Fişa suspiciunii de plagiat / Sheet of plagiarism's suspicion	Indexat la: 74/07

Opera suspicionată (OS) Suspicious work	Opera autentică (OA) Authentic work

OS	D.M. Pleşan, M.Georgescu, C.V.Georgescu, N.Pătrană, T.Nină, C.Pleşan, Immuno-histochemical evaluation of hormone receptors with predictive value in mammary carcinomas, In: Rom J.Morphol Embryol, 2011, 52(4):1331-1336.
OA	D.M.Pleşan, C.V.Georgescu, S.Ciobotea, N.Pătrană, M.Laura, Pleşan C., Immuno-histochemical evaluation of hormone receptors with predictive value in mammary carcinomas, In: Current Health Sciences Journal, vol.35, No.3, 2009, p.184-189.

Incidența minimă a suspiciunii / Minimum incidence of suspicion	
p.1331:01 - p.1336:45d	p.184:01 - p.189:56d
p.1332s:Table 1	p.185d: Tabel fn
p.1332d:Table 2	p.185d: Tabel fn
p.1332d:Table 3	p.187s: Tabel fn
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Precizare:

Prin notația p.1331:01 - p.1336:56 se înțelege că fragmentul de text preluat fără indicarea provenienței în opera suspicionată este cuprins integral între rândul 1 al pag.1331 şi rândul 45 al pag.1336.

Rom J Morphol Embryol 2011, 52(4):1331–1336

http://www.rjme.ro/RJME/resources/files/52041113311336.pdf

ORIGINAL PAPER

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

Immunohistochemical evaluation of hormone receptors with predictive value in mammary carcinomas

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Abstract

Aim: Immunohistochemical evaluation of hormone receptors (ER, PR) and correlation of immunohistochemical and morpho-clinical data. *Materials and Methods*: The study was performed on paraffin-embedded and HE-stained tissues originating from 100 cases of invasive mammary carcinoma. Monoclonal antibodies, anti-estrogen and anti-progesterone receptors, were used for the immunohistochemical study. The detection system was EnVision HRP and the visualization system was 3,3'-diaminobenzidine tetrahydrochloride (DAB). The evaluation of the result was performed using the Allred score. *Results*: The majority of the studied cases (57%) expressed both types of hormone receptors and in 32% of the cases the hormone receptors were completely absent. The rest of the cases presented a heterogeneous phenotype: 7% presented the ER-/PR+ phenotype and 4% the ER+/PR- phenotype. Compared with the classical phenotype (ER+/PR-), ER+/PR- tumors were more frequent at patients over 50-year-old. The tumors with ER+/PR- were larger than the ER+/PR+ and they were of the invasive ductal carcinoma type with an Allred score for ER under 6. *Conclusions*: The predictive value is amplified when the ER status is correlated with the PR status because the heterogeneous phenotypes are identified, especially the ER+/PR- phenotype, which have an aggressive behavior and the lowest response to tamoxifen therapy.

Keywords: mammary carcinoma, hormone receptors, immunohistochemistry, predictive factors.

Introduction

Hormone receptors for estrogen (ER) and for progesterone (PR) are biomarkers with prognostic and predictive value in mammary carcinoma therapy. ER and PR are commonly used for more than 30 years to conduct the therapy of mammary carcinoma [1].

The cellular effects of estrogens are the result of their action on two receptors: ERα and ERβ. Estrogen receptors are members of a larger class of nuclear receptors called ligand-inducible transcription factors [1]. The factors that modulate the transcriptional activity of α-receptors are used today for the therapy of various diseases such as mammary carcinoma, osteoporosis and cardiovascular diseases [2]. Synthetic ligands such as tamoxifen and raloxifen belong to a group of molecules known as selective modulators for estrogen receptors that act as estrogen antagonists [3]. The discovery of the second receptor, known as ERβ, indicates that the estrogen acting mechanism is more complex than anticipated. The human receptor ERβ has a very similar structure to ERα. ERβ is expressed in the normal mammary epithelium and in most mammary carcinomas [4]. The vast majority of ERβ+ mammary carcinomas are also ERα- and PR+, without lymph node metastases, well differentiated and with low proliferative activity [5].

