Original article

Neoadjuvant Chemotherapy Affects TFF3 Peptide Expression in Luminal B Subtype of Breast Cancer – A Pilot Study

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Abstract

Aim: Trefoil factor family 3 (TFF3) peptide is normally expressed by epithelial cells in breast ducts, but it is also associated with different pathological conditions, including breast cancer. It is considered a marker of poor prognosis and associated with increased resistance to chemotherapy. Data on the effect of chemotherapy on TFF3 peptide expression are scarce. The aim of this pilot study was to assess suitability of research on this topic for large-scale studies.

Methods: Formalin-fixed, paraffin-embedded samples of core biopsies and of surgically removed tumors from patients with luminal B subtype of breast cancer were used for immunohistochemical analysis. Changes in TFF3 peptide and Ki-67 expression and microvessel density (MVD) values before and after chemotherapy were analyzed, as well as the association between TFF3 peptide expression and Ki-67 expression and MVD values.

Results: Significant reduction in TFF3 and Ki 67 expression was observed after chemotherapy, while MVD values did not differ significantly before and after chemotherapy. The association of TFF3 peptide expression and Ki-67 expression and TFF3 peptide expression and MVD values was not significant before or after chemotherapy.

Conclusion: The data obtained in this pilot study suggest that a large-scale study is justified, and it other breast cancer subtypes should be included.

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Introduction

Early detection of breast cancer enables better treatment. Also, adequate methods for monitoring response to treatment help in planning further treatment. Modern breast cancer diagnostics include core biopsy with immunohistochemical characterization of tumor tissue. According to St. Galen consensus, breast cancer is classified into following surrogate subtypes: luminal A, Her2-positive luminal B, Her2-negative luminal B, Her2-enriched and triple negative breast cancer. Such classification helps with prognosis and choice of therapy and is based on expression of estrogen receptor, progesterone receptor, Her2 and Ki 67 in the tumor [1]. Still, there is a need for further characterization of oncogenes and molecular markers connected to the onset, progression and relapse of breast cancer [2].

In modern breast cancer therapy, neoadjuvant chemotherapy is considered a standard approach in an increasing number of clinical settings (e. g. in patients with tumor subtypes that are highly sensitive to chemotherapy and in patients that have tumors > 2 cm and/or metastases in axillar lymph nodes), also allowing for cancer down-staging and helping with breast conservation [3,4]. It also alters expression of different markers in cancer cells, such as hormone receptors, Her2, claudin-1 and claudin-3 [5,6].

Trefoil Factor Family (TFF) is a group of three small secretory peptides – TFF1, TFF2 and TFF3 peptide, mostly secreted by epithelial cells of various mucosal sites. Their major role is mucosal protection, and they are known to affect cell migration, apoptosis, angiogenesis and immune response [7]. Of the three peptides, TFF3 peptide is the most widespread in the human organism, and is normally expressed by epithelial cells in breast ducts [8,9]. However, presence of TFF peptides is also associated with different pathological conditions, including breast cancer. TFF3 peptide is expressed in different subtypes of breast cancer, most predominantly in hormone receptor-positive subtypes and is considered a marker of poor prognosis because it promotes metastatic seeding and neoangiogenesis [10,11]. Our previous research showed that TFF3 peptide expression was the highest in luminal B subtype, moderately differentiated cancers (grade II) and in tumors with moderate expression of Ki 67, implying that TFF3 peptide might be considered a possible marker for determining tumor status, which might influence the choice of therapy [12]. Presence of TFF3 peptide in breast cancer cells is associated with increased resistance to chemotherapy, while cancers with complete response to chemotherapy have lower or no TFF3 peptide expression [13]. Despite the growing interest in TFF3 peptide research in the context of breast cancer diagnostics and therapy, there are no data on the effect of chemotherapy on TFF3 peptide expression in breast cancer tissue. Therefore, we launched a pilot study in order to assess the suitability of a large-scale study.

