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Case Report

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OCT-4 and DAZL expression in benign and malignant breast lesions: a prospective case control study

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Abstract

Purpose: The impact of cancer stem cells on breast cancer tumor biology has been poorly investigated during the last years. In the present study we sought to identify the expression of OCT-4 and DAZL, two prominent stem cell biomarkers, on malignant breast lesions.

Methods: A retrospective analysis of patients that underwent simple or radical mastectomy was performed. Immunohistochemical expression of OCT-4 and DAZL was sought, and real time PCR was also applied in deparaffinized tissue samples retrieved from the pathology bank of our hospital.

Results: Eighteen women aged 43-84 years old (median 57 years) were included in the present study. Of those, 10 women had invasive ductal carcinoma, 4 women had in situ ductal carcinoma and 4 women had invasive lobular carcinoma. Immunohistochemistry of tissue samples did not reveal expression of OCT-4, whereas DAZL was expressed in only 3 samples. Real time PCR was available in 13 samples as the control gene (G6PD) was not expressed in 5 samples. The 13 studied breast cell samples showed positive expression for the Oct-4 gene. In the same population the expression of DAZL varied significantly with 9 samples showing complete absence of expression and 4 providing a small signal in the last cycles, indicating barely detectable expression. The expression was significant in lobular carcinoma cases compared to cases with invasive or in situ ductal carcinoma.

Conclusion: The findings of our study indicate the difficulty of retrieving reliable tissue samples following the process of deparaffinization and points towards the need for prospective case control studies that will be based on fresh tissue samples for the analysis of stem cell markers. Nevertheless, it also indicates a potentially increased expression of stemness markers in lobular carcinoma samples compared to ductal cancer cases, an observation that requires further research in the future.

Key words: breast cancer; stem cells; OCT-4; DAZL; DCIS; lobular carcinoma

Introduction

Breast cancer is the most frequent gynecologic malignancy that accounts for approximately 12.5% of new cancer cases worldwide. It is estimated that in 2020 breast cancer accounted for 2.3 million cases of whom 685.000 died from the disease[1]. Early diagnosis of the disease is feasible with current screening techniques which include mammography as well as magnetic resonance imaging (MRI) which is used for cases that are with a hereditary background as well as those with dense breasts in whom standard mammography cannot reliably rule out the presence of disease[2,3]. Several factors predispose to the condition including obesity, early menarche, delayed menopause, advancing age, smoking as well as hormonal previous hormonal therapy[4].

Hereditary breast cancer is encountered in approximately 5-10% of cases and breast cancer 1 and 2 (BRCA1 and BRCA2) genes account for the majority of these cases[5,6]. Other tumor suppression genes, including the TP53, the checkpoint kinase 2 (CHECK 2)and the phosphatase and tensin homolog (PTEN) genes have been also implicated in the pathogenesis of breast cancer, however, to date, they do not constitute targets for potential screening as their penetrance in breast cancer patients is limited[5].

The expression of stemness markers has been considered in tumor biology, as it is believed that some forms of cancer may be triggered by the presence of stem cells that originate in the tissue microenvironment and which undergo a process of differentiation that is considered to be responsible for tumorigenesis[7,8]. In previous experiments we observed the presence of Octamer-binding transcription factor 4 (OCT-4) and Deleted in Azoospermia (DAZL) genes in premalignant and cancerous cervical lesions[9,10], indicating that these genes may be considered potential targets for screening. To date, current research in the field of breast lesions remains extremely limited, however, breast cancer cell lines seem to express OCT-4 in experimental in vitro models[11]; therefore, indicating that stem cells might actually originate in the mammary gland and can potentially become targets for potential future treatment modalities. This study aims to detect the presence and expression of the Oct-4 and Dazl genes in cancerous breast lesions using two different laboratory methods.

