MOLECULAR DIAGNOSIS OF THE *AVEN* GENE AS A PROGNOSTIC FACTOR IN BREAST CANCER PATIENTS

M. MOAWAD[1], E. A. MOHAMED[2,3], GHADA M. NASR[4] AND A. H. H. EZZAT[5]

1. *Department of Surgical Pathology, National Cancer Institute, Cairo University, Egypt*
2. *Biotechnology and Genetic Engineering Research Unit, Deanship of Scientific Research, Taif University, Kingdom of Saudi Arabia*
3. *Department of Genetics, College of Agriculture, Cairo University, Cairo, Egypt*
4. *Department of Molecular Diagnostics, Genetic Engineering & Biotechnology Research Institute, Sadat City University, Egypt*
5. *Department of Surgical Oncology, National Cancer Institute, Cairo University, Egypt*

Cancer is a leading cause of death worldwide, accounting for 7.6 million deaths in 2008. About 70% of all cancer deaths in 2008 occurred in low- and middle income countries. Deaths from cancer worldwide are projected to continue rising, with an estimated 13.1 million deaths in 2030 (WHO, 2013). Breast cancer is the second most common cancer in women in the world. The incidence rate of breast cancer has been rising both in the developed and developing countries and it is becoming frequent in some developing countries including Egypt. Breast carcinoma constitutes 33% of all females' cancers in Egypt (El-Bolkainy *et al.*, 2013). Immunohistochemistry (IHC) techniques are widely used in diagnostic histopathology to help re-differentiate the light microscopically undifferentiated tumors (Ross and Fletcher, 2000). Vascular endothelial growth factor (*VEGF*) is a key mediator of angiogenesis in numerous solid tumors, including breast cancer. Furthermore, higher levels of VEGF protein in breast tumors have been shown to be associated with poor prognosis of the disease (Konecny *et al.*, 2004). Ki67 antigen is expressed in all proliferating cells and recently there is a great interest in its role as a marker of proliferation in breast cancer and its assessment by immunohistochemistry (IHC) has become the most widely used method for comparing proliferation between tumor samples (Yerushalmi *et al.*, 2010).

AVEN, a protein designated *Aven* (Aven tine, a Roman stronghold) was first discovered as an inhibitor of caspase activation that interacts with the antiapoptotic BCl-xL protein by Chau *et al*. (2000). The first implication of AVEN in cancer was reported by Paydas *et al*. (2003) as they showed that AVEN is overexpressed at the mRNA level in acute leukemias. Kutuk *et al*. (2010) reported the implication of AVEN in breast cancer as they describe decreased nuclear expression of AVEN in breast cancer tissue microarrays,

Egypt. J. Genet. Cytol., 44:93-102, January, 2015
Web Site (*www.esg.net.eg*)

in particular in infiltrative ductal carcinoma and papillary carcinoma compared to non-neoplastic breast tissue and infiltrating lobular breast cancer. Moreover, they suggested that AVEN might be an important mediator in DNA damage-induced apoptotic signaling and its nuclear down regulation in breast cancer can lead to genomic instability. A more recent study by Ouzounova *et al.* (2013) suggested that *Aven* gene might be an important mediator in DNA damage-induced apoptotic signaling and its nuclear down-regulation in breast cancer that can lead to genomic instability; thus, overexpression of AVEN is important for breast tumor growth.

This study aimed to assess the molecular diagnosis of the *Aven* gene as a prognostic factor in breast cancer patients. Investigation of the histopathological parameters of breast cancer cases using light microscope, assessment of the *Vegf* and *Ki67* genes expression, ER and PR status by Immunohistochemical analysis in cancer tissues and study the co-expression of these known prognostic factors with the *Aven* gene using (RT-PCR technique) in breast cancer tissues.

MATERIALS AND METHODS

Patients

The present study was performed on 40 diagnosed female breast cancer patients, and 10 cases of benign breast tissues, presented to National Cancer Institute (NCI), Cairo University. The age of patients ranged from 27 to 60 with a mean of 44.74 ± 1.432, median of 44 years.

The fresh tumor tissues were divided into two fragments; one fragment was fixed in 10% neutral buffered formalin (18 to 24 hours), and processed for histological, and immunohistological analysis, the second fragment of the tissue was frozen in a dry ice and stored at –80°C. The latter was used for RT-PCR.

