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The role of Ki-67 in the proliferation and prognosis of breast cancer molecular classification subtypes

George P. Stathopoulos[a], Nikolaos A. Malamos[b], Christos Markopoulos[c], Athanasios Polychronis[a], Athanasios Armakolas[a], Sotirios Rigatos[b], Anna Yannopoulou[a], Maria Kaparelou[a] and Photini Antoniou[b]

The Ki-67 antigen was identified in the early steps of polymerase I-dependent ribosomal RNA synthesis. Although it seems that this protein has an important function in cell division, its exact role is still unclear and there is little published work on its overall function. The aim of the present study was to evaluate the contribution of the level of Ki-67 with respect to tumor recurrence in molecularly classified groups of breast cancer patients. Ki-67 was divided into the percentage levels up to and including 20% and over 20%. Immunohistochemistry and fluorescence in-situ hybridization are described for the results of estrogen receptor, progesterone receptor, c-erb-B2, and Ki-67 biomarkers. Formaldehyde-fixed breast samples were paraffin wax embedded and processed for paraffin sections. The protocol of the present study started in 1995 and finished in 2010. Nine hundred and sixteen patients with breast cancer were examined: 291 were grouped as luminal A, 228 as luminal B, 221 as the Her-2 subtype, and 107 as basal cell (triple negative). Follow-up ranged from 3 to 15 years following diagnosis. It was found that in luminal A patients, only one had a Ki-67 level higher than 20%. In luminal B, the Ki-67 was higher than 20% in 51.16% of the patients and recurrence occurred in 23.68%. In the Her-2 subtype, the Ki-67 level was more than 20% in 48.63%. In basal cell triple-negative patients, Ki-67 was more than 20% in 63.86%. The data presented here indicate that the level of Ki-67 may be considered one of the valuable biomarkers in breast cancer patients with respect to process and recurrence. *Anti-Cancer Drugs* **25:950–957**
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[a]First Oncology Department, Errikos Dunant Hospital, [b]Oncology Department, Elenas Hospital and [c]Surgery Department, Iatriko Kentro, Athens, Greece

Correspondence to George P. Stathopoulos, MD, Semitelou 2A, 115 28 Athens, Greece
Tel: + 30 210 7752600; fax: + 30 210 7251736; e-mail: dr-gps@ath.forthnet.gr

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Introduction

Over the last two decades, considerable new knowledge of breast cancer has been gained. Treatment, including targeting agents, has improved. The data on breast cancer-related genes seem to predict treatment and prognosis. The data have also led to the division of breast cancer into certain groups of molecular classification. This division is made on the basis of histological details mainly hormonal receptors, tumor grade, and the c-erb-B2 level. This molecular classification is a very important new framework for the study of breast cancer. It is appropriate to consider that breast cancer is no longer a single disease with heterogeneous estrogen receptor (ER) and Her-2 expression [1]. It is worth mentioning that there are at least three molecularly and clinically clearly distinct diseases that perhaps arise from different precursor cells in breast cancer [1]. According to one study, all luminal cancer types were ER-positive and 63% of low or intermediate grade in contrast to 95% of basal-like cancers that were ER-negative and 91% of high grade [1]. The development of superior technology, particularly the microarray, provides the opportunity to understand the molecular profile of cancer [2]. With the use of the cDNA microarray, the feasibility and usefulness of this method to study variations in the genetic pattern of cancer expression are supported [3]. Using the hierarchical cluster, it is possible to differentiate genomic signatures in breast cancer, similar to those found in lymphocytes and in epithelial, adipose, and stromal cells [4]. Through the pattern of genetic expression, one can provide the basis for improving the molecular taxonomy of breast cancer and the classification of breast cancer tumors [2,5].

The need for dividing the breast tumor into heterogeneous subtype groups and the gene signature led to molecular classification. This classification was considered to mainly aid clinicians to better approach the prognosis and also to formulate treatments for different prognoses [6,7]. It has been determined that there are at least four subtypes of the breast tumor. There are two luminal types, A and B, within the luminal cluster. Where

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hormone receptors are expressed, the difference is that the proliferative genes are lower in luminal A than in luminal B [6,8]. Luminal A, grade I or grade II, and luminal B, grade III, are ingrate in the percentage of proliferation. Between these two subtypes, there is a different prognosis that has persisted in primary breast cancers as well as in their metastases [9]. Hormone receptor-negative breast cancer comprises two distinct subtypes, the Her-2 subtype and the basal-like subtype [6,8], the latter with a poorer prognosis. The HER-2 subtype is characterized by a high expression of the *c-erb-B2* (*HER-2*) gene. The basal cell subtype has ER, progesterone receptor (PR) and is c-erb-B2 negative, the greater percentage of which is the triple negative type [10,11].