Material and Methods

Study design and material

This was a cross-sectional pilot study performed on tissue samples archived at the Department of Pathology and Cytology, University Hospital Centre Zagreb. Tissue samples of luminal B subtype of breast cancer were used in the study. They were taken during routine diagnostic and therapy procedures from women whose treatment included neoadjuvant chemotherapy. There were 22 tumor samples overall; 10 were Her2-negative, and 12 were Her2-positive. Also, samples from 5 normal breast tissue controls were included for comparison of TFF3 peptide expression. For each tumor, a core biopsy sample (pre-chemotherapy) and a sample of surgically removed tumor (post-chemotherapy) were used. Chemotherapy protocols of each patient are available in the Supplemental table. Changes in TFF3 peptide and Ki-67 expression and in microvessel density (MVD) values before and after chemotherapy were analyzed, as well as the association between TFF3 peptide expression and Ki-67 expression and MVD values.

Immunohistochemical staining

For each patient, archived immunostained slides (hormone receptor positivity, Ki-67 status and Her2 status) were revised in order to confirm the cancer subtype. Paraffin blocks of samples that met the criteria to be included in the study (luminal B subtype) were cut on a microtome (Leica, SM2000R, Leica Biosystems, Nussloch, Germany) into 3-5-µm-thick slides and mounted onto adhesive slides suitable for immunohistochemistry. Slides were stained for TFF3 peptide and CD34 endothelial marker, and Ki-67 staining scores (share of immunopositive nuclei) were obtained from the aforementioned archived slides. Antigen retrieval was performed using the Dako PT Link device (Dako, Agilent Technologies, Santa Clara, USA) with the EnVision FLEX Target Retrieval Solution, Low pH for TFF3 peptide and Ki-67 staining, and the Dako EnVision FLEX Target Retrieval Solution, High pH for CD34 staining. After that, the slides were stained in the Dako TechMate device using standard protocols as per the manufacturer's instructions. Purified monoclonal mouse anti-human primary antibody to TFF3 peptide was used at 1:1000 dilution (Sigma-Aldrich, St. Louis, USA), for Ki-67 monoclonal mouse, anti-human antibody was used at 1:75 dilution (Dako), and for CD34 monoclonal mouse, anti-human antibody was used at 1:50 dilution (Dako). For immunostaining visualization, the Dako EnVision FLEX kit was used. All slides were counterstained with hematoxylin, dehydrated and coverslipped using the Entellan covering medium (Merck, Darmstadt, Germany). For TFF3 peptide staining, appendix tissue was used as positive control, while placenta tissue and the appendix tissue with primary antibody omitted were used as negative control. The appendix tissue was used as positive and negative control for CD34 (primary antibody omitted on negative controls).

TFF3 peptide tissue expression level was assessed using a modified Quick score method that incorporates the share of immunopositive cells as well as staining intensity and reports the staining on a scale of 0 to 7 [12,14]. CD34 staining was used to obtain MVD (number of small blood vessels per surface unit under a 10 × objective) [15]. Digital photographs were taken using the Axiocam 305 color camera mounted on a Zeiss Axio imager A2 light microscope and ZEN 2.3 lite (blue edition) software (Carl Zeiss Microscopy GmbH, Jena, Germany).

The investigations were carried out following the rules of institutional and national ethical standards and the rules of Helsinki Declaration of 1975, revised in 2013. The study has been approved by the ethical committees of the University Hospital Centre Zagreb (Class: 8.1-18/256-2, No.: 02/21 A6) and the Faculty of Medicine Osijek (No. 2158-61-07-19-10).

Statistical analysis

Patient age was expressed using mean values and standard deviation, while all other data were expressed using the median and interquartile range. For comparison of dependent samples, the non-parametric Wilcoxon test was used, and the Kendall's Tau test was used for the analysis of the association of numerical variables. Coefficient of determination was obtained by linear regression after correlation testing. For comparison of independent samples, the Mann-Whitney U test was used.

Data on Ki 67 expression after chemotherapy were unavailable for two patients, and these were excluded from the analyses that included Ki 67 expression. The statistical analysis was done using the MedCalc software (18.11.6, MedCalc Software bvba, Ostend, Belgium) and a P-value less than 0.05 was considered statistically significant.

Results

The mean age of cancer patients was 53.1 years (st. dev. 13.2) at the time of diagnosis; the mean age of subjects with no cancer was 55.6 years (st. dev. 9.07). TFF3 peptide staining was detected both in breast cancer tissue and in normal breast tissue.

