

# Embryonic Markers association in Mice Bearing Mammary Adenocarcinoma (AN3) transplantable tumor model

Ahmed Majeed Alshammari<sup>1</sup>, Marwa Ibrahim Salman<sup>2</sup>, Aous Kahtan Almzaien<sup>1</sup>, Laith Husian Alwan<sup>1</sup>, Shalal Murad Al-Atabi<sup>1</sup>, Adnan Khazaal Ajeel<sup>1</sup>, Aseel Khalid<sup>1</sup>, Ahlam Duwich<sup>1</sup> and Nahi Y.Yaseen<sup>1</sup>

<sup>1</sup> Experimental therapy department, Iraqi center for cancer and medical genetic research, university of Mustansiriyah  
<sup>2</sup> Department of Biotechnology, College of science, Baghdad University

## Abstract:

AN3 tumor model had special importance as breast cancer model to evaluate new and novel therapeutic agents and there is need to study the tumor associated markers presence during AN3 tumor growth. Most common markers are Carcino-embryonic antigen (CEA) and  $\alpha$ -fetoprotein (AFP) which are cells surface glycoproteins. Oncofetal antigens involved in cell adhesion marker for cancers diagnosis and prognosis and normally they are not present in the blood of healthy adults. AN3 is transplantable mammary adenocarcinoma tumor used as in vivo model for years in cancer research for human breast cancer. Hence studying the presence of associated tumor antigens that extensively used as tumor markers (AFP and CEA) is necessary to characterize the model and determine the usefulness to use them as follow up markers after testing therapeutic agents in this model. Healthy and pregnant healthy female mice were used as control groups and AN3 transplanted mice measured after tumor grow to reach at least 0.5 cm. The results showed that CEA is markedly high in mice bearing tumors while no presence of it in both control groups. AFP had a different picture as the higher percentage was in the pregnant females, which is very normal because AFP is a fetal protein, but mice bearing tumor significantly higher concentration than healthy mice. In conclusion, AFP and CEA are useful markers to be used for AN3 model in experimental evaluation of novel therapies follow up.

**Key word:** Carcinoembryonic antigen, CEA, animal model, AFP, malignancy, mammary adenocarcinoma, breast cancer

## Introduction:

Tumor Markers are biochemical substances elaborate by tumor cells either due to the cause or effect of malignant process, they may be present as intracellular substances in tissues or may be released into the circulation and appear in serum(1). Several serum markers have been developed in different types of cancer as tools for non-invasive assessment of the tumor burden. Application of these secreted substances can be used as, Population Screening tests, Diagnosis and identification of individuals with the disease in the early stages of the disease, evaluation and Prognosis of the disease and follow up and assessment of disease recurrence (2-4). The tumor associated antigens expressed on the malignant cells could be detected and characterized in depth by using specific

monoclonal antibodies against newer epitopes(5-8). Tumor markers such as alpha fetoprotein (AFP), carcino embryonic antigen (CEA), have been widely used for the diagnosis of different types of cancers (9,10). The first tumor marker used for diagnostic purposes of different cancer (colorectal, pancreatic, breast, ovary, head and neck, bladder, kidney, and prostate cancers) was the CEA antigen, found over expressed in serum of oncological patients compared to healthy individuals (11). The AFP concentration increases above non-pregnant level at about tenth to twelfth week of pregnancy and reaches a peak between 30 and 32 weeks. Sudden decline in AFP level is noted shortly before term (12). CEA is expressed only in cancer cells, especially adenocarcinomas, such as those arising in the colon, lung, breast, stomach and pancreas (13). The aim of our work is to studying the presence of associated tumor antigens that extensively used as tumor markers (AFP and CEA) to characterize the AN3 model and determine the usefulness to test the levels of these proteins as follow up markers after testing therapeutic agents in AN3 tumor model.

### Corresponding address:

**Ahmed M. Al-Shammari**

Iraqi center for cancer and medical genetic research, Baghdad, Iraq.

**Email:** ahmed.alshammari@iccmgr.org

## Methods:

### Experimental animal:

Twenty inbred Albino Swiss females mice, aged 6-8 weeks, supplied and housed at Iraqi Center for Cancer and Medical Genetic Research (ICCMGR) animal house unite. The animals were divided into two groups, one of them is control and the other is transplanted with AN3 tumor cells. Each ten animals were housed in a plastic cage containing hard-wood chip as bedding. The housing conditions followed the guidelines of the ICCMGR.