Histological diagnosis

For histological studies, following fixation, specimens were dehydrated through ascending series of alcohol, cleared in two changes of xylene and embedded in molten paraplast. Sections of 5 micron thickness were cut using rotary microtome, mounted on clear glass slides without using any adhesive media and stained with hematoxylin and eosin (Bancroft and Gamble, 2002).

Immunostaining

Immunohistochemical reaction was performed using an avidin biotin complex immunoperoxidase technique on paraffin sections. ER, PR and Ki67 were detected using an anti-human ER, PR and *Ki67* monoclonal antibody (Dako A/S, Glostrup, Denmark), respectively. The mean percentages of ER, PR and *Ki67* positive tumor cells in all major foci of cancer were used as immunohistochemical scoring system (Hsu *et al*., 2000). The expression of VEGF protein was examined immunohistochemically using autostainer machines. VEGF was categorized into negative showing homogenous or heterogeneous tumor membrane and cytoplasm immunoreactivity was utilized

to indicate tumor VEGF expression (Park *et al.*, 2005).

Detection of Aven gene expression by RT-PCR

RNA extraction and primers design

Frozen tumor tissues stored at −80°C were used for RNA extraction from cancer breast tissue and normal tissue as control using RNA extraction kit. Specific primers for and *Aven* gene and *β-actin* gene (as a control reaction) were designed based on the mRNA sequence of the *Aven* and *β-actin* genes deposited in Genbank with accession numbers, AF283508 and NM_001101, respectively. For *Aven* gene, the sequence of the sense strand and the Anti-sense strand primers were 5'-GATTTCAGTGTCCTCCTTAG-3' and 5'-CCTTGCCATCATCAGTTCTC-3', respectively, and amplify a fragment of 252 bp. The *β-actin* gene had the following sequence for the sense strand and the Anti-sense strand primers, 5' GTGGGGCGCCCCAGGCACCA 3' and 5' GTCCTT AAT GTC ACG CAC GAT TTC 3', respectively, and amplify a fragment of 300 bp. All primers were supplied by (Operon primers).

RT-PCR

The RT-PCR reactions to detect the transcript of *Aven* gene and *β*-actin (as a control reaction) were carried out using the RT-PCR kits: Qiagen (Catalog number: 12110007) and according to the manual supplied with the kit. Briefly, reaction tubes were transferred to a thermal cycler (Roobycycler gradient 96 stratagene) and incubated at 55°C for 30 minutes for c-DNA synthesis then, incubated at 95°C for 15 minutes to inactivate the reverse transcriptase and to completely denaturant the template. For PCR amplification of *Aven*, denaturation was done at 94°C for 60 sec followed by annealing at 60°C for 60 sec and polymerization at 72°C for 60 sec. This profile was repeated for a total of 32 cycles and ended with final extension at 72ºC for 10 min. Another set of PCR master mix was used for the amplification of *β-actin*, denaturation: 94°C for 60 sec. annealing: 55°C for 60 sec. polymerization: 72°C for 60 sec. final extension 72°C for 10 min. All amplification reactions were performed in final volume of 50 µl that contain 12 µl of master mix, 8 µl of c-DNA template and 1 µl of each primers. Amplified material was stored at -20°C until gel electrophoresis was performed in 1% agarose gel. The size of the amplified product was determined using a DNA marker of different molecular weight (100 bp ladder).

Statistical analysis

The results were annualized using Graph pad prism computer program (Graph pad software, San Diego, USA). Chi-square and simple correlation test were used to test the correlation between the studied parameters. Correlation is considered significant when $p \leq 0.05$ (Everitt and Dunn, 1998).

RESULTS AND DISCUSSION

Invasive duct carcinoma cases with malignant ductal cells can usually be de-

tected by both light and electron microscopy. However, the clinical and pathological parameters alone cannot predict survival or disease free duration in certain cancer cases. Other non-subjective and more biologically oriented parameters such as hormone receptor studies and molecular studies are becoming more and more important as a prognostic adjuvant to histological evaluation (Omar *et al*., 2010).

Histopathology

In the present study, malignant breast tissues invasive duct carcinoma of 40 female breast cancer patients were confirmed by hematoxelin and eosin stain using light microscope. The stained tissues showed groups and clusters of malignant ductal cells, of highly anaplasia and mitosis (Fig. 1).