The analysis of Quick score values for TFF3 peptide expression in patients with luminal B subtype of breast cancer showed a significant

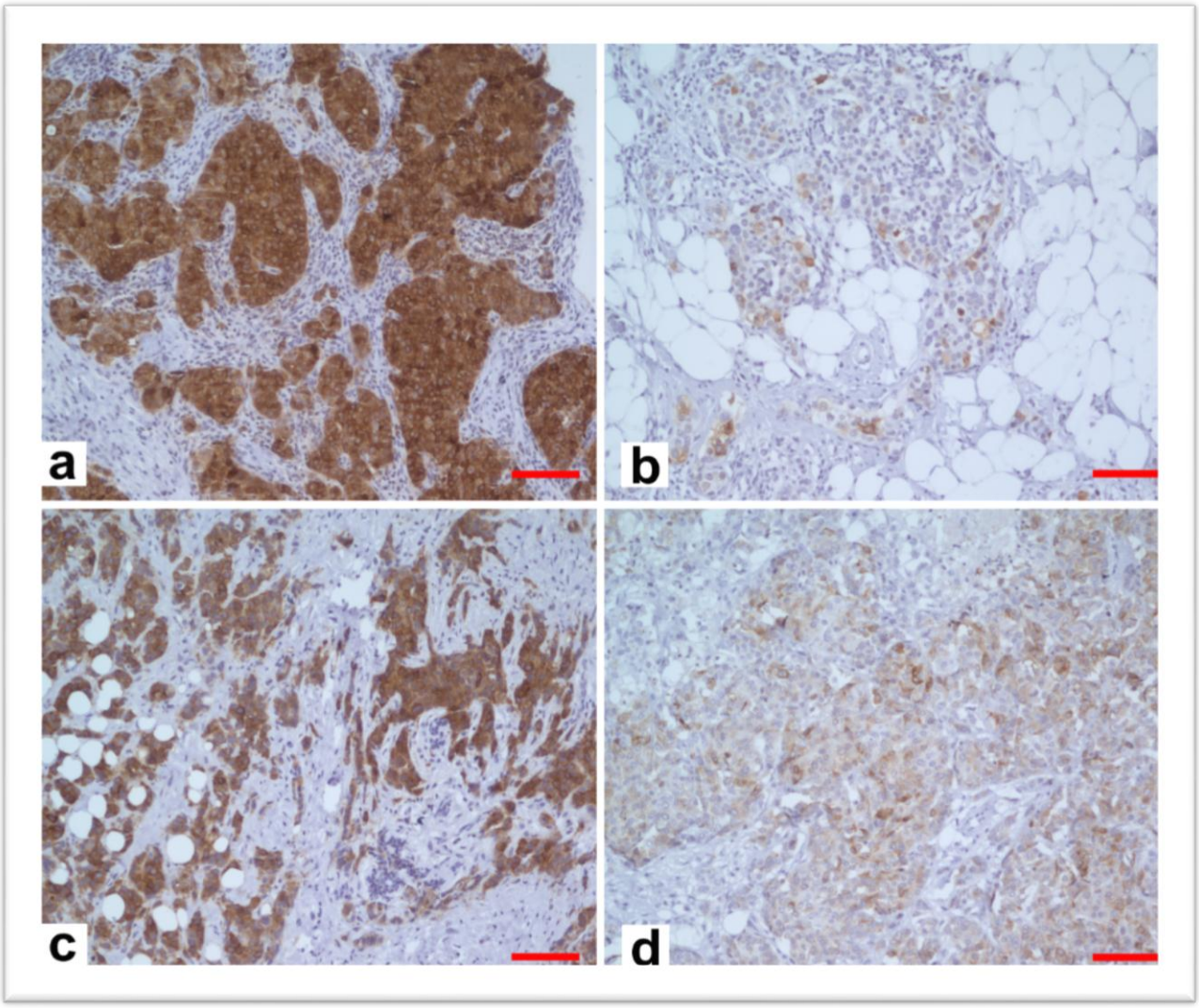
reduction in TFF3 peptide immunostaining after chemotherapy (Table 1, Figure 1).

