Original Article

Ag-NOR and P53 Expression of the breast cancer in correlation with tumor grade

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ABSTRACT

***Objective*:** Breast cancer comprise approximately one third of malignant cases in women and is considered as the most common invasive condition in women at an age range of 15-54 years and as the second most prevalent cause of mortality at an age range of 55-74 years. Tumor inhibiting factor p53 is a vital homeostatic regulator and its inactivation at the related gene or molecule could lead to tumor growth and development in various tissues. Therefore, in this research study it was tried to evaluate the diagnostic methods Ag-NOR and p53 immunohistochemistry in malignancy of mammary gland using cytochemical staining methods and its relationship with tumor grade.

Materials and Methods: In this research study, 50 referred breast specimens to Deaprtment of Pathology (Sina Hospital, Hamedan) were studied. They were processed as usual and 3 micrometer sections were prepared from related blocks. Then, staining methods for nucleolus organizing regions (Ag-NOR) and p53 immunohistochemistry were applied. Out of these specimens, 41 had malignancy (40 cases of invasive ductal carcinoma and 1 case of invasive lobular carcinoma) and 9 cases were normal. The latter cases were compared with malignant ones. Tumor grade in studied individuals was I (3 cases; 7.3%), II (23 cases; 56.1%), and III (15 cases; 36.6%) respectively.

Results: Statistical analysis of data showed that there is only a significant difference regarding frequency distribution of cluster shape and there is no such difference for satellite shape, satellite size, and cluster size. In addition, staining intensity for $p53^{+}$, $p53^{++}$, and $p53^{+++}$did not show any significant difference in various grades of the disease. Using Spearman regression analysis, it was found out that there was a relationship between p53 negative and $p53^{+}$ ($r = 0.723$) ($p<0.01$) and between $p53^{+}$ and $p53^{++}$ ($r = 0.78$). Furthermore,

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it was found out that a higher expression of p53 protein is negatively correlated with darkly-stained granules using silver nitrate method.

Conclusion: These findings showed that higher expression of p53 protein is negatively correlated with darkly-stained granules using Ag-NOR method and this may indicate its anti-tumor activity. It appears that this method is an essential tool for evaluation of normal and malignant cases of breast tissue regarding its replication pattern and intensity and expression of those chromosomal segments which are involved in encoding of ribosomal RNA**.**

Key words: Breast, p53 Protein, Immunohistochemistry, Nucleolus Organizing Regions

Introduction

Breast cancer comprise approximately one third of cases in women and is considered as the most common invasive condition in women at an age range of 15-54 years and as the second most prevalent cause of mortality at an age range of 55-74 years. There have been many screening and preventive strategies for early diagnosis of this condition, each one with its special limits and faults. Therefore, finding new diagnostic methods with a high sensitivity and specificity is of high value in clinical practice (1-11).

Tumor inhibiting factor p53 is a vital homeostatic tissue factor and its inactivation at the related gene or molecule could lead to tumor growth and development in various tissues and its mutation has been observed in more than 50% of such conditions in human beings and considered as a prognostic factor in various tumors. A mutation at its gene is regarded as an independent risk factor and could lead to therapy resistance (6-12). P53 is a multifunctional protein (containing 392 amino acids) within nucleus. It can inhibit transcription process at some genes and in some cases can inhibit translation process for mRNA regarding some proteins. Its gene is continuously replicated and translated and its product is immediately degraded in proteosome. Therefore, p53 concentration is low in most tissues and its measurement is difficult to perform. The production of this protein is post-translationally activated following various kinds of stresses and its degradation is simultaneously attenuated. In addition, its structural conformation is altered and the active form of protein is available. Meanwhile, its stability makes the opportunity for its accumulation within nucleus (13-16).

Silver nitrate staining is a new method for Ag-philic areas within nucleolus which can mark non-histone proteins (sometimes active regions of ribosomal DNA replication). Therefore, it is a marker of protein synthesis and cell replicative activity at metaphase and interphase stages of division. NOR-containing regions appear as small and dark spots (granules) following such staining. These spots increase in tumor cells (7-9).