Molecular analysis of Aven gene expression

RT-PCR of the studied cases using the specific primers for *β-actin* gene resulted in the amplification of the expected 300 bp fragment in all cases (Fig. 2-a). These results indicated that all the kit components and RNA samples were working properly.

Association of *Aven* gene expression with poor prognosis in several cancers has been reported including childhood acute lymphoblastic leukemia, acute myeloid leukemia (Paydas *et al*., 2003) and breast cancer (Kutuk *et al*., 2010). Our results of the *Aven* gene expression at the mRNA level showed that: out of 40 female breast cancer patients with invasive duct carcinoma, only 20 cases (50%) showed positive *Aven* gene amplification of the expected 252 bp fragment corresponding to the *Aven* gene cDNA, the other 20 cases (50%) showed negative *Aven* gene amplification (Fig. 2-b and Table 1).

Ozgur *et al*. (2010) demonstrated the enforced expression of Aven blocks UV-irradiation-, SN-38- or cisplatin-induced apoptosis upstream of mitochondria by stabilizing Bcl-xL protein levels in breast cancer cells. Moreover, they suggested that Aven is an important mediator in DNA damage-induced apoptotic signaling in breast cancer cells and its nuclear expression is altered in breast cancer tissues, which may contribute to genomic instability in breast cancer tumors.

Immunohistochemistry

Immunohistochemistry assay was used to correlate the expression of *Aven* genes with other well characterized prognostic factors: the ER & PR status and the expression of *Ki67* and *Vegf* genes in all of the IDC cases confirmed tissues.

The application of immunohistochemical methods has become popular in assessing the state of cellular proliferation in histologic material, as it particularly allows the preservation of architectural and cytological configuration, moreover, it is much easier to perform and interpret than other methods (Eissa and Shoman, 1998).

Protein expression of PR and ER was indicated by nuclear staining of tumor cells. While, specific ER staining was characterized by brown immuno-precipitate in the nucleus of malignant cells (Fig. 3) and PR protein expression was indicated by nuclear staining of tumor cells (Fig. 4).

Ki-67 immunostaining was nuclear and varied in intensity (Fig. 5), VEGF predominantly showed a cytoplasmic staining pattern of invasive tumor cells, all tumors were positively stained, and staining level varied between weak and strong (Fig. 6).

The relationship between Aven gene expression and other prognostic factors

The relationship between the expression of *Aven* gene at the transcription level (detected by RT-PCR) and the expression of the other well established prognostic factors ER, PR status, *Ki67* and *Vegf* genes in IDC confirmed tissues was studied. Data in Table (1) revealed the presence of direct statistically significant positive correlation between the amplification of *AVEN* gene transcript detected by RT-PCR and the expression of ER, PR, Ki67 and VEGF detected by immunohistochemistry, respectively. Values of 37.5, 37.5, 50.0 and 42.2% of positive *AVEN* amplification cases showed simultaneous positive gene expression of the prognosis factors ER, PR, Ki67 and VEGF, respectively. While 25, 17.5, 22.5 and 22.5% of *Aven* gene negative amplification cases showed positive amplification of the prognosis factors ER, PR, Ki67 and VEGF, respectively. These results were in concordance with that reported by Ouzounova *et al.* (2013) as shown in Table (1).

Moreover, a direct significant association was revealed between expression of the *Aven* and *Ki67* genes, where 69% of breast cancer patients showed simultaneous expression of the two genes. Similar results were reported by (Jeffrey *et al*., 2003). In addition, a highly statistically significant correlation was observed between *Aven* gene expression and *Vegf* gene, as the *Aven* gene expression was found in 47% of *VEGF* expressed tissues and the results obtained by Lee and Rosen (2002) was in agreement with our results.

In conclusion, the molecular diagnosis of the expression of the *Aven* gene at the level of mRNA and the positive correlation of its expression with the expression of the other well characterized prognostic factors in the studied cases reported in this study support the hypothesis of using *Aven* gene as a prognosis factor in breast cancer. However, quantitative study of *Aven* gene mRNA and protein in the breast cancer cases are needed to further explore its role as a prognosis factor.