Methods

Patients – surgical specimens

The present study was based on a retrospective analysis of patients that were offered simple or radical mastectomy for breast cancer between 2007 and 2013 in Metropolitan Hospital, Greece. Surgical preparations were initially fixed in 10% neutral formaldehyde buffer, which is widely used for sterilization and temporary preservation of human tissues. Then, the breast tumors were embedded in paraffin blocks, and the paraffin sections were stained with hematoxylin-eosin and observed under a microscope. This allow to have at our disposal samples of the tumors and their adjacent normal tissues in paraffin blocks, and by searching the medical records and reading the existing histological reports (tumour size, grade of malignancy, status of surgical margins) we were able to review the representative histological sections and select the appropriate ones to carry out expression of the investigated genes. All women were followed-up at 5-years following the procedure and also asked to provide blood samples which were used for the determination of hormonal index by the enzyme immunoassay ELISA method. The study was approved by the institutional review board of our hospital and is in accordance with the declaration of Helsinki for medical research involving human subjects. Informed written consent was provided by all participants.

Immunohistochemistry

Immunohistochemistry of available tissue samples

was performed following deparaffinization and a 64 minute duration of cell conditioning under the influence of the ULTRA CC1 solution, which is a prediluted tissue pretreatment solution with the main objective of revealing antigenic epitopes. Following that titration was performed with addition of the antigen (100μl) which was suitable diluted and incubated for a period of 32 minutes. Counterstaining of histological samples with hematoxylin eosin was used to allow nucleus staining. Following that we used, post counterstain, with bluing reagent for 4 minutes, which is an aqueous solution of lithium carbonate buffer intended to blue stain the spots stained with hematoxylin on the slides. Staining was performed with the use of Benchmark Ultra Ventanta (Roche™) using the Ultra View DAB kit (Roche Diagnostics™) and the anti-Oct3/4 and anti-DAZL antibodies provided by SIGMA Diagnostics Inc™ in a dilation of 1:100 and 1:200 respectively.

RNA extraction

In the present work, the total RNA isolation method using the RNeasy FFPE kit (Qiagen) was used for the samples we had from the paraffin cubes. Consequnetly, 150 μl of PKD and 10 μl of Proteinase K were added to the sample. Following that, an incubation period of 10-15 minutes in water bath at 55 °C and 15 minutes at 80 °C took place. At this temperature and with the help of the PKD lysis solution the tissue was lysed and nucleic acid modifications by the formaldehyde present were prevented. The samples were then transferred and centrifuged for 15 minutes at 13,500 rpm. The supernatant liquid was collected in an elution tube and 320μl of the RBC debinding solution were added containing salt and guanidine solution and 720μl of pure ethanol, to create optimal binding conditions. The samples were then collected and 700μl were transferred to the RNeasy microcolumn which were centrifuged for 1 minute at 10,000 rpm. The process was followed by two consecutive washes with 500μl RPE, centrifuging at 10,000 rpm for 1 and 2 minutes respectively and a centrifugation at maximum speed for 5 minutes to prevent contamination with unwanted substances and extract the total RNA sample. Finally, samples were transferred to new tubes and 14μl of RNase free water were added to collect the RNA samples.

Reverse transcription and real time PCR

The RNA extracted from cervical cells was used to obtain complementary DNA (cDNA) with reverse transcription, using Super Script First-Strand Synthesis System for RT-PCR (Invitrogen, USA) according to a standardized protocol that has been previously described[12]. Briefly, quantitative Real-time PCR was performed with Light Cycler 480 II (Roche Molecular Biochemicals, Mannheim, Germany) using the SYBR Luna Universal qPCR Master Mix kit (New England Biolabs Inc.). The solution for OCT4, DAZL, Nanog, and G6PD as control gene consisted of 10 μL Master mix, 1 μL of Forward Primer (10 pm/μL), 1 μL of Reverse Primer (10 pm/μL), 3 μL H_2O. The primers used for this trial were provided by Eurofins Genomics. The primers and the hybridization probes used for this trial were provided by TIB-MOLBIOL (Table 1). The qRT-PCR reaction cycling profile was 30 s at 95 °C, 1 cycle, 5 s at 95 °C, and 30 s at 60 °C, 40 cycles. The 2−ΔΔCT method was used to calculate the relative transcript abundance. All qRT-PCR reactions were repeated twice.

Results

Overall, 18 women aged 43-84 years old (median 57 years) were included in the present study. Of those, 10 women had invasive ductal carcinoma, 4 women had in situ ductal carcinoma and 4 women had invasive lobular carcinoma. The 5-year survival rates for in situ ductal carcinoma was 75%, of invasive ductal carcinoma 80% and of invasive lobular carcinoma 100%.