Using Ag-NOR counting is appropriate in predicting vitality in gross morphology and malignancy histopathology. Ag-NOR grading shows a relationship with malignancy degree. There is also such an association for tumor metastasis regarding axillary lymph node involvement (4-6).

Therefore, in this research study it was tried to evaluate the diagnostic method of Ag-NOR and p53 immunohistochemistry in breast cancer using cytochemical staining methods and its relationship with tumor grade.

Materials and Methods

In this research study, 50 specimens (referred to dept. Pathology, Sina Hospital, Hamedan) were studied. They were processed as usual and 3 micrometer sections were prepared from related blocks. Then, staining methods for nucleolus organizing regions (Ag-NOR) and p53 immunohistochemistry were applied.

For Ag-NOR staining, sections were transferred

to alcohols 100%, 95%, and 70% (each one for 5 min), rinsed with distilled water, immersed in working solution (containing silver nitrate and gelatin) for 30-45 min, rinsed again, and placed in sodium thiosulphate solution for 5 min. For dehydration, alcohols 70, 95, and 100% were used. Thereafter, slides were placed in xylene (5-10 min) and finally coverslipped.

For p53 immunohistochemistry, slides were coated with an adhesive. Then, sections were collected on slides, paraffinized for 24 h at 37 °C, transferred to xylene, alcohols 70%, 95%, and 100% for dehydration, placed in hydrogen peroxide solution for 20-30 min, transferred to citrate buffer solution, placed at microwave oven for 10-15 min, albumin was added, p53 primary antibody was added for 1 h, placed in biotin solution (Dako Co.) for 30 min, rinsed in Tris buffer solution, and Streptoavidin solution was added for 10-15 min, and rinsed again in Tris buffer. Finally, DAB solution was added for 10-15 min and slides were placed in Hematoxylin solution for 1-30 s. At last, slides were rinsed with distilled water, dehydrated, and coverslipped for microscopic analysis.

Results

In this research study, 50 referred breast specimens to Deaprtment of Pathology (Sina Hospital, Hamedan) were studied. They were processed as usual and 3 micrometer sections were prepared from related blocks. Then, staining methods for nucleolus organizing regions (Ag-NOR) and p53 immunohistochemistry were applied. Out of these specimens, 41 had malignancy (40 cases of invasive ductal carcinoma and 1 case of invasive lobular carcinoma) and 9 cases were normal. The latter cases were compared with malignant ones. Tumor grade in studied individuals was I (3 cases; 7.3%), II (23 cases; 56.1%), and III (15 cases; 36.6%) respectively.

After counting cell granules, the average number of granules was 0.37 and 5.6 in normal and tumor cells respectively with a significant statistical difference (t=26.8, p=0.000). In addition, average number of granules was not statistically different between two kinds of studied carcinoma. The maximum averaged number of granules was observed for grade III of the condition (5.4) and then for grades II (4.88) and grade I (4.08) respectively). Meanwhile, averaged number of granules was not statistically different for all three grades of the condition ($p = 0.09$, $F = 2.48$).

Regarding frequency distribution of satellite shape, out of 50 studied cases, 9 cases were normal with shape 1 and out of 40 cases of invasive ductal carcinoma, 16, 19, and 5 cases had satellite shapes 1, 2, and 3 respectively. In addition, the only case of invasive lobular carcinoma had satellite shape 1.

Regarding frequency distribution of satellite size, out of 50 studied cases, 9 cases were normal with size 1 and out of 40 cases of invasive ductal carcinoma, 24, 12, and 2 cases had satellite sizes 1, 2, and 3 respectively. In addition, the only case of invasive lobular carcinoma had satellite size 2.

Regarding frequency distribution of cluster shape, out of 50 studied cases, 9 cases were normal with shape 1 and out of 40 cases of invasive ductal carcinoma, 10, 24, and 6 cases had cluster shapes 1, 2, and 3 respectively. In addition, the only case of invasive lobular carcinoma had cluster shape 2.

Regarding frequency distribution of cluster size, out of 50 studied cases, 9 cases were normal with size 1 and out of 40 cases of invasive ductal carcinoma, 27, 11, and 1 case had cluster sized 1, 2, and 3 respectively. In addition, the only case of invasive lobular carcinoma had cluster size 2.