SUMMARY

In the present study, we investigated the possible role of *Aven* gene and its expression at the transcription level detected by RT-PCR technique as a prognostic factor in breast cancer to confirm the prognosis assessed by the immunohistochemical analysis of *Vegf* and *Ki67* genes,

and ER, PR status.

The present study was performed on 40 diagnosed female breast cancer patients and 10 cases benign breast tissues. The fresh tumor tissues were divided into two fragments for histological, immunohistological analysis and for the RT-PCR analysis.

In this study, we evaluated a conventional RT-PCR assay for *Aven* gene as a detection marker of 40 IDC patients. The findings were analyzed to assess the prognostic significance of *Aven* gene expression-detection in the breast tissues in association with the other known prognostic factors. Statistically significant association was found between positive *Aven* gene expression in the IDC and well established prognostic parameters; namely, *Ki67*, *Vegf* and hormonal status (P value <0.0001).

In breast cancer, *Aven* gene is considered responsible for circulating tumor tissues and its expression might constitute a biologically active subset of breast cancer patients with high tumor burden and bad prognosis. As the expression of *Aven* gene is easily determined by conventional RT-PCR, it may be useful marker to predict prognosis of breast cancer.

REFERENCES

Bancroft, J. D. and M. Gamble (2002). Theory and Practice of Histological techniques, Fifth Edition; Elserviser Science Limited, 689-691.

Chau, B. N., E. H. Cheng, D. A. Kerr and J. M. Hardwick (2000). Aven a novel inhibitor of caspase activation binds Bcl-xL and Apaf-1. Mol. Cell, 6: 31-40.

El-Bolkainy, M., A. Noh, T. El-Bolkainy and M. O. Badawy (2013). Pathology of Cancer, 4th Edition. Medical bock library, Cairo, Egypt, 298-299.

Eissa, S. and S. Shoman (1998). Markers of invasion and metastasis and markers of tumor proliferation and apoptosis in: Tumors markers. Chapman and Hall, Lippincott-Raven Publisher Inc., London, UK, p. 131-153.

Everitt, B. and G. Dunn (1998). Statistical analysis of medical data new developments, US Oxford University press Inc., New York.

Hsu, S. M., L. Rsine and H. Fanger (2000). The use of avidin biotin peroxidase techniques complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. J. Histochem. Cytochem., 29: 577-581.

Jeffrey, S. R., P. L. Gerald, S. James, C. Edwin, A. Mark, W. Nick, S. Fraser, N. H. Gabriel and P. Lajos (2003). Breast cancer biomarkers

and molecular medicine. Expert Rev. Mol. Diagn., 3: 573-585.

Konecny, E., G. Meng and M. Untch (2004). Association between Her-2/neu and Vascular Endothelial Growth Factor expression predicts clinical outcome in primary breast cancer patients. Clin. Cancer Res., 10: 1706-1716.

Kutuk, O., S. G. Temel, S. Tolunay and H. Basaga (2010). Aven blocks DNA damage-induced apoptosis by stabilising Bcl-xL. Eur. J. Cancer, 46: 2494-2505.

Lee, S. and M. Rosen (2002). Clinical Experience With Angiogenesis Signaling Inhibitors: Focus on Vascular Endothelial Growth Factor (VEGF) Blockers. Cancer Control March/April, 9, No. 2, Supplement.

Omar, S., G. Contesso and H. Khaled (2010). Breast Cancer 3rd Edition, Publ. By Association Medical Franco Egyptienne, p. 33-87.

Ouzounova, M., T. Vuong, P. B. Ancey, M. Ferrand, G. Durand, F. Le-Calvez Kelm, C. Croce, C. Matar, Z. Herceg and H. Hernandez-Vargas (2013). Micro RNA 30 familly regulates non all atchments growth of breast cancer cells. BMC Genomics, Feb. 28; 14:139. doi: 10.1186/1471-2164-14-139.

Ozgur, K. A., G. M. Sehime, T. Sahsine and B. Huveyda (2010). Aven blocks DNA damage-induced apoptosis by stabilising Bcl-xL. European J. Cancer, 46: 2494-2505.

Park, S. S., Y. A. Kim, J. E. Kim, Y. C. Kim and S. W. Kim (2005). Caveolin-1 is down- regulated and inversely correlated with HER2 and EGFR exepression status in invasive ductal carcinoma of the breast. Histopatology, 47: 625-630.