The progesterone receptors belong to the same class of nuclear receptors, the "ligand-inducible transcription factors". There are two forms PR-B and PR-A, transcription products of the same gene, but by using different promoters [6]. Molecular analysis has proven that although some genes are regulated through both isoforms, the majority of them are regulated through only one isoform, predominantly through PR-B [6].

The quantification of ER and PR is a controversial problem [1, 7–9]. Initial studies that validated the evaluation of estrogenic receptors through immunohistochemistry established a level of 10% positive cells that correlate with 10 fmol/mg of biochemically detected protein. The positivity level of 10%, irrespective of the immunomarker intensity, has been accepted and has been the most used level to immunohistochemically interprets ER and PR [10]. Despite this, following studies have shown that patients with tumors that express ER in more than 1% of neoplastic cells, with moderate or strong intensity, are responsive to anti-estrogenic therapy [2].

The score recommended now to interpret the hormonal receptor immunomarking is the one Allred had suggested, according to which the cases that have a total score of ≥3 are considered positive.

Materials and Methods

This study was conducted on a number of 100 cases with invasive mammary carcinoma. The tissues were fixed in 10% neutral formalin and included in paraffin blocks. Serial sections were cut and initially stained with Hematoxylin–Eosin (HE), and afterwards using immunohistochemistry.

The immunohistochemical technique was performed on 4 μm thick sections that were placed on superfrost slides. Sections were then dewaxed in three xylene baths (5 minutes each), hydrated in successive baths of absolute alcohol, 96%, 90% and 75% (5 minutes each) and a distilled water bath for 5 minutes. Antigen retrieval was performed using microwaves and EDTA buffer (pH 8), for 20 minutes. This stage was followed by the inhibition of the endogenous peroxidase with 6% hydrogen peroxide for 5 minutes. After washing them with plenty of water, the sections were washed for 5 minutes with PBS and incubated with the primary antibody for one hour at 37^{0}C.

The primary antibodies used were ER (monoclonal mouse anti-human estrogen receptor α, clone 1D5; DAKO Cytomation, Denmark) and PR (monoclonal mouse anti-human progesterone receptor, clone Pgr 636; DAKO Cytomation, Denmark) in a 1:50 dilution.

After washing them with PBS–Tween, the sections were incubated with the EnVision HRP detection system for 30 minutes at room temperature. After being washed with water, the signal was detected using 3,3'-diaminobenzidine (DAB).

The counterstaining was performed using Mayer Hematoxylin, and then the sections were dehydrated in ethanol, clarified and mounted with Canada balm. In each determination, both positive and negative external controls were included control.

Evaluation method

For the assessment of the immunohistochemical stains, only the nuclear labeling was taken into consideration. In order to quantify the hormonal status the Allred score was used. This takes into consideration both the percentage of labeled cells and the medium intensity of the nuclear labeling.

The Allred score is the sum of the percentage score (percentage of labeled cells) and the intensity score (labeling intensity) (Tables 1 and 2).

Table 1 – *Percentage score*

Positive cell percentage	Proportion score
0	0
0–1%	1
1–10%	2
10%–1/3	3
1/3–2/3	4
2/3–100%	5

Table 2 – *Intensity score*

Labeling intensity	Intensity score
No labeling	0
Low intensity	1
Moderate intensity	2
High intensity	3

Tumors with an Allred score ≤2 were considered negative, and the ones with an Allred score >2 were positive.

Results

In the present study, 100 cases of invasive mammary carcinoma were analyzed. The patients were aged between 22 and 75 years (average age 53 years). From these, 37% were under 50-year-old and 63% were at least 50-year-old. The sizes of the primary mammary tumors were smaller or equal with 2 cm in 35% of the cases and larger than 2 cm in 65% of the cases. The examination of the HE stained slides under the light microscope, led to the identification of 90 cases of invasive ductal mammary carcinoma and 10 cases of invasive lobular mammary carcinoma (Table 3). From the 90 cases with invasive ductal mammary carcinoma, 47 showed areas of intraductal carcinoma. The assessment of hormonal receptors was performed according to the existing guidelines in the literature, only in areas of invasive carcinoma.