Statistical analysis of data showed that there is only a significant difference for cluster shape in various grades of the condition and there are no such differences for other parameters.P53 staining intensity in different grades was 1^+, 2^+, and 3^+ which were statistically non significant for

$p53^{+}$, $p53^{++}$, and $p53^{+++}$ using Kruskal-Wallis test.

Using Spearman regression analysis, it was found out that there was a relationship between p53 negative and $p53^{+}$ ($r = 0.723$) ($p<0.01$) and between $p53^{+}$ and $p53^{++}$ ($r = 0.78$). Furthermore, it was found out that a higher expression of p53 protein is negatively correlated with darkly-stained granules using silver nitrate method. (Table 1)

Table 1:Relationship between P53 expression and granule number ratio

P53	Neg	+	++	+++	Granule number ratio
Neg	1	0.723**	0.429**	0.085-	0.058-
+	0.723**	1	0.78**	0.1	0.004-
++	0.495**	0.780**	1	0.449	0.130-
+++	0.58 -	0.150	0.449**	1	0.359-
Granule number ratio	0.058-	0.004-	0.130-	0.359*	1

* $p < 0.05$

** $p < 0.01$

Discussion

Invasive ductal carcinoma is the most common (70%) kind of breast cancer and its prognosis is dependent on the following factors: 1) Size of primary tumor, 2) involvement of lymph nodes,the absolute number & the amount of metastatic tumor 3)cytoarchitectural type & microscopic grade 4) presence or absence of estrogen and progesterone receptors, 5) rate of proliferation and anaploidal intensity, 6) overexpression of C-ERB B2, and 7) excessive growth of blood vessels within and around tumor.

In this study, the relationship between intensity of cellular dysplasia and tumor grade was evaluated with regard to important tumor suppressor p53. Its gene is located on the short arm of chromosome 17. This gene is essential for normal growth and functions of living animals. In this respect, offspring of mice with an inactive gene are very predisposed to a variety of tumors and are alive for a maximum of 6 months and have a higher rate of malignant lymphoma. In 50% of malignant conditions, point mutations or deletion are observed regarding p53 gene. Sorlie et al (14) found out that p53 is a regulating agent for DNA damages at phase G1 and following injury, its activity increases which can prevent cell division.

Although P53 gene is continually transcribed and translated, but the resulted protein is immediately degraded within cytoplasm. Therefore, its concentration is low in most tissues and for this reason, its measurement is difficult to perform. Activation of p53 production can lead to a delay in cell cycle progression and may lead to apoptosis.

In addition, activation of p53 protein may occur due to various stresses including DNA damage following chemical and physical agents, a defect in the regulation of production and degradation of microtubules, oncogenes activation, hypoxemia, hyperthermia, and etc and this may stop cell cycle and lead to apoptosis (3, 17).

In this study, using p53 immunohistochemistry it was found out that there is no positive staining around tumorous tissues. In a study on p53 immunohistochemistry by Stephenson et al on 46 fine needle aspiration specimens from marked breast tissue, it was found out that 26 cases were benign and out of these, 23 cases were p53 negative and the remaining 3 were positive. Meanwhile, out of 20 malignant cases, 6 and 14 cases were p53 negative and positive respectively. In the latter study, the sensitivity and specificity of p53 immunohistochemical staining was 70% and 88% respectively (7).

In another study by Miller et al on 251 patients, it was found out that p53 state is associated with estrogen receptors and tumor grade. A mutation in p53 gene is observed in 79% and 14% of tumors with a grade of III and I respectively. In this study, p53 staining intensity in different grades was not statistically significant that is consistent with previous studies and indicated antitumor effect of p53 and its

mutation is involved in breast cancer (10).

In addition, Takikawa et al found out that there is no association between expression of p53 and/or C-erbB-2 genes and Ag-NOR regarding breast cancer. Meanwhile, both genes are closely associated with Ag-NOR and involvement of axillary lymph nodes. Therefore, expression of these genes is of a high prognostic value regarding breast cancer (3).

On the other hand, p53 protein has pronounced effect on tumor development. In this respect, its intactness is usually indicative of a low invasion of tumor and the tumor is highly sensitive to p53 protein. It has also been shown that p53 protein can reduce the incidence and development of metastatic tumors (2, 3) through reducing the essential mutations for tumor growth and its metastasis.