There are several other parameters: on the one hand, new gene signatures and on the other, those that are already known such as *BRCA1*, *BRCA2*, and *TP53*, which led to further subdivision of each one of the four molecular types, as well as the expression of basal cytokeratins 5, 6, 17 [2,7,12,13]. Testing multiple variables from microarray or other experiments adds to the methodology to carry out prognostic studies [5,14].

The Ki-67 antigen [15] has been identified in the early steps of polymerase I-dependent ribosomal RNA synthesis. Although it seems that the protein has an important function in cell division, its exact role is still unclear and there is little published work on its overall function [16,17].

The protocol of the present trial started in 1995 and finished in 2010. The aim of the present study was to evaluate the contribution of the level of Ki-67 with respect to tumor recurrence and patients' survival. The Ki-67 percentage has, in other studies, been divided into two or three categories: less than 14%, higher than 14%, or up to 10%, and up to 20% or higher than 20%. In our study, the cut-off point was 20%. Apart from in-vivo investigation in humans, in-vitro investigation of mice and breast cell lines was also conducted.

Patients and methods

Breast cancer tumor samples were examined for histological confirmation and for estrogen and PRs, c-erb-B2 expression, proliferation with grade and Ki-67, and also for p53. Ki-67 was divided into percentages of up to and including 20% and over 20%. Immunohistochemistry and fluorescence in-situ hybridization (Supplemental digital content 1, *http://links.lww.com/ACD/A65*) are described with respect to the results of ER, PR, c-erb-B2, Ki-67, and p53 biomarkers. This IC had been approved by the Institutional Ethics Committee and all the experimental procedures conformed to the Declaration of Helsinki. An informed consent form was signed by all the patients who participated in this study.

Immunohistochemistry

Formaldehyde-fixed breast samples were paraffin wax embedded and processed for paraffin sections. Microtome sections of 3 μm were allowed to adhere to glass slides, dried at 37°C overnight, dewaxed in xylene, and rehydrated in serial dilutions of ethanol. The sections were then incubated with the primary antibody. The primary antibodies used were the monoclonal antibody 1D5 (M7047; DakoCytomation, Carpinteria, California, USA) for the detection of ER (dilution 1 : 25). For the detection of PR, the monoclonal anti-PR antibody636 (M3569; DakoCytomation) was used (dilution 1/100). For the detection of p53, we used monoclonal mouse anti-human p53 (DO-7, M7001; DakoCytomation) at a dilution of 1/25, and for the detection of Ki-67, we used monoclonal mouse anti-human Ki-67 MIB-1 (M7240; DakoCytomation) at a dilution of 1/100. All dilutions were performed in PBS.

Incubation with the primary antibodies was carried out at 4°C overnight. Secondary biotinylated goat anti-mouse IgG antibody (Dako Real EnVision, Glostrup, Denmark) was then added and tissue sections were visualized under light microscopy. Negative control staining procedures were also included in all immunohistochemical analyses, as described elsewhere [18,19].

Cell lines

MCF-7 breast cancer cells were obtained from the American Type Culture Collection (ATCC, Bethesda, Maryland, USA). Cells were maintained in a 5% CO_2 incubator at 37°C. The culture media was Dulbecco's modified essential medium supplemented with 2 mmol/l L-glutamine (Invitrogen, Carlsbad, California, USA) and 15% fetal bovine serum (Invitrogen). MCF-7 cells were selected as this cell line expresses Ki-67 at about 90% and although it has been characterized by molecular staging as luminal A, it has been associated with metastases in the lungs and liver [20,21].

Subcutaneous inoculation in severe combined immunodeficient mice

All in-vivo experiments were conducted according to approved protocols from the mouse handling and experimental procedures were approved by the Hellenic Ministry of Rural Development and Food, General Directorate of Veterinary. Animal handling and experimental procedures were conducted in the Experimental Surgery Laboratory of the Athens Medical School. MCF-7 and MCF-7 Ki-67 knock out (KO) cells (1×10^8 cells) in PBS were implanted subcutaneously in 6-week-old to 8-week-old female nude mice (Ekefe Dimokritos, Athens, Greece). Severe combined immunodeficient (SCID) mice were injected subcutaneously with wild-type MCF-7 cells and with MCF-7 Ki-67 KO cells.