Table 1. Oligonucleotides used during Real Time PCR

	DELETED IN AZOOSPERMIA-LIKE (DAZL) GENE
Primers	**Sequence**
DAZL S	gCTATgTTgTACCTCCggTTA
DAZL A	gCCCgACTTCTTCTAAAgATg
Probes	**Sequence**
DAZL FL	TTTCAgAgggTggAgTAgCTTCATg-FL
DAZL LC	640-ACTgAACATTCATTTggACAACTTCAgCT p
	OCT-4
Primers	**Sequence**
OCT-4 S	AAgCAgAAACCCTCgTg
OCT-4 A	ACTCggACCACATCCCT
Probes	**Sequence**
OCT-4 FL	AACAAATTCTCCAggTTgCCTC-FL
OCT-4 LC	640-CACTCggTTCTCgATACTggTTCgC p

Immunohistochemistry of tissue samples did not reveal expression of OCT-4, whereas DAZL was expressed in only 3 samples. The expression of the G6PD gene following RT-PCR in most of the women's samples was relatively low, while in five of them no expression was found even after repeated attempts. These numerical data partly suggest the difficulty of isolating genetic material from paraffin-embedded tissues and the eventual loss of gene expression over time. The quantitative real time PCR provided results for 13 women which were included in the final analysis. The mean body mass index of included patients was 31.2 kg/m^2 (range 23-38). Six active smokers were identified in the dataset and 4 more past smokers. No participants reported a history that could reveal a hereditary background of breast cancer.

Amplification curves for Oct-4 and DAZL genes are depicted in Figure 1. The 13 studied breast cell samples showed positive expression for the Oct-4 gene. This expression fluctuated in the price range with the lowest value at 140 copies and the highest at 2001 copies. In the same population the expression of DAZL varied significantly with 9 samples showing complete absence of expression and 4 providing a small signal in the last cycles, indicating barely detectable expression.

Discussion

The findings of our study suggest the existence of

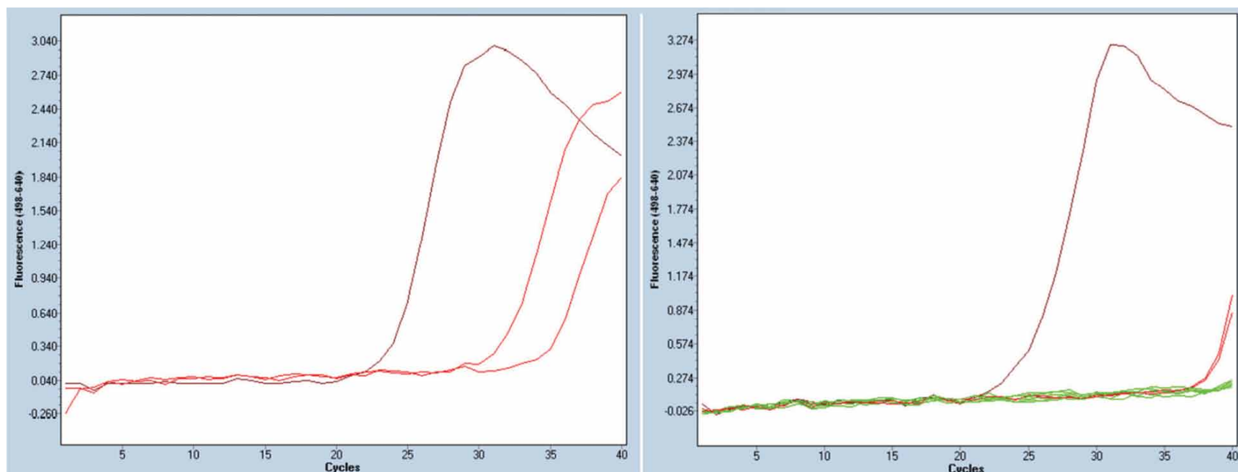

Figure 1. Left side. Amplification curve of OCT-4 revealing expression range of the gene. Right side. Amplification curve of DAZL revealing lack of expression for the majority of included samples and a small, barely detectable signal in the four samples.