Table 3 – *Characteristics of patients and tumors*

Characteristics	No. of patients	Percentage [%]
Age [years]:		
▪ <50	37	37
▪ ≥50	67	67
Size of tumor [cm]:		
▪ ≤2	35	35
▪ >2	65	65
Histological type:		
▪ Invasive ductal carcinoma	90	90
▪ Invasive lobular carcinoma	10	10

The estrogen receptors (ER) were positive (Allred score ≥3) in 63% of the cases, and the progesterone receptors (PR) in 64% of the cases. Most cases expressed the hormonal receptors in a heterogeneous manner, thus a very careful evaluation of the entire histological product was required. Therefore, in same cases, the labeling of tumor cells had different intensities from one area to another, and the percentage of positive cells also varied from area to area. The labeling heterogeneity was more obvious in the case of progesterone receptors. In all cases, a nuclear positivity was noticed in the normal ductal epithelial cells adjacent to the tumor (internal control), validating the accuracy of the technique used and the results that were obtained.

In relation to the histological type, the invasive ductal carcinoma expressed estrogen receptors in 53 cases (58.88%), and progesterone receptors in 57 cases (63.33%), while the invasive lobular carcinomas expressed estrogen receptors in eight cases (80%), and progesterone receptors in seven cases (70%).

Most cases (57%) presented both types of receptors with an ER+/PR+ phenotype (Figures 1–4). 32% of the

cases had no hormonal receptors with an ER-/PR- phenotype (Figures 5 and 6). The rest of the cases (11%) had a heterogeneous phenotype. Thus, 7% of the cases were ER-/PR+ (Figures 7 and 8), and 4% of the cases were ER+/PR- (Figures 9 and 10, Table 4).

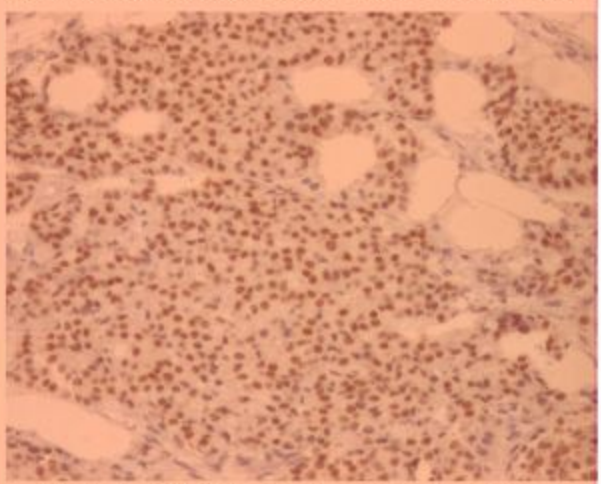

Figure 1 – *ER+/PR+ phenotype. ER positive in tumor (ER immunolabeling, ×200).*

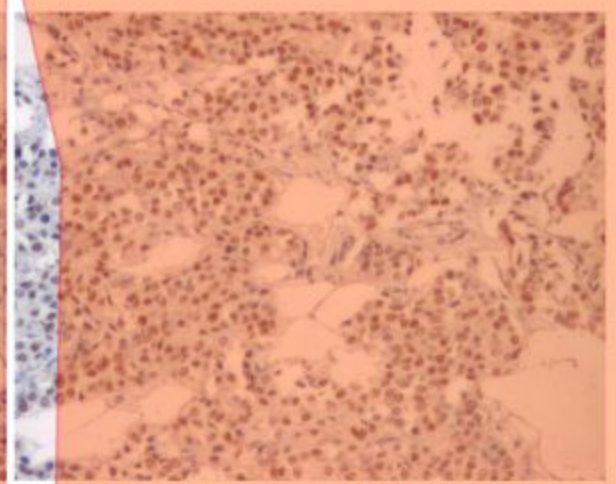

Figure 2 – *ER+/PR+ phenotype. PR positive in tumor (PR immunolabeling, ×200).*

Figure 3 – *ER+/PR+ phenotype. ER positive in tumor: another case (ER immunolabeling, ×200).*