### Tumors:

Ahmed Majeed 2003 (AM3) also named (AN3) mammary adenocarcinoma transplantable tumor line (14) was supplied from ICCMGR, Experimental Therapy Department.

Transplantation of tumor cell in mice:

Single tumor (mammary adenocarcinoma) bearing mouse, was used to obtain tumor cells which were later transplanted into adult female albino mice. The following protocol was followed to perform the transplantation process (15), which was done under sterile conditions:

1. The tumor mass region well disinfected with 70% ethyl

alcohol.

2. By using 10ml disposable syringes, the contents of tumor mass tissue withdraw into sterile flask.

3. The solid content was allowed to settle down while the supernatant discarded.

4. The residues washed 2-3 times with sterile PBS by final wash, appropriate amount of PBS was stayed. This amount was comparable with the number of animals which prepared to transplantation. Generally, the withdrawing content from tumor mass of single mouse was adequate for transplantation of on average 10 mice.

5. Separations of the tumor material into cells suspension made through mechanical disaggregation of cells in the withdrawing materials, by vigorous pipetting (withdraws and return of contents several times).

6. Each adult female albino mice (6-8 wk. old) became ready to tumor cell transplantation; 0.1ml of tumor cell suspension was transplanted through insertion of a needle (gage NO.18) subcutaneously from thigh region toward the shoulder region where the injection was performed. (Figure.1)

7. Tumor growth volumes measurement were observed and recorded by using vernier to one month.



Figure 1: Showing adult female albino mice (6-8 wk. old) transplanted with AN3 transplantable tumor model, tumor growth can be easily recognized

### Specimen collection and preparation:

Blood was collected after 30 days of tumor transplantation by heart puncture after anesthesia by xylazin. The blood was centrifuged (1500 rpm/min for 10 min) for serum isolation, which was then divided into two tubes, one to measure the AFP and the other to measure the CEA using ELISA kits.

### Quantitative determination of tumor markers (CEA and AFP) by ELISA

The procedure was done according to manufacturer protocol (Cortez Diagnostic, CA, USA). Briefly, Twenty to fifty microliters of standard, samples, and controls were dispensed into appropriate wells and then 100µl of enzyme conjugate reagent was added to each well and incubated at room temperature for 60 minutes. After washing, 100µl of TMB solution was dis-

pensed into each well and incubated at room temperature for 20 minutes. The reaction was stopped and the optical density readied at 450nm with a microtiter plate reader (Assayhitech, Austria) within 15 minutes.

## Results:

As shown in Table 1, we obtained a continuously increasing percentage of standard that used to make standard curve with an increasing number of concentrations (fig 1, 2). The test done for two serum tumor markers (CEA and AFP) in mice bearing AN3 murine mammary adenocarcinoma group, pregnant health mice group and healthy control group. The results showed the high concentration of serum tumor marker

CEA in AN3 transplanted mice group measured after tumor grow to reach at least 0.5 cm. The results showed that a serum level of CEA is markedly high in mice bearing tumors while no presence of it in both control groups. Whereas serum levels of AFP had elevated percentage in the pregnant females, the average serum level and the positive rate of AFP, CEA, and cancer group were higher than that in the control group and the healthy control groups. CEA for healthy mice was 0.033

and pregnant health mice was zero, while CEA concentration for mice bearing AN3 murine mammary adenocarcinoma transplantable cell line was 1.7 ng/ml (Fig. 3). Alphafeto protein for healthy mice was 0 and pregnant health mice was 294.1, while AFP concentration for mice bearing AM3 murine mammary adenocarcinoma transplantable cell line was 1.1 ng/ml (Fig.4).