stemness markers in the mammary gland of patients with malignant breast lesions. However, the amplification strategy and the performance of real time PCR seems to be limited by the process of deparaffinization as the retrieval of genetic material from paraffin blocks seems to be problematic. It should be, therefore, considered to base further research in fresh frozen sections that might be more reliable. On the other hand, the actual large range of fluctuations in the expression of Oct-4 renders possible the existence of a strong correlation between cancerous lesions and the expression of stemness markers. It should be noted that of the 13 samples that were analyzed all cases that referred Oct-4 expression the highest expression was observe in lobular carcinoma samples, whereas ductal carcinoma samples had lower expression values and in situ ductal lesions even lower, although the relatively small sample size of this group should be considered a strong limitation.

The presence of breast cancer stem cells is not an entirely novel issue, as previous studies have observed the existence of stemness markers in malignant breast lesions[13-15]. Currently, research is mainly focused on the expression of CD44 and ALDH1 which seem to be prominent cancer stem cell biomarkers in several tissue samples and that seem to be potential therapeutic targets, primarily in solid tumors[16,17]. It should be noted that breast tumors that express these biomarkers seem to be more aggressive, as they have a larger tumor size and are more likely to be associated with higher stage at presentation as well as lymphovascular space involvement, a feature that indicates an aggressive tumor phenotype[15,18].

The rationale for searching the expression of OCT-4 and DAZL in the present study relies in their universal expression in cancer stem cells of solid tumors[19,20] as well as in the fact that at least OCT-4 expression seems to be related to chemoresistance which directly affects patients survival[21]. Considering the useful features of those genes, and the absence of scientific data we chose to evaluate their expression in breast cancer samples that were retrospectively retrieved from paraffin embedded blocks. The minimal expression detected in ductal breast carcinomas can be explained by looking at their origin in general. Ductal carcinomas arise from breast duct cells that multiply uncontrollably. In contrast, lobular carcinomas arise from an excessive proliferation of the cells of the glandular lobules of the breast. Considering this it may be speculated that the DAZL gene is expressed in focused anatomical sites of the breast and that it is related to the uncontrolled proliferation that leads to carcinogenesis.

Study limitations

The present study is limited by the relatively small number of included patients that prohibits a direct comparison of stemness markers among patients with pre-invasive and invasive breast lesions or even among patients with ductal and lobular carcinomas. Nevertheless, our findings may support future research as they clearly depict the difficulties in collecting appropriate RT-PCR samples from paraffin embedded blocks, indicating the need for analysis of fresh tissue samples. Furthermore, they indicate the potential expression of OCT-4 which seems to be particularly expressed in lobular carcinomas, hence denoting the need to evaluate its diagnostic accuracy in detecting patients with risk factors for disease relapse in this particular group of patients.

Conclusion

The present work showed that the results of immunohistochemistry and RT-PCR concerning the expression of the OCT-4 and DAZL genes in paraffin embedded samples of malignant breast lesions do not agree. However, real-time qPCR was able to detect a minimal expression of the DAZL gene and a higher expression of the OCT-4 gene in grade 2 invasive lobular carcinoma samples, which was

considerably higher compared to ductal carcinoma samples. Further research is needed to evaluate the performance of RT-PCR in fresh tissue samples and to evaluate the differential expression of OCT-4 in patients with lobular cancer compared to those with ductal cancer as well as to evaluate its potential association with histology features that predict an aggressive tumor biology, including stage and grade of lesions as well as the presence of lymphovascular space involvement.