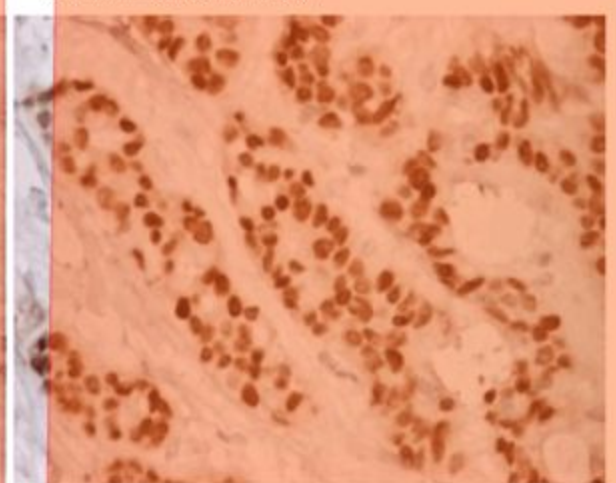

Figure 4 – *ER+/PR+ phenotype. PR positive in tumor: another case (PR immunolabeling, ×200).*

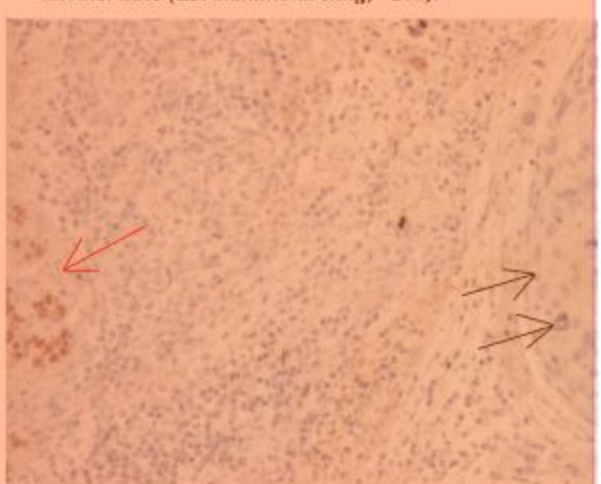

Figure 5 – *ER-/PR- phenotype. ER negative in tumor (double arrow) and positive in internal control (normal ducts – simple arrow) (ER immunolabeling, ×100).*

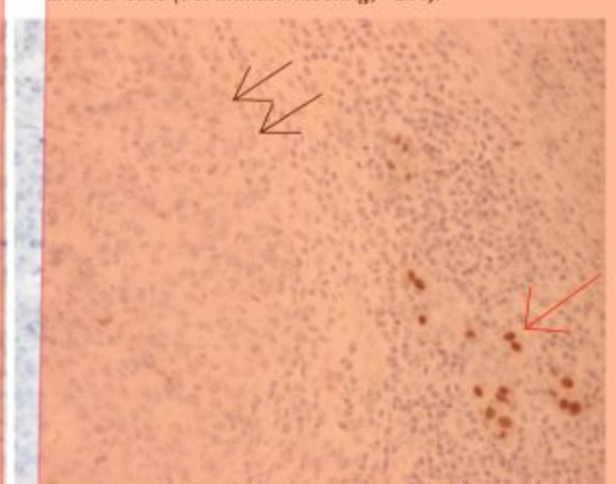

Figure 6 – *ER-/PR- phenotype. PR negative in tumor (double arrow) and positive in internal control (normal ducts – simple arrow) (PR immunolabeling, ×100).*