**Table (1)** Standard levels of AFP and CEA in the study subjects

CEA ng/ml	Absorbance	AFP ng/ml	Absorbance
0	0.063	0	0.080
3	0.135	5	0.108
12	0.37	20	0.142
30	0.739	50	0.516
60	1.199	150	0.815
120	1.887	300	2.932

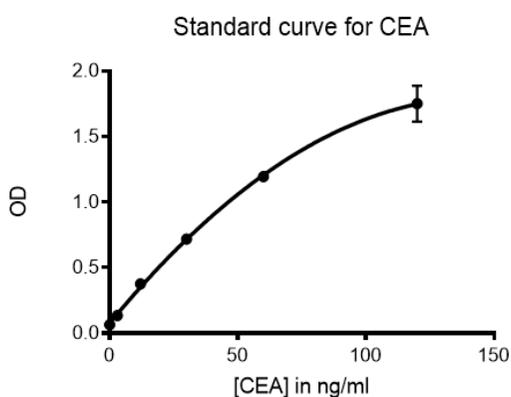


Figure 1. Standard curve of concentration of (0, 3,12, 30, 60, 120) ng/ml for CEA of optical assay with 100µlof TMB solution for 20 min.

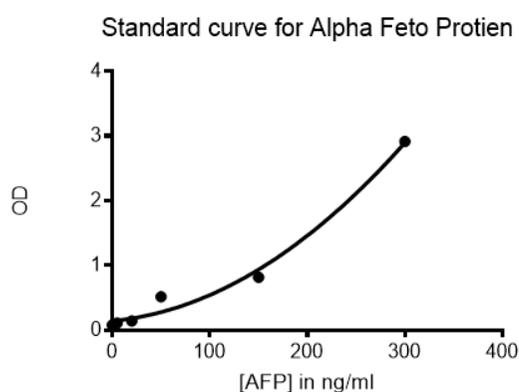


Figure 2. Standard curve of concentration of (0, 5,20 ,50,150,300 ) ng/ml for AFP of optical assay with 100µlof TMB solution for 20 min.

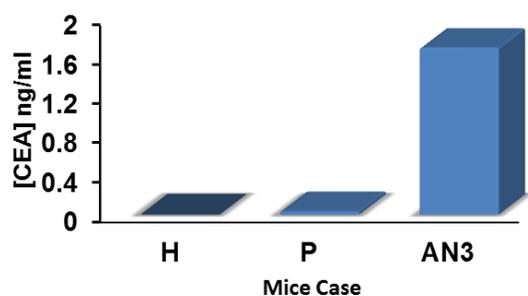


Figure 3: The serum CEA level for healthy mice (H) was 0.033 and pregnant health mice (P) was zero, while CEA concentration for mice bearing AM3 murine mammary adenocarcinoma transplantable cell line was 1.7 ng/ml.

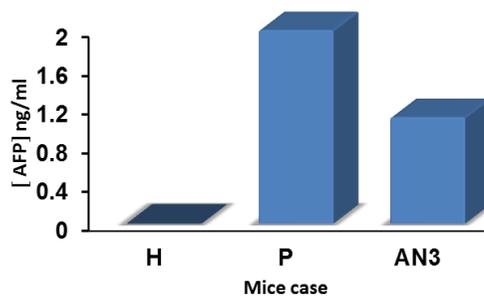


Figure 4: the serum Alphafeto protein level for healthy mice (H) was 0 and pregnant health mice (P) was 294.1, while AFP concentration for mice bearing AM3 murine mammary adenocarcinoma transplantable cell line was 11 ng/ml.

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## Discussion:

AN3 transplantable tumor line (formally known as AM3), is important tumor model in Iraqi Center for Cancer and Medical Genetic Research where used as model for 15 years in hundreds of publications. This study aimed to establish a follow up markers can be used for this model and CEA and AFP was used as tumor markers. Tumor markers that could be made of protein and carbohydrate or glycoprotein nowadays have an important role in the diagnosis and treatment of patients in different tumors, benign and malignant (16-18). In this study, we compared between AN3 bearing mice and normal healthy and pregnant health to see if the fetus proteins could affect the results and to create precise measurements for the serum tumor markers. Serum tumor markers are molecules or substances shed by a tumor into the circulation where they can be detected and quantitated, they required by clinical oncologists as an economic and noninvasive test for patient management during follow up after primary breast cancer therapy for an early detection of recurrence or metastases (19,20). They should be useful to discriminate those patients at risk to have a recurrence after primary breast cancer treatment; in this study, we measured value of two common clinical serum tumor markers AFP and CEA. Measurement of serum levels of CEA marker in the mice bearing mammary adenocarcinoma group, pregnant group and healthy group showed that CEA is obviously high in mice bearing tumors whereas no presence of it in both control groups. However, in case of symptomatic breast cancer patients CEA sensitivity increases and some authors evidenced that CEA levels at diagnosis are able to correlate with the stage

of disease (21, 22). Additionally, as a prognostic tool, the positive pretherapeutic levels of CEA may be useful to highlight those patients with a worse prognosis and at risk to have a recurrence after primary therapy (23, 24). AFP had elevated percentage in the pregnant females, which is very normal for the reason that AFP is a fetal protein. Mice bearing tumor had significantly higher concentration than healthy mice, and that make sure the AFP gene is almost completely repressed in fully matured fetus leading to disappearance of the protein soon after birth (25). Our results agree with authors whom reported that tumor marker concentrations in healthy individuals is low or zero, and its increasing suggest the incidence of related Tumor (26-29).