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Statistical analysis

Data are described by mean ± SD and median value with interquartile range (Q1–Q3). The Kruskal–Wallis test was used to compare the four groups, followed by the Mann–Whitney *U*-test for two-group comparisons. The results obtained by the trypan blue and MTT assays were assessed using the two-tailed equal variance Student's *t*-test. A *P* value less than 0.05 was considered significant. The Kaplan–Meier method was used for survival distribution and the log-rank test for comparison of the groups. (All the tests were performed using the SPSS v. 11 statistical package; SPSS Inc., Chicago, Illinois, USA.) For the FISH method, the trypan blue, MTT assay, qPT PCR, and Ki-67 silencing, see Supplementary data (Supplemental digital content 1, *http://links.lww.com/ACD/A65*).

Results

Nine hundred and sixteen patients were examined and evaluated for the majority of the data. Ki-67 was found to have different percentage levels on comparing the four molecular groups. The patients' characteristics are shown in Table 1.

In luminal A patients, the Ki-67 level was higher than 20% in only one patient (0.36%) of 275 patients. In 31 patients with recurrence, six had a Ki-67 level of 20% and the remaining 25 had less than 20%; of the latter, 14 had less than 10%. In luminal B, in 203 patients, the majority had a Ki-67 level higher than 20% (56.16%) and the rest lower than 20% (43.84%). In the 54 patients with recurrence, 33 (61.11%) had a Ki-67 level higher than 20% and the remaining 21 patients had a Ki-67 level lower than 20% (38.89%). In patients with the Her-2 subtype, 48.63% had a Ki-67 level higher than 20% and 51.37% less than 20%. In patients of the same group with recurrence, 78.94% had a Ki-67 level higher than 20% and in the remaining patients had a Ki-67 level lower than 20%, found in 21.05%. In the basal cell subtype, triple-negative group, a Ki-67 level over 20% was detected in 63.86% of patients and lower than 20% in 36.14%. In patients with recurrence, the Ki-67 level was over 20% in 65.62% and less than 20% in 34.37%. Table 2 shows the Ki-67 cut-off point 20%/ > 20% of the patients in each group.

Table 1 Demographics and clinical characteristics of all evaluated patients

Patients	*n* (%)
Evaluable	916 (100)
Sex	
Female	916 (100)
Age (years)	
Median	59
Range	22–90
Cancer type	
Adenocarcinoma of the breast	916 (100)
Molecular classification	
Luminal A	291 (34.36)
Luminal B	228 (26.92)
Her-2 subtype	221 (26.09)
Basal cell (triple negative)	107 (12.63)
Premenopausal	272 (29.69)
Postmenopausal	644 (70.31)
Treatment	
Surgery	916 (100)
Adjuvant chemotherapy	800 (87.34)

The statistical difference in the Ki-67 level was compared between among all groups. The *P* values of luminal A versus luminal B, luminal A versus the Her-2 subtype, and luminal A versus basal cell were significant, as were luminal B versus the Her-2 subtype and luminal B versus basal cell. The *P* value was not significant between the Her-2 subtype and basal cell (Table 3).

Recurrence

The total number of patients with recurrence was 155 of 916 (16.92%); on the basis of molecular classification, disease recurrence was as follows: luminal A, 31/291 patients (10.65%), luminal B, 54/228 (23.68%), the Her-2 subtype, 38/221 (17.19%), and the basal cell type including triple negative, 32/107 (29.91%).

There were a total of 272 premenopausal and 644 postmenopausal patients. Disease recurrence in premenopausal women versus postmenopausal was as follows: in luminal A, premenopausal patients 9/31 (29.03%), and in postmenopausal 22/31 (70.97%); in luminal B, 13/54 (24.07%) and 41 (75.93%), respectively; in the Her-2 subtype, 8/38 (21.05%) and 30/38 (78.95%), respectively; and in the basal cell type, 10/32 (31.25%) and 22/32 (68.75%), respectively.

Grade II is not a persuasive predictor as it was found in a rather high percentage of all the groups. Ki-67 is more precise as it is more accurate when luminal A is compared with the other three groups.