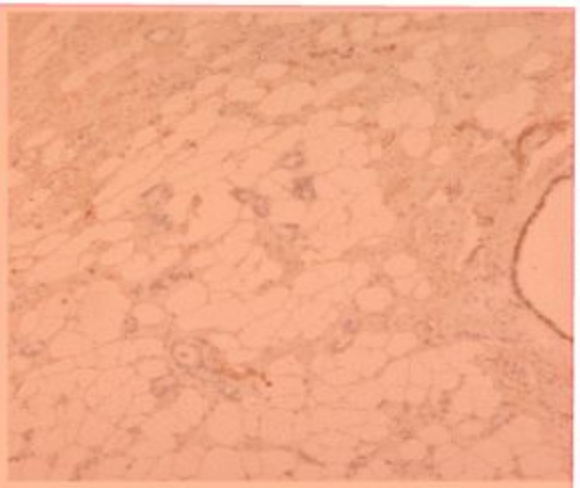

Figure 7 – *ER-/PR+ phenotype. ER negative in tumor and positive in normal ducts (ER immunolabeling, ×100).*

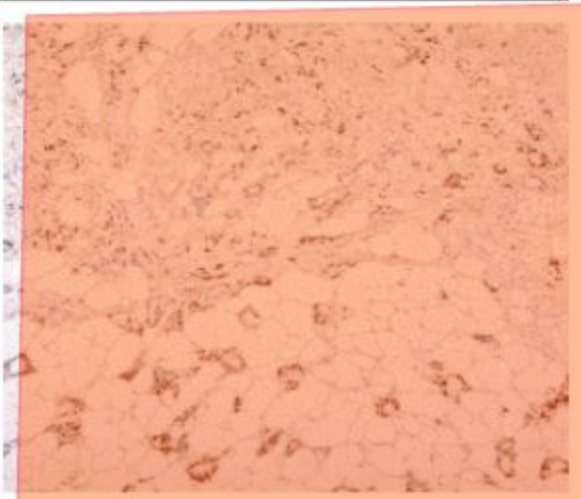

Figure 8 – *ER-/PR+ phenotype. PR positive in tumor (PR immunolabeling, ×100).*

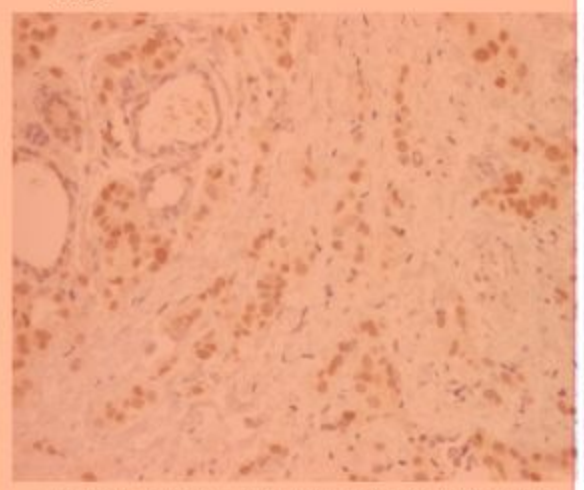

Figure 9 – *ER+/PR- phenotype. ER positive in tumor and internal control (ER immunolabeling, ×200).*

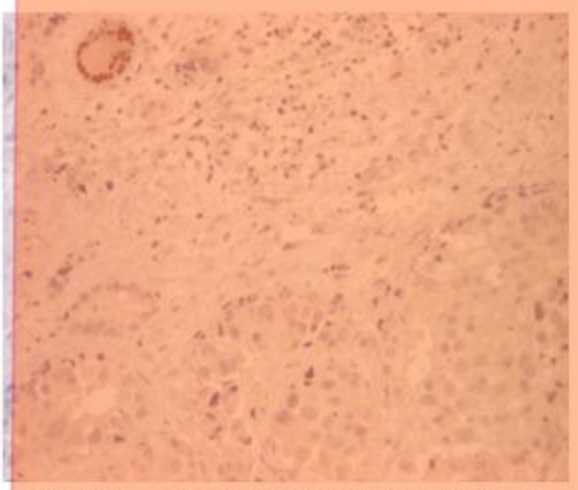

Figure 10 – *ER+/PR- phenotype. PR negative in tumor and positive in internal control (normal ducts) (PR immunolabeling, ×200).*