Table 1. TFF3 peptide expression, Ki 67 expression and MVD values in luminal B breast cancer subtype before and after chemotherapy

	N	Before chemotherapy	After chemotherapy	P-value*
TFF3 Quick score	22	6 (6 – 7)	5 (4 – 6)	0.005
Ki-67 score (%)	20	31 (25 – 38.5)	11.5 (4.8 – 21)	< 0.001
MVD value (/mm²)	22	79.9 (62.3 – 96.3)	71.3 (54.8 – 99.4)	0.59

* Wilcoxon test

Figure 1. Immunohistochemical staining of luminal B breast cancer tissue for TFF3 peptide



a) Her2-positive cancer before chemotherapy (core biopsy), Quick score = 7; b) the same tumor after chemotherapy (surgical excision), Quick score = 3; c) Her2-negative cancer before chemotherapy (core biopsy), Quick score = 6; d) the same tumor after chemotherapy (surgical excision), Quick score = 4. Scale bar: 0.1 mm

Compared to normal breast epithelium, Quick score values did not differ significantly in breast cancer before chemotherapy, while after chemotherapy, the median Quick score value was lower and close to significant values (P = 0.92 and 0.08, respectively; Mann-Whitney U test). Ki 67 expression in patients with breast cancer was significantly lower after chemotherapy (Table 1). Microvessels were identified by CD34 staining. On several slides, tissue fibroblasts caused some false positivity, which was excluded from MVD calculation. MVD values did not differ significantly before and after chemotherapy (Table 1).

Supplemental Table 1. The table shows all cancer patients with their corresponding neoadjuvant therapy regimens and durations used in this study.

Patient code	Neoadjuvant therapy regimen (type and duration; notes)
2 HER2-	AC-T 6 months
3 HER2-	AC-T 6 months
4 HER2-	AC-T 6 months
5 HER2-	AC-T 6 months
6 HER2-	AC-T 5 months
7 HER2-	AC-T 7 months
9 HER2-	AC-T 6 months
10 HER2-	AC-T 6 months
11 HER2-	AC-D (3 x AC + 1 x D) 2 months; discontinued due to intolerance
12 HER2-	AC-D 2 months
1 HER2+	ACdd 2 months + PHP
2 HER2+	ACdd-THP* 6 months
3 HER2+	AC-T 5 months**; trastuzumab 7 months
4 HER2+	AC-T 6 months
6 HER2+	AC-THP 6 months
8 HER2+	AC-PHP 6 months
9 HER2+	AC-PHP 5 months
10 HER2+	AC-TH 7 months
11 HER2+	AC-PHP 6 months
12 HER2+	AC-T + 4 PH 7 months
13 HER2+	AC-T + PH duration unknown
14 HER2+	AC-THP 6 months

* trastuzumab taken for only 3 months

** regimen discontinued after the 4th paclitaxel dose

Abbreviations: AC-D – doxorubicin, cyclophosphamide, docetaxel; ACdd – dose-dense AC regimen; AC-T – doxorubicin, cyclophosphamide, paclitaxel; AC-PH – doxorubicin, cyclophosphamide, pertuzumab, trastuzumab; AC-PHP: doxorubicin, cyclophosphamide, paclitaxel, trastuzumab, pertuzumab; AC-TH – doxorubicin, cyclophosphamide, paclitaxel, trastuzumab; AC-THP – doxorubicin, cyclophosphamide, docetaxel, trastuzumab, pertuzumab

The association of TFF3 peptide expression and Ki 67 expression, as well as of TFF3 peptide expression and MVD values, was not significant before or after chemotherapy, although the P-value for the association of TFF3 peptide expression and MVD values before chemotherapy was close to the significance level (Table 2).

Table 2. Association of TFF3 peptide with Ki-67 expression and MVD values in luminal B breast cancer subtype before and after chemotherapy

		Tau	95% CI of Tau	P-value*	R^2
Before chemotherapy					
TFF3	Ki-67	0.04	-0.27 – 0.40	0.84	0.017
	MVD	0.28	-0.19 – 0.58	0.07	0.090
After chemotherapy					
TFF3	Ki-67	-0.25	-0.52 – 0.16	0.11	0.031
	MVD	0.25	-0.11 – 0.53	0.11	0.074

* Kendall's Tau test

Discussion

This pilot study revealed a significant reduction in TFF3 peptide and Ki 67 expression after neoadjuvant chemotherapy, while no significant changes in MVD values were found. Until now, no data have been available on the effect of chemotherapy on TFF3 peptide expression in breast cancer cells or its association with Ki-67 and MVD before and after chemotherapy. Since it is known that overexpression of TFF3 peptide affects proliferation of breast cancer cells, stimulates angiogenesis and contributes to chemotherapy resistance, information about the effect of chemotherapy on TFF3 peptide expression should be helpful in understanding its role in breast cancer [11,13].

