing method, RNA from exosomes secreted by human breast cancer cells can be characterized [27]. Endogenous p53 protein in exosomes can be transferred to p53-deficient cells and consecutively can suppress growth and proliferation of p53-deficient cells [28].

Concerning the tumor stroma of our investigated cases of invasive ductal mammary carcinoma, the most interesting original ultrastructural aspect observed here is related to the telocytes, a new cell phenotype reported to be present inside of the almost normal tissues as stromal/interstitial cells [29, 30], as well as inside of some tumors as cutaneous basal carcinoma tumor stroma [22] and mammary carcinoma [31, 32]. A plethora of reports emphasize that telocytes as a new described interstitial cell type play an important role in tissue homeostasis maintenance, intercellular signaling, tissue regeneration, angiogenesis, as well as in the pathogenesis of different diseases (telocytopathies) [29, 30, 33–36], so that there

are many reasons to include telocyte phenotype in the histological nomenclature [37]. Telocytes' role is still a matter of discussion, but mention must be made that preliminary observations are very exciting [29, 38]. Recently, we identified telocytes inside of the tumor stroma associated to mammary carcinoma and we described especially homo- and heterocellular junctions which telocytes performed [31, 32]. In this study, we observed that many telocytes become by their telopodes in very close vicinity with tumor cells *per se* of invasive ductal mammary carcinoma and very often established heterocellular communications with other tumor stroma cell types (fibroblasts, small blood vessels).

Here, we emphasize the ability of telocytes also to deliver extracellular vesicles inside of mammary tumor stroma. Taken in consideration results issued from many published papers that extracellular vesicles contain an appreciable diversity of epigenetic (macro)molecules (proteins, segments of genomal DNA, multiple forms of RNA, including miRNA, lipids, metabolites) all mediators involved in cell–cell and cell–extracellular matrix interactions [30, 34, 39–41], we underline also their potential as important players in mammary tumor progression. We may only speculate that, to some extent, could be an inter-play between *TP53* gene alterations and telocytes status as tumor-associated cell component.

Conclusions

In our study group of breast cancer patients, the frequency of *TP53* somatic mutations was similar with that published in *International Agency for Research on Cancer* (IARC) database (22.8%). The mutation type distribution showed a predominance of deletions and a reduced frequency of substitutions comparing with *IARC* database. We intend to extend the investigations of the *TP53* alterations in breast cancer and their clinical significance. Particular infrastructural abnormalities were also recorded by TEM analysis, especially shedding membrane vesicles delivered by mammary tumor cells. Moreover, our observations concerning homo- and heterocellular communications established by telocytes as tumor stroma component underline putative role of telocytes in invasive growth of the mammary tumor.

Conflict of interests

The authors declare no conflict of interests.

Acknowledgments

This work was supported by the Romanian Academy, Project No. RO1567/IBB07/2017. A part from this paper was presented at the 11th Annual Congress of the Romanian Medical Association, Bucharest, 20–22 April 2017 and is a part from a PhD Thesis elaborated by Corina Elena Mihalcea at the School of Advanced Studies of the Romanian Academy (SCOSAAR)/2017.

Electron microscopic investigations were performed by a JEOL JEM 1400 transmission electron microscope, supported by DIBIOCLIM Project.

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Received: June 19, 2017

Accepted: September 5, 2020