Paydas, S., K. Tanriverdi, S. Yavuz, U. Disel, B. Sahin and R. Burgut (2003). Survivin and aven: two distinct antiapoptotic signals in acute leukemias. Annals Oncol, 14: 1045-1050.

Ross, J. S. and J. A. Fletcher (2000). The HER2/neu oncogene in breast cancer: prognostic factor, predictor factor and target for therapy. Stem Cell, 16: 413-428.

WHO report (2013). Relative Frequency of Major Cancers in Men and Women Worldwide 2012.

Yerushalmi, R., S. Woods and M. Ravdin (2010). Ki67 in breast cancer: Prognostic and Predictive Potential. Lancet Oncol, 11: 174-183.

Table (1): Correlation between *Aven* gene by RT-PCR and prognostic factors.

Prognostic factors		*Aven* gene expression In IDC By RT-PCR				
		+ve		-ve		P. Value
		No.	%	No.	%	
ER	-ve	5	12.5	10	25.0	< 0.0001***
	+ve	15	37.5	10	25.0	
PR	-ve	5	12.5	13	32.5	< 0.0001***
	+ve	15	37.5	7	17.5	
Ki67	-ve	0	0.0	11	27.5	< 0.0001***
	+ve	20	50.0	9	22.5	
VEGF	-ve	3	7.5	11	27.5	< 0.0001***
	+ve	17	42.5	9	22.5	

***: P. Value< 0.0001

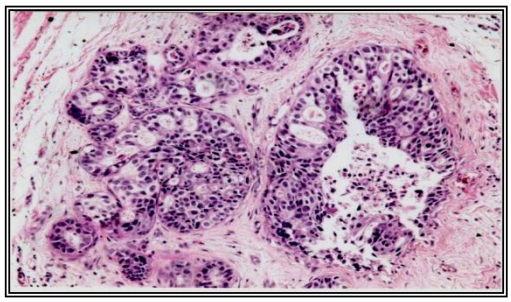


Fig. (1): Case of invasive duct carcinoma showing tubular and glandular formations (H&E x400).

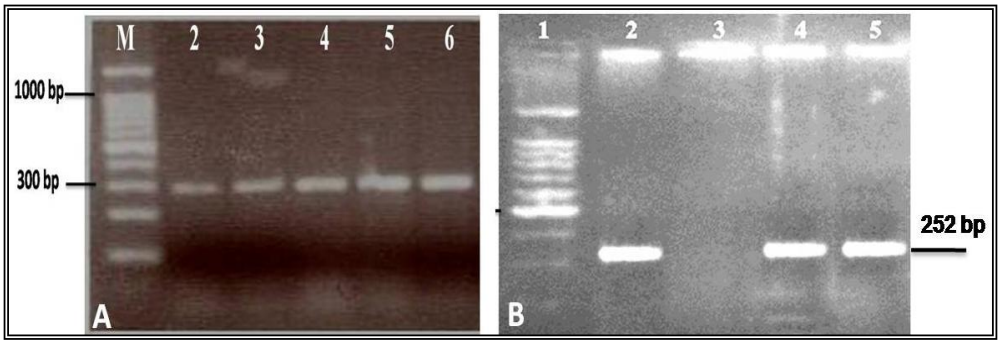
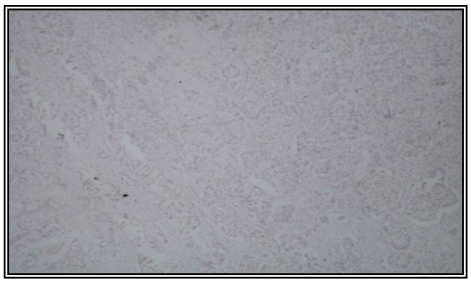
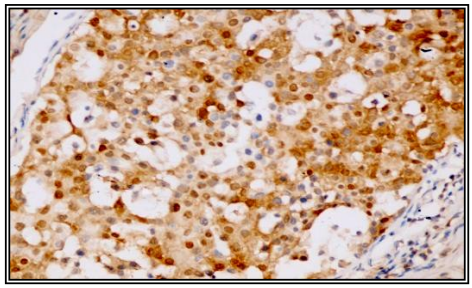


Fig. (2): RT-PCR analysis of IDC patients. A: shows RT-PCR product of *β-actin* (300 bp): Lanes 2, 3, 4, 5 and 6: *β-actin* of IDC patient. Lane 1: Marker ladder (100-1000). B: shows PCR product of *Aven* (252 bp): Lanes 2, 4 and 5: show *Aven* positive expression of IDC patients. Lane 3: shows *Aven* negative expression. Lane 1: Marker ladder (100-1000).