Although expression of TFF3 peptide increases the chances of local invasion and metastatic seeding, its role in breast cancer is still not fully understood [10]. Some researchers argue that it should not be considered an oncogene, but a normal breast protein "abused" by cancer cells, and similar conclusions could be drawn from other diseases involving TFF3 peptide expression [9,16]. Due to its effect on disease progression and chemotherapy results, TFF3 peptide is considered a potential therapeutic target in certain types of breast cancer, and current research looks promising [13,17,18]. Nevertheless, since TFF3 peptide has important physiological roles in the human organism, a careful risk/benefit assessment is needed. For example, TFF3 peptide is important for maintenance of intestinal mucosa, which is often affected by chemotherapy, hence targeting TFF3 peptide might worsen certain chemotherapy side-effects [19].

Newly-formed small blood vessels can be an early predictor of metastatic potential, and CD34 is one of the most important endothelial markers used for small vessel detection [20]. MVD values did not change significantly in this research, and the correlation between MVD and TFF3 Quick score was weak. Hence, although TFF3 peptide stimulates angiogenesis, it seems that a reduction in TFF3 expression after chemotherapy did not affect tumor vascularity. A reduction in Ki 67 expression was expected, although it was interesting to see that there was no significant association between TFF3 peptide and Ki 67 expression before or after chemotherapy, probably due to different mechanisms regulating their expression.

Limitations to this pilot study were the analysis of only one subtype of breast cancer and sample size. Nevertheless, based on the data obtained, we believe a large-scale study including other breast cancer subtypes is justified. Since TFF3 is a secretory protein found in different body liquids, there is also a possibility

of a quantitative evaluation of TFF3 levels (e.g. in serum) by diagnostic methods. Such sampling is less invasive than a core or surgical biopsy and could be used as a screening method in certain conditions, which should be further investigated in the future.

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Competing interests. None to declare.

References

1. Goldhirsch A, Winer EP, Coates AS, Gelber RD, Piccart-Gebhart M, Thürlimann B, Senn H-J, Panel members. Personalizing the treatment of women with early breast cancer: highlights of the St Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2013. Ann Oncol 2013; 24: 2206–23. doi: 10.1093/annonc/mdt303

2. Inoue K, Fry EA. Novel Molecular Markers for Breast Cancer. Biomark Cancer 2016; 8: 25–42. doi: 10.4137/BIC.S38394

3. Bartsch R, Bergen E, Galid A. Current concepts and future directions in neoadjuvant chemotherapy of breast cancer. Memo 2018; 11: 199–203. doi: 10.1007/s12254-018-0421-1

4. Cardoso F, Kyriakides S, Ohno S, Penault-Llorca F, Poortmans P, Rubio IT, Zackrisson S, Senkus E. Early breast cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol 2019; 30: 1194–220. doi: 10.1093/annonc/mdz173

5. Skálová H, Hájková N, Majerová B, Bártů M, Povýšil C, Tichá I. Impact of chemotherapy on the expression of claudins and cadherins in invasive breast cancer. Exp Ther Med 2019; 18: 3014–24. doi: 10.3892/etm.2019.7930

6. Li C, Fan H, Xiang Q, Xu L, Zhang Z, Liu Q, Zhang T, Ling J , Zhou Y, Zhao X , Cui Y. Prognostic value of receptor status conversion following neoadjuvant chemotherapy in breast cancer patients: a systematic review and meta-analysis. Breast Cancer Res Treat 2019; 178: 497–504. doi: 10.1007/s10549-019-05421-7

7. Bijelić N, Belovari T, Tolušić Levak M, Baus Lončar M. Localization of trefoil factor family peptide 3 in epithelial tissues originating from the three germ layers of developing mouse embryo. Bosn J Basic Med Sci. 2017; 17: 241-47. doi: 10.17305/bjbms.2017.1838.