Table 4 – *Expression of hormonal receptors according to the histological type*

	Invasive ductal carcinoma No. (%)	Invasive lobular carcinoma No. (%)	Total cases No. (%)
ER+/PR+	50 (55.55)	7 (70)	57 (57)
ER-/PR-	30 (33.33)	2 (20)	32 (32)
ER-/PR+	7 (7.77)	0 (0)	7 (7)
ER+/PR-	3 (3.33)	1 (10)	4 (4)

The ER+/PR+ phenotype was seen in 55.55% (50 cases) of the invasive ductal carcinomas *vs.* 70% (seven cases) of the invasive lobular carcinomas, and the ER-/PR- phenotype was present in 33.33% (30 cases) of the ductal carcinomas *vs.* 20% (two cases) of the lobular carcinomas. The heterogeneous ER-/PR- phenotypes were detected in 7.77% (seven cases) of the invasive ductal carcinomas *vs.* no case of invasive lobular carcinomas, and ER+/PR- phenotype was present in only 3.33% (three cases) of the ductal carcinomas *vs.* 10% (one case) of lobular carcinomas. We can see that in lobular carcinomas the estrogen receptors are expressed at a much higher rate than in the ductal ones (80% of cases *vs.* 58.88%), while the expression of progesterone receptors was relatively similar in the two histological types (70% *vs.* 63.32%).

From the point of view of the response to anti-hormonal therapy, evolution and prognosis, a particular phenotype is the ER+/PR- one. Therefore, we analyzed this phenotype in relation to the classical ER+/PR+ phenotype according to tumor and patient characteristics (Table 5).

Table 5 – *Characteristics of cases with ER+/PR+ vs. ER+/PR- phenotype*

Characteristics	ER+/PR+ 57 cases	ER+/PR- 4 cases
Age [years]:		
• <50	18 (31.58%)	1 (25%)
• ≥50	39 (68.42%)	3 (75%)
Size of tumor [cm]:		
• ≤2	33 (57.89%)	2 (50%)
• >2	24 (42.11%)	2 (50%)
Histological type:		
• Invasive ductal carcinoma	50 (87.72%)	3 (75%)
• Invasive lobular carcinoma	7 (12.28%)	1 (25%)

Thus, the ER+/PR- phenotype was more commonly seen in patients over the age of 50 years, as compared to the ER+/PR+ phenotype (75% *vs.* 68.42%). ER+/PR- tumors were larger (>2 cm) than the ER+/PR- tumors (50% of cases *vs.* 42.11%). Also, the majority of ER+/PR- tumors were invasive ductal carcinomas, expressing much more frequently the ER+/PR- phenotype than lobular carcinomas (75% of cases *vs.* 25% of cases). All ER+/PR- cases had low Allred score values for estrogens, this score being below 6.

Discussion

Because the hormonal receptors are well-known predictive factors for the response to hormonal therapy in mammary carcinoma, their evaluation through the actual immunohistochemical methods is absolutely necessary.

In this study, 61% of invasive mammary carcinomas had estrogen receptors, while progesterone receptors were detected in 64% of cases, confirming the recent data in the literature stating the presence of ER in 63% of the patients and of PR in 65% of them [11].

Both types of receptors showed, in most cases, a heterogeneous labeling pattern. The presence this labeling heterogeneity seems to partially explain the weak response to the hormonal therapy of some tumors expressing hormonal receptors. It is a known fact that 30–40% of mammary carcinomas do not respond to therapy. The absence of the response is insufficiently understood, but it seems that the steroid-depending growth factors (i.e., via Her2-neu), the deficient functioning of ER, and tumor heterogeneity are involved [12]. As we have seen in this study, the heterogeneity of the immunolabeling was more obvious in the case of progesterone receptors. The nuclear labeling for PR is generally more heterogeneous than the one for ER, and can be a source of false negative results [13].