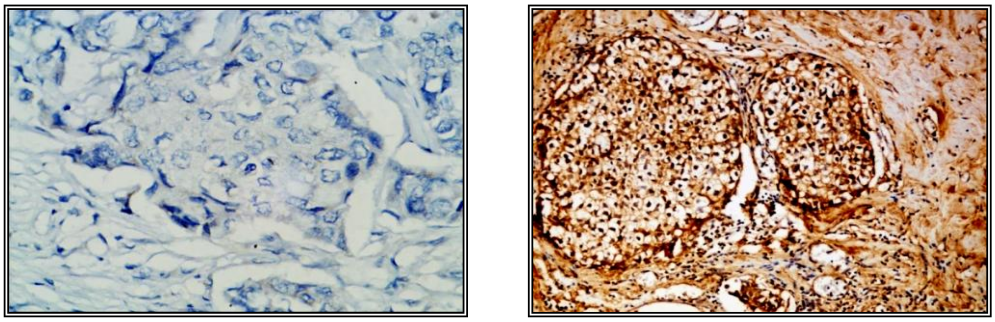


Negative



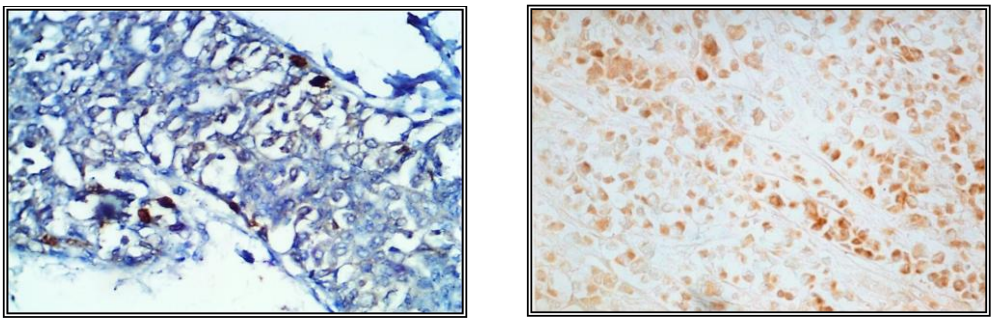
Positive

Fig. (3): IDC case stained with ER immunostaining characterized by brown immunoprecipitate in the nucleus of malignant cells (X400).



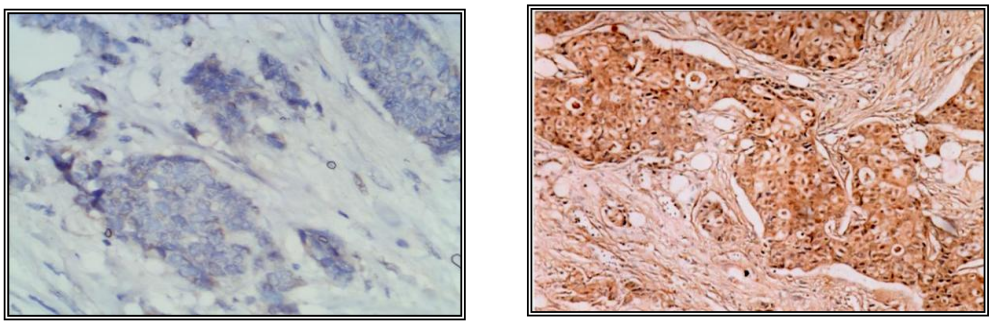
Negative Positive

Fig. (4): A case of IDC stained with PR immunostaining (X400).



Negative Positive

Fig. (5): A case of IDC showed distinct nuclear positive reaction to Ki-67 antibody, IHC technique, counterstained with DAB (X400).



Negative Positive

Fig. (6): A case of IDC showed a cytoplasmic staining pattern of invasive tumor cells and positive reaction to VEGF (X400).