8. Madsen J, Nielsen O, Tornoe I, Thim L, Holmskov U. Tissue Localization of Human Trefoil Factors 1, 2, and 3. J Histochem Cytochem 2007; 55: 505–13.

9. Ahmed ARH, Griffiths AB, Tilby MT, Westley BR, May FEB. TFF3 is a normal breast epithelial protein and is associated with differentiated phenotype in early breast cancer but predisposes to invasion and metastasis in advanced disease. Am J Pathol 2012; 180: 904–16. doi: 10.1016/j.ajpath.2011.11.022

10. Pandey V, Wu Z-S, Zhang M, Li R, Zhang J, Zhu T, Lobie PE. Trefoil factor 3 promotes metastatic seeding and predicts poor survival outcome of patients with mammary carcinoma. Breast Cancer Res BCR 2014; 16: 429. doi: 10.1186/s13058-014-0429-3

11. Lau W-H, Pandey V, Kong X, Wang X-N, Wu Z, Zhu T, Lobie PE. Trefoil Factor-3 (TFF3) Stimulates De Novo Angiogenesis in Mammary Carcinoma, both Directly and Indirectly via IL-8/CXCR2. PloS One 2015; 10: e0141947. doi: 10.1371/journal.pone.0141947

12. Tolušić Levak M, Mihalj M, Koprivčić I, Lovrić I, Novak S, Bijelić N, Baus-Lončar M, Belovari T, Kralik K, Pauzar B. Differential Expression of TFF Genes and Proteins in Breast Tumors. Acta Clin Croat 2018; 57: 264–77. doi: 10.20471/acc.2018.57.02.06

13. Al-Salam S, Sudhadevi M, Awwad A, Al Bashir M. Trefoil factors peptide-3 is associated with residual invasive breast carcinoma following neoadjuvant chemotherapy. BMC Cancer 2019; 19: 135. doi: 10.1186/s12885-019-5316-y

14. Detre S, Saclani Jotti G, Dowsett M. A "quickscore" method for immunohistochemical semiquantitation: validation for oestrogen receptor in breast carcinomas. J Clin Pathol 1995; 48: 876–8.

15. Svagzdys S, Lesauskaite V, Pavalkis D, Nedzelskienė I, Pranys D, Tamelis A. Microvessel density as new prognostic marker after radiotherapy in rectal cancer. BMC Cancer 2009; 9: 95. doi: 10.1186/1471-2407-9-95

16. Belovari T, Bijelić N, Levak MT, Baus Lončar M. Trefoil factor family peptides TFF1 and TFF3 in the nervous tissues of developing mouse embryo. Bosn J Basic Med Sci 2015; 15: 33–7. doi: 10.17305/bjbms.2015.251

17. Chong Q-Y, You M-L, Pandey V, Banerjee A, Chen Y-J, Poh H-M, Zhang M, Ma L, Zhu T, Basappa S, Liu L, Lobie PE. Release of HER2 repression of trefoil factor 3 (TFF3) expression mediates trastuzumab resistance in HER2+/ER+ mammary carcinoma. Oncotarget 2017; 8: 74188–208. doi: 10.18632/oncotarget.18431

18. Poh HM, Chiou YS, Chong QY, Chen R-M, Rangappa KS, Ma L, Zhu T, Prem Kumar A, Pandey V, Soo-Chin Lee B, Lobie PE. Inhibition of TFF3 Enhances Sensitivity—and Overcomes Acquired Resistance—to Doxorubicin in Estrogen Receptor-Positive Mammary Carcinoma. Cancers 2019; 11: 1528. doi: 10.3390/cancers11101528

19. Beck PL, Wong JF, Li Y, Swaminathan S, Xavier RJ, Devaney KL, Podolsky DK. Chemotherapy- and radiotherapy-induced intestinal damage is regulated by intestinal trefoil factor. Gastroenterology 2004; 126: 796–808.

20. Zaha DC. Significance of immunohistochemistry in breast cancer. World J Clin Oncol 2014; 5: 382–92. doi: 10.5306/wjco.v5.i3.382

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