Bodansky (30) pointed out that a major contribution of the work on CEA is that it emphasized the clinically common solid cancers (digestive tract, lung, breast, prostate, etc.). Another studies suggested very significant elevation of serum AFP is documented rarely in malignancies of gastrointestinal tract, pancreas, lungs, kidney, and breast (31-34). Our results suggest that measuring of both tumor markers (AFP and CEA) could promote the in vivo screening and detection of tumor in our breast cancer model, the murine mammary adenocarcinoma tumor line AN3. This can help to create a follow up profile when creating an experimental protocol for novel experimental therapeutics.

### Acknowledgments

This research project was funded by Iraqi Center for Cancer and Medical genetic Research/ Mustansiriyah University Ministry of Higher Education and scientific research Iraq.

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## المعلقات الجنينية المصاحبة لأورام الغدة اللبنية المغروسة في الفئران لخط AN3

احمد مجيد الشمري<sup>1</sup>، مروى إبراهيم سلمان<sup>2</sup>، أوس قحطان المزين<sup>1</sup>، ليث حسين علوان<sup>1</sup>، شلال مراد العتايبي<sup>1</sup>، عدنان خزعل عجيل<sup>1</sup>، اسيل خالدا، احلام دويج<sup>1</sup>

1 قسم العلاج التجريبي المركز العراقي لبحوث السرطان والوراثة الطبية/ الجامعة المستنصرية  
2 قسم الكيمياء الحياتية/ كلية العلوم/ جامعة بغداد

### الخلاصة:

يمتلك خط ال AN3 أهمية خاصة كونه نموذج لسرطان الثدي في الانسان لتقييم فعالية العلاجات الجديدة والمبتكرة وهناك حاجة لدراسة المعلقات الورمية المصاحبة للورم خلال نمو السرطان في الفئران. أكثر المعلقات شيوعا هي CEA و AFP والتي هي بروتينات تتواجد على سطح الخلية. المستضدات الورمية الجنينية تشترك في معلمات الالتصاق الورمية لتشخيص الأورام، عاداتا لا تتواجد في الدم للأشخاص الأصحاء. خط ال AN3 هو ورم الغدة اللبنية الذي يستخدم في الموديلات الورمية في الحيوانات المختبرية منذ سنوات طويلة كنموذج لسرطان الثدي البشري. وهنا تبرز أهمية دراسة تواجد المستضدات الورمية التي تستخدم كمعلقات ورمية لتمييز النموذج الورمي AN3 وتحديد فائدة استعمالها كمعلقات للمتابعة بعد اختبار علاجات جديدة على هذا الموديل الورمي. التقدير الكمي والنوعي لهذه المعلقات ممكن من خلال اختبار الاليزا باستعمال الاجسام المضادة أحادية النسيلة او متعددة النسيلة. تم قياس الفئران الاناث الاصحاء التي استعملت كمجموعة سيطرة ومجموعة الفئران المغروسة بخط AN3 وذلك بعد نمو الأورام لتصل نصف سنتيمتر على الأقل. اظهرت النتائج ارتفاع ال CEA في الفئران الحاملة للأورام في حين انعدامه في كلا مجموعتي السيطرة. ال AFP يظهر صورة مختلفة تتمثل بارتفاع نسبه في مجموعة السيطرة للفئران الحوامل والتي فسرت بانها طبيعيه لانه أصلا بروتين جنيني ولكن الفئران الحامله للأورام أيضا اظهرت ارتفاع معنوي عن مجموعة السيطرة السليمه. كاستنتاج يظهر لنا ان كلا من ال CEA و AFP هما معلقات مهمه للاستعمال مع نموذج ال AN3 في تقييم التجارب على العلاجات الحديثه ومتابعتها.