In this study, through evaluation of association between granule density and p53 staining it was found out that the higher the expression of p53 protein as a tumor-inhibiting factor (in other words, the higher the staining intensity for p53), the lower is Ag-philic granules using Ag-NOR staining technique. This may indicate anti-tumor activity of p53. It appears that this method is an essential tool for evaluation of normal and malignant cases of breast tissue regarding its replication pattern and intensity and expression of those chromosomal segments which are involved in encoding of ribosomal RNA and this could differentiate normal tissues from pathological ones.

References

1. Sammaratunga H, Clark B, Owen L, Bryson G, Swanson C. Phyllodes tumors of the breast: correlation of nucleolar organizer region with histopathological malignancy grading, flow cytometric DNA analysis and clinical outcome. Pathol mt. 2001 Nov; 51(11): 866-73

2.Brawijumachaia M, Musiatowics B, Cyiwik J, et al. Ag-NOR, KI-67and PCNA expression in fibriepithelial tumors of the breast in correlation with morphological features. Folia Morphol. 2004 Feb; 63(1): *133-5*

3. Takikawa Y, Noguchi M, Kitagawa H, Thomas M. Immunohistochemical detection of p53 and c-erbB-2 prognostic significance in operable breast cancer. Breast Cancer. 1994 Jul 30;p (p): 17-23

4. Koyama H, Inaji H, Yamamoto H, et al. Nucleolar organizer regions in intraductal lesions associated with invasive ductal carcinoma of the breast. Oncology 1993 Mar-Apr; 50(2): 116-20

5. Eskelien M, Lipponen PK, Collari Y, Syrjanen KJ. The role of nucleolar organizer regions as prognostic factors in breast cancer. Eur J Cancer 1991 27(8): 989-92

6. Smith R, Crocker J. Evaluation of nucleolar organizer region-associated proteins in breast malignancy. Histopathol 1988 Feb; 12(2): 113-25

7. Stephenson TJ, Royds JA, Silcocks PB, et al.Diagnostic associations of p53 immunostaining in fine needle aspiration cytology of the breast. Cytopathology 1994 Jun; 5(3): 146-53

8. Gao RJ, Bao HZ, Yang Q, Ct al.The presence of serum anti p53 antibodies from patients with invasive ductal carcinoma of breast: correlation to other clinical and biological parameters. Breast Cancer Res Treat 2005 Sep; 93(2): 111-5

9. Choi DH, Kim S, Rimm DL, et al. Immunohistochemical biomarkers in patients with early onset breast carcinoma by tissue microarray. Cancer J 2005 pay Sep-Oct; 11(5): 404-11

10. Miller LD, Smeds J, George J, et al. An expression signature for p 53 status in human breast cancer predicts mutation status, transcriptional effects, and patient survival. N Eng J Med 2005 Sep; 102(38): 13550-55

11. Goldhirsch A, Glick JH, Gelber RD, Coates AS, Senn Hi. Meeting highlights: International Consensus Panel on the Treatment of Primary Breast Cancer: Seventh International Conference on Adjuvant Therapy of Primary Breast Cancer. J

Clin Oncol 200119:3817-3827

12. lsaacs C, Steams V, Hayes DF. New prognostic factors for breast cancer recurrence. Semin Oncol 2001 28:53-67

13. Perou CM, Sorlie T, Eisen MB, et al. Molecular portraits of human breast tumors. Nature 2000 406:747-752

14. Sorlie T, Perou CM, Tibshirani R, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci USA 2001 98:10869-10874

15. Hedenfalk I, Duggan D, Chen Y, et al. Gene-expression profiles in hereditary breast cancer. N Engl J Med 2001 344:539-548

16. Van't Veer U, Dai H, van de Vijver MJ, et al. Gene expression profiling predicts clinical outcome of breast cancer. Nature 2002 415:530-536

17. West M, Blanchette C, Dressman H, et al.predicting the clinical status of human breast cancer by using gene expression profiles. Proc Nati Acad Sci USA 2001 98:11462-11467