Grade was divided into three categories I, II, and III. In the 276 luminal A patients, 53 (19.20%) were grade I, 223 (80.80%) were grade II, and none were grade III. In the 232 luminal B patients, none were grade I, 65 (28.02%) were grade II–III, and 167 (71.98%) were grade III. In the 217 Her-2 subtype patients, three (1.38%) were grade I, 96 (44.24%) were grade II, and 118 (54.38%) were grade III. In the 106 basal cell-type patients, two (1.89%) were grade I, 34 (32.08%) were grade II, and 70 (66.04%) were grade III.

Table 2 Ki-67 cut-off point 20%/ > 20%

	Molecular classification [*n* (%)]			
	Luminal A	Luminal B	Her-2 sub	Basal cell
≤ 20%	274 (99.64)	89 (43.84)	94 (51.37)	30 (36.14)
> 20%	1 (0.36)	114 (56.16)	89 (48.63)	53 (63.86)
Total	275	203	183	83

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Table 3 Pairs' comparison of protein Ki-67, Mann–Whitney *U*-test, *P* value

Groups	*P* value
Luminal A vs. luminal B	< 0.001
Luminal A vs. Her-2	< 0.001
Luminal A vs. basal cell	< 0.001
Luminal B vs. Her-2	0.018
Luminal B vs. basal cell	0.042
Her-2 vs. basal cell	0.840

Table 4 Metastatic site

Site	Luminal A	Luminal B	Her-2 subtype	Basal cell
Skeleton	11	15	6	6
Lung	9	6	5	3
Local	4	6	5	8
Liver	3	6	5	4
Abdomen	1	–	2	1
Bone, lungs	1	2	2	1
Bone, liver	1	6	2	3
Mediastinal	1	1	–	–
Lung, liver	–	7	1	–
Brain	–	2	4	1
Liver, abdomen	–	2	–	–
Lung, brain	–	1	–	2
Local, bone	–	1	1	1
Bone, lung, brain	–	1	–	1
Skin	–	–	2	–
Liver, lung, bone, brain	–	–	1	–
Bone, liver, lung	–	–	1	1

The site of metastasis per group by molecular classification is shown in Table 4.

Ki-67 silencing

MCF-7 stable transfectants with the Ki-67 KO vector (MCF-7 Ki-67 KO) presented significant silencing compared with the wild-type MCF-7 cells as presented by quantitative RT-PCR (Fig. 1a).

MTT and trypan blue assays

MCF-7 cells presented a 37% increase in cell number at 24 h and 40% at 48 h ($P = 0.003$ and 0.005) (Fig. 1b) in comparison with those that with a silenced *Ki-67* gene, suggesting a possible role of the *Ki-67* gene in cellular proliferation.

Inoculation into severe combined immunodeficient mice

SCID mice were injected subcutaneously with wild-type MCF-7 cells and with MCF-7 Ki-67 KO cells. MCF-7 tumors were palpable 6 weeks after implantation, whereas MCF-7 Ki-67 KO were palpable during eighth week after implantation. Tumors were removed 2 months after injections and were measured along their longest dimension and weighed. Wild-type MCF-7 tumors were shown to be statistically significantly larger than MCF-7 Ki-67 KO tumors ($P < 0.001$) (Fig. 1c).

Tumors generated in SCID mice by MCF-7 cell inoculation had an elevated labeling index of both HER-2 and Ki-67, whereas tumors generated by the MCF-7 cells that had their *Ki-67* gene silenced presented – as expected – a low Ki-67 labeling index, which was associated with the low HER-2 labeling index in all the tumors examined ($P < 0.001$). This was also the case for PR, but not ER (Fig. 2).

Discussion

Ki-67 is considered to be of prognostic value as a proliferative marker. It is also considered to be modulator and has been shown to be an appropriate end point for preoperative studies involving hormonal therapies [16,22,23]. A decrease in Ki-67 presurgically serves as an appropriate surrogate marker for outcome in patients who are administered antiestrogen therapy [24].

There is variability in Ki-67 staining [17,25]. Ki-67 staining by pathologists estimates the percentage of nuclei staining and other investigators count hundreds of consecutive nuclei to determine an overall average index [26].

Breast cancer is a heterogeneous disease and this has led to molecular classification. The subgroups may, sooner or later, be multiplied by microarray research.

Hormone receptor status, a target for endocrine therapies, has been considered to be the standard for prediction of response to treatment [27,28]. Some of the features, such as tumor size, histological grade, comedo, necrosis, and the influence of the margin, may be a risk for recurrence [29–32].