The lobular carcinomas analyzed expressed ER in a much greater proportion than ductal carcinomas (80% *vs.* 58.88%). In accordance with the observations from the literature, around 70–95% of lobular carcinomas are ER-positive, the rate of positivity being greater than the one of 70–80% seen in invasive ductal carcinomas, and the positivity for progesterone is of 60–70% in both histological types [14].

Most mammary carcinomas expressed both types of hormonal receptors, with an ER+/PR+ phenotype (57% of cases), followed by the tumors without hormonal receptors and an ER-/PR- phenotype (32% of cases). Other studies found that approximately 50% of invasive mammary carcinomas express both types of hormonal receptors, and 25% have no estrogen or progesterone receptors [15].

The heterogeneous phenotype, in which one of the receptors was absent, was seen in 11% of the cases, of which 7% had an ER-/PR+ phenotype, and 4% an ER+/PR- phenotype.

Knowing that the presence of estrogen receptors is necessary for progesterone receptors to be positive, it seems that the ER-/PR+ phenotype is due to the fact that estrogen receptors are incapable of linking the circulating hormone or to be recognized by the monoclonal antibodies used in immunohistochemical techniques, but also that they can still be functional in regard to the stimulation of progesterone receptor formation. It is also possible that the estrogen receptors are present at a level below the detectable threshold for IHC methods [15].

The cases with a heterogeneous phenotype are still widely debated now because the benefit of hormone-therapy diminishes almost by half in the cases in which there is one lacking receptor, in comparison to the ones that have both. The ER+/PR- phenotype is a sub-group of mammary carcinomas, because they possess aggressive clinical and biological features, benefiting less than the other phenotypes from the hormonal therapy [16].

In the present study, the ER+/PR- phenotype was detected in 4% of the tumors. It seems that the loss of progesterone receptors is caused by the loss of activity of estrogen receptors (or by a low blood level of estrogen in some older women, or due to non-functioning of intracellular pathways of estrogen receptors). This theory does not, however, explain why some ER+/PR- tumors respond to the endocrine therapy, even though the response is diminished compared to the ER+/PR+ phenotype [16].

It was later proven that the status of hormone receptors is not a stable phenotype and can be modified during the natural evolution of the disease or because of endocrine therapy. During the tamoxifen treatment, the levels of estrogen and progesterone receptors diminish, but the one of progesterone drops, and almost half of the tumors lose the PR expression and become tamoxifen-resistant. In such cases, the loss of PR expression leads to a more aggressive evolution suggesting that other alterations of the tumor growth process accompany the loss of PR [16–18]. The cumulated data suggest that the loss of PR could be a marker for excessive activation of the growth factors (Her-1 and Her-2), leading to the tamoxifen resistance.

In comparison to the ER+/PR+ phenotype, the ER+/PR- phenotype was more frequent in patients over 50-year-old, with tumors larger than 2 cm, which is in accordance to the results of a major study performed on 40 000 patients with mammary carcinoma.

As we have seen in the present paper, the ER+/PR- cases had low values on the Allred scale (below 6), the results being similar to those obtained through other methods (dextran-coated charcoal – DCC), according to which the average level of estrogen receptors in ER+/PR- tumors is only half of the one in ER+/PR+ tumors [16].

Conclusions

The correlated evaluation of estrogen and progesterone receptor immunolabeling improves their predictive value by identifying the tumors that have a heterogeneous phenotype. In comparison with the classical ER+/PR+ phenotype, a distinctive sub-group of invasive carcinomas is the ER+/PR- phenotype, which is more frequent in the case of patients over

50 years of age and with tumors larger than 2 cm, an invasive ductal carcinoma and with an Allred score lower than 6. The detection of ER+/PR- tumors allows the selection of cases that have aggressive clinical and biological characteristics, which will have the fewest benefits from hormonal therapy.

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Received: September 3rd, 2011

Accepted: December 22nd, 2011