Conflict of Interest

None for all authors

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None

References

1. Arnold M, Morgan E, Rumgay H, Mafra A, Singh D, Laversanne M, et al. Current and future burden of breast cancer: Global statistics for 2020 and 2040. Breast. 2022;66:15-23.
2. Gunduru M, Grigorian C. Breast Magnetic Resonance Imaging. [Updated 2022 Aug 29]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2023 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK539727/.
3. Ren W, Chen M, Qiao Y, Zhao F. Global guidelines for breast cancer screening: A systematic review. Breast. 2022;64:85-99.
4. Fakhri N, Chad MA, Lahkim M, Houari A, Dehbi H, Belmouden A, et al. Risk factors for breast cancer in women: an update review. Med Oncol. 2022;39:197.
5. Mahdavi M, Nassiri M, Kooshyar MM, Vakili-Azghandi M, Avan A, Sandry R, et al. Hereditary breast cancer; Genetic penetrance and current status with BRCA. J Cell Physiol. 2019;234:5741-50.
6. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA Cancer J Clin. 2021;71:209-49.
7. Guo W, Lasky JL, 3rd, Wu H. Cancer stem cells. Pediatr Res. 2006;59:59r-64r.
8. Bajaj J, Diaz E, Reya T. Stem cells in cancer initiation and progression. J Cell Biol. 2020;219.
9. Stefanidis K, Patta J, Pergialiotis V, Stefanidi D, Loutradis D. Imiquimod treatment effectively reduces the percentage of viable cells in a cervical carcinoma cell line but does not affect the expression of HLA-G or OCT-4. J Stem Cells. 2015;10:217-23.
10. Stefanidis K, Pergialiotis V, Christakis D, Patta J, Stefanidi D, Loutradis D. OCT-4 and DAZL expression in precancerous lesions of the human uterine cervix. J Obstet Gynaecol Res. 2015;41:763-7.
11. Tai MH, Chang CC, Kiupel M, Webster JD, Olson LK, Trosko JE. Oct4 expression in adult human stem cells: evidence in support of the stem cell theory of carcinogenesis. Carcinogenesis. 2005;26:495-502.
12. Pittokopitou S, Mavrogianni D, Pergialiotis V, Pappa KI, Antsaklis P, Theodora M, et al. Expression of Stemness Markers in the Cervical Smear of Patients with Cervical Insufficiency. Cells. 2023;12.
13. Ricardo S, Vieira AF, Gerhard R, Leitão D, Pinto R, Cameselle-Teijeiro JF, et al. Breast cancer stem cell markers CD44, CD24 and ALDH1: expression distribution within intrinsic molecular subtype. J Clin Pathol. 2011;64:937-46.
14. Rabinovich I, Sebastião APM, Lima RS, Urban CA, Junior ES, Anselmi KF, et al. Cancer stem cell markers ALDH1 and CD44+/CD24- phenotype and their prognosis impact in invasive ductal carcinoma. Eur J Histochem. 2018;62.
15. Kapucuoğlu N, Bozkurt KK, Başpınar Ş, Koçer M, Eroğlu HE, Akdeniz R, et al. The clinicopathological and prognostic significance of CD24, CD44, CD133, ALDH1 expressions in invasive ductal carcinoma of the breast: CD44/CD24 expression in

breast cancer. Pathol Res Pract. 2015;211:740-7.

16. Wei Y, Li Y, Chen Y, Liu P, Huang S, Zhang Y, et al. ALDH1: A potential therapeutic target for cancer stem cells in solid tumors. Front Oncol. 2022;12:1026278.
17. Tomita H, Tanaka K, Tanaka T, Hara A. Aldehyde dehydrogenase 1A1 in stem cells and cancer. Oncotarget. 2016;7:11018-32.
18. Currie MJ, Beardsley BE, Harris GC, Gunningham SP, Dachs GU, Dijkstra B, et al. Immunohistochemical analysis of cancer stem cell markers in invasive breast carcinoma and associated ductal carcinoma in situ: relationships with markers of tumor hypoxia and microvascularity. Hum Pathol. 2013;44:402-11.
19. Zhang Q, Han Z, Zhu Y, Chen J, Li W. The Role and Specific Mechanism of OCT4 in Cancer Stem Cells: A Review. Int J Stem Cells. 2020;13:312-25.
20. Zhang F, Liu R, Zhang H, Liu C, Liu C, Lu Y. Suppressing Dazl modulates tumorigenicity and stemness in human glioblastoma cells. BMC Cancer. 2020;20:673.
21. Mohiuddin IS, Wei SJ, Kang MH. Role of OCT4 in cancer stem-like cells and chemotherapy resistance. Biochim Biophys Acta Mol Basis Dis. 2020;1866:165432.

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