Tumor size, as well as the axilliary lymph node infiltrated by the disease, are two important baseline prognostic determinants [33]. Tumor size may not play an important role as very small tumors with four positive lymph nodes may be a predictor for higher breast cancer-specific mortality compared with larger tumors [34]. It has been shown that in one of the subgroups (basal cell triple negative) of molecular taxonomy, the number of positive lymph nodes infiltrated by the disease may not play a role as a prognostic factor. The conclusion of one study [35] was that the prognosis may not be affected by the number of positive lymph nodes.

With respect to the patient's future outcome, tumor grade is important in the prognosis. Grade plays a prognostic role in tumor proliferation, where a higher grade may lead to a worse prognosis for the patient. Grade is divided into three categories: I, II, and III. Slow proliferation is indicated by grade I and high proliferation by III, whereas grade II is considered to be medium. On the basis of molecular classification, grade is commonly used. Often, the pathological examination of grade is controversial. We may find that in patients with basal cell triple negative or the Her-2 subtype, the grade may be II, which does not indicate high tumor proliferation. In this study, on examining luminal A, grade I was 19.20% and

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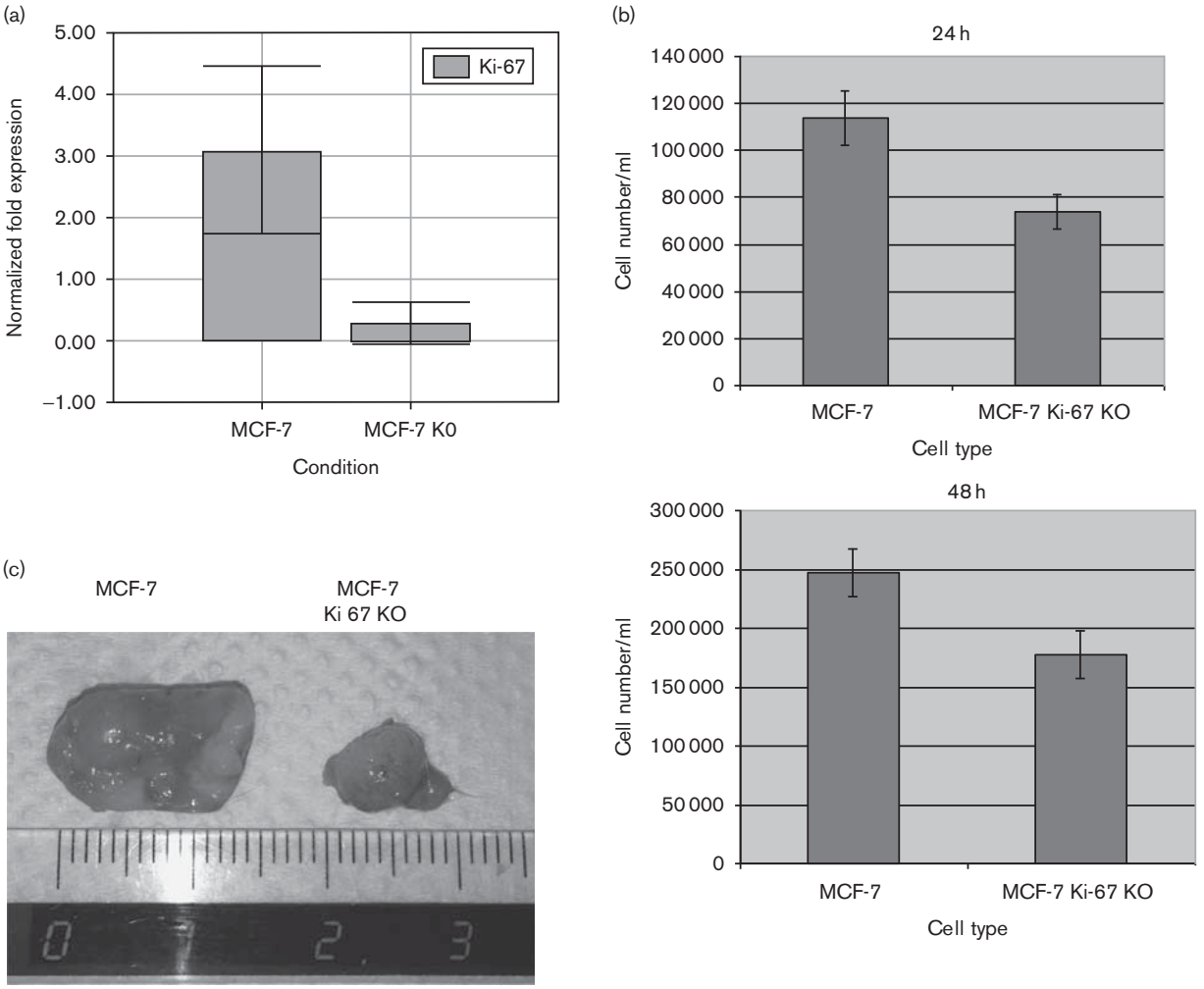
Fig. 1



Ki-67 silencing characterization and effects. (a) Characterization of the degree of reduction of Ki-67 expression in wild-type MCF-7 breast cancer cells and in MCF-7 Ki-67 knock out (KO) cells as observed by qRT-PCR. Note that the degree of reduction of Ki-67 expression in the MCF-7 Ki-67 KO cells is about 80% in respect to the Ki-67 levels observed in the wild-type (wt) MCF-7 cells ($P < 0.05$). (b) Proliferation assays of wild-type MCF-7 cells versus MCF-7 Ki-67 KO cells suggest that the wt MCF-7 cells present a 40% increase in the proliferation rate when compared with that of MCF-7 Ki-67 KO cells at 24 and 48 h. (c) Tumors extracted from SCID mice 2 months after subcutaneous inoculation with MCF-7 or MCF-7 Ki-67 KO cells. MCF-7-generated tumors were found to be significantly larger compared with those generated by MCF-7 Ki-67 KO cells.

grade II was 80.80%. In luminal B, grade II–III was 28.02% and grade III was 71.98%. Luminal B has a worse prognosis with a higher percentage of tumor recurrence than luminal A. Morphological assessment of the degree of differentiation has been shown in numerous studies and it provides useful prognostic information in breast cancer [36]. Up to a few years ago, grading had not been accepted as a routine procedure mainly because of perceived problems with reproducibility and consistency. Over the last few years, the technique has been revised involving semiquantitative evaluation of three morphological features: (a) the percentage of tubule formation, (b) the degree of nuclear pleomorphism, and (c) an accurate mitotic count using a defined field area [36].

Ki-67 is also considered to be a prognostic factor. Whether Ki-67 is a more precise prognostic biomarker cannot, as yet, be established. Both grade and Ki-67 levels should be used for prognosis. In our study, on examining the Ki-67 levels in each of the molecular classification subgroups, we found important results: in luminal A, the percentage of Ki-67 up to 20% was found in 99.64% of the patients and over 20% in one patient (0.36%). In luminal B, the Her-2 subtype and basal cell, a Ki-67 level higher than 20% was detected in 56.16, 48.63, and 63.86%, respectively.

The heterogeneity of breast cancer has been sustained by the development of microarray-based prognostic gene

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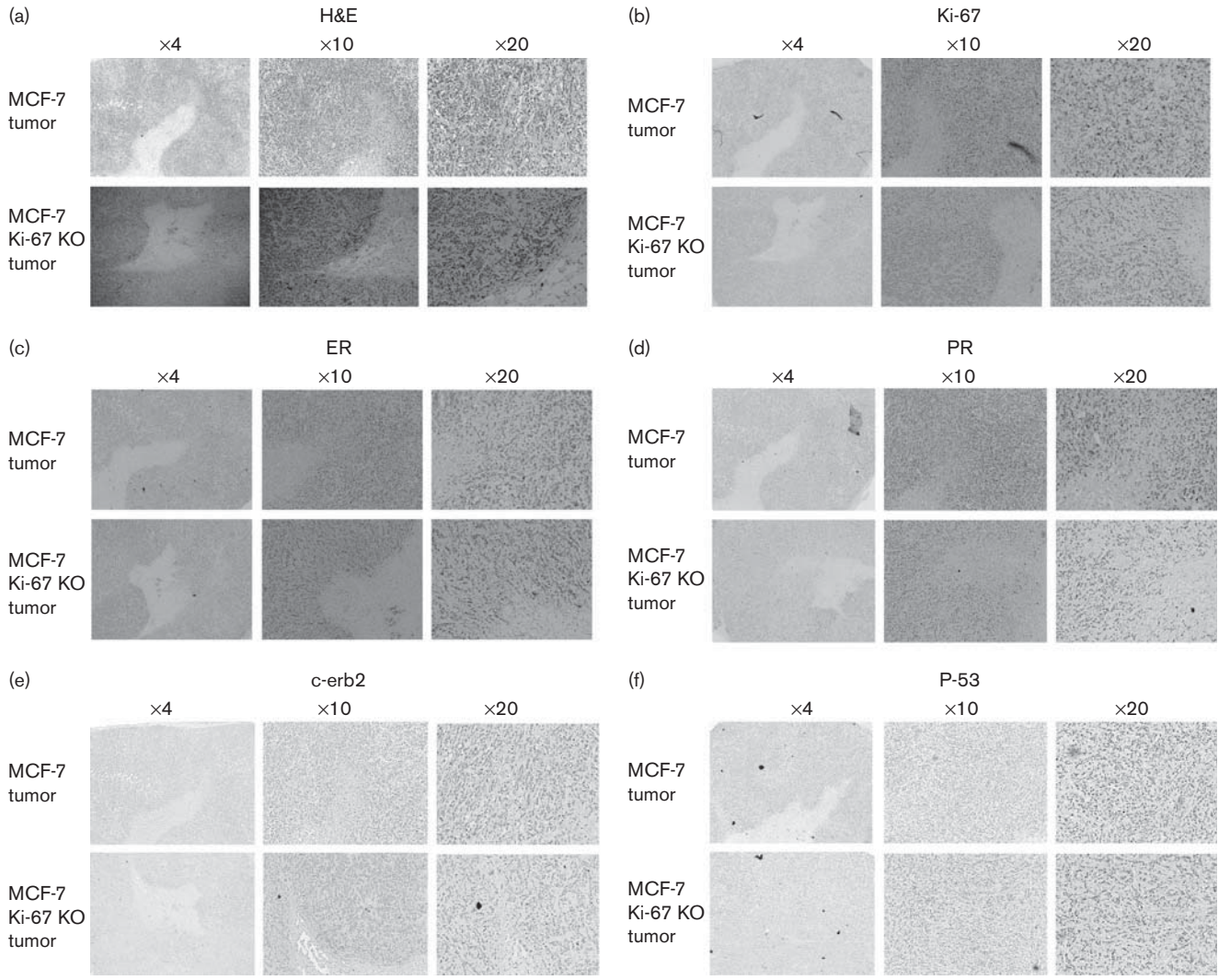
Fig. 2



Immunohistochemical analysis of the tumors generated by the MCF-7 and the MCF-7 Ki-67 KO cells. As expected, tumors generated by the MCF-7 cells that had their Ki-67 gene silenced showed a decreased expression of the Ki-67 protein compared with the wild-type MCF-7 (b). They also presented decreased levels of PR (d) and to a further extent of c-erb2 (e). (a, c, and f) The rest of the proteins examined (ER and p53) seemed to present similar expression levels in both cell lines.

signatures. This was heralded as a major breakthrough for the management of breast cancer patients [37,38]. The initial data of studies on cancer prognosis with microarrays have shown that the overlap between gene signatures was not stable in terms of their gene composition [39,40].

In our study, the value of Ki-67 as a prognostic factor is shown in luminal A, where the recurrence of the breast tumor was 10.65%, whereas in the other three groups, it was approximately double, triple, or higher.

Recent evidence suggests that HER2 overexpression in breast cancer tumors in postmenopausal women is associated with a high Ki-67 labeling index, but not in premenopausal women [41]. Furthermore, the fact that Ki-67 silencing was associated with low HER2 and PR labeling index suggests that Ki-67 expression may affect the tumor's molecular classification.

It seems that our knowledge of breast cancer molecular classification is at a premature stage. Further studies including microarray analysis at mRNA, protein, and miRNA levels will be useful in our quest for more precise prognosis and individualized therapies.

Conclusion

The main finding of the present study was that Ki-67 was found to have different percentage levels on comparing the four molecular groups. Grade II is not a persuasive predictor as it was found in a rather high percentage of all

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the groups. Ki-67 is more precise as it was more accurate when luminal A was compared with the other three subgroups. In addition, it was also found that Ki-67, besides being a precise predictor for luminal A subtype in breast cancer patients, is also involved in the breast cancer cellular proliferation process and is associated with elevated levels of Her-2 in the tumors.

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Conflicts of interest

There are no conflicts of interest.

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