Establishment and characterization of AN3 first murine mammary adenocarcinoma transplantable tumor line in Iraq

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Abstract:

Spontaneous murine mammary adenocarcinoma of aged female mouse was allotransplanted into immunosuppressed mice and successfully adapted for growing in immunocompetant mice for more than 50 passages *in vivo.* Take rate, tumor doubling time and growth rate were evaluated, cross examination showed no invasion or infiltration and highly angiogenesis nature of the tumor with milky like secretion. Histopathological examination performed at each passage showed mammary adenocarcinoma picture which characterized with presence of glandular structures, special stain as Mallory triple stain and PAS stain was performed to study the general tumor structure. Late passages (after passage 25) showed highly metastatic nature especially in to the liver. Ultrastructural study proved the secretory nature of that tumor by presence of secretory vacuoles. Chromosomal analysis showed polyploidy chromosomes in the most cells.

AN3 transplantable tumor line may be used as animal tumor model in the development and testing of new anticancer agents (chemotherapy, radiotherapy and biological therapy), and it is useful in tumor pathology and biology studies.

Introduction:

Few cancer patients benefit substantially from chemotherapy, the majority shows little or no useful antitumor effect but nevertheless suffer from the toxic effects of that drugs they are given, and sometimes die from them. Therefore, there is a pressing need for methods to determine the most effective drug against cancer.

This problem may be approached by observing the effects of drugs on tumor cells grown *in vitro* or on tumors transplanted to laboratory animals (1).Various animal models (transplantable rodent tumors) have been used in the development and testing of new anticancer agents (2). Also many human tumors have been grown either in congenitally athymic (nude) mouse or in rodents rendered immunodeficient by various techniques (3, 4).

Animal tumors used for screening procedures in the selection of cytotoxic agents with potential clinical application are in general rapidly growing (5).Transplanted tumors in animals are used extensively in research to simulate the condition in humans, They provide cell-cell and host-cell interactions which are absent in studies using cancer cells in tissue culture, large numbers can be obtained of same size, growth rat and in the same position (6). Compounds that are active in a number of screening systems in rodents could have more likelihood of demonstrating activity against hematologic malignancies and solid tumors in the clinic (7).

In general, animal tumor models can be divided into either spontaneous or artificially transplanted systems. Solid tumors are usually transplanted by the inoculation of cell suspensions by the subcutaneous (SC), intradermal (ID),

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intramuscular (IM), intraperitoneal (IP), or intravenous (IV) route (8).

The aim of this work was to establish animal tumor model to be used in drug testing and development in Iraq, because there is no transplantable tumor in mice found in Iraq before, also it will be useful in tumor biology and pathology studies.

Materials and methods:

Mice:

BALB/C female mice (6-10) weeks old were purchased from Al-Razi Company/ Iraq for diagnostic tools and bred at ICCMGR (Iraqi Center for Cancer and Medical Genetic Research) animal house, Baghdad, Iraq.

Immunosuppression of mice:

Immunosuppression of mice was done by two ways:

1- Immunosuppressive drugs: Cyclosporine A (CsA) (Novartis Pharmaceuticals, Basel, Switzerland). CsA was subcutaneously (S.C) administered in a dose of 30 mg/kg; CsA was administered daily beginning 24 h prior to tumor inoculation. CsA used to suppress cellular immunity (9). Methyl prednisolon sodium (Rotexmedica, Germany) was administered intramuscularly (I.M) at dose of 30 mg/kg. Methyl prednisolon sodium was administered daily beginning 24 hours prior to tumor inoculation. Methyl prednisolone sodium used to suppress humoral immunity (10).

2- Neonatal Thymectomy: Thymectomy was performed at 6 hours old neonatal mice as described in (10); briefly, the mouse was kept in the refrigerator (4C°) until it stopped moving. then the animal was support in a harness, longitudinal incision was made in the mid line overlying the sternum and a triangular cut was made in the rib cage at the anterior end of the sternum .The underlying fibrous tissue was removed to expose the thymus and thymic lobes were sucked out using a fine –glass tube attached to a vacuum line, The wound was closed with 2 or 3 fine –silk sutures. 6-8 weeks later they treated with 500 rad whole body irradiation from a cobalt-60 source (38 rad/ min) (Shimadza-Japan) .Then, one week later the tumor was inoculated (3).

Immunosupressed mice care:

The animals were kept in a separate animal house, on a heat -sterilized bedding and autoclaved water to prevent contamination, Neomycin or gentamycin was added to the drinking water for 14 days to prevent septicemia.

Tumor:

Tumor tissue was obtained by surgical excision from a spontaneous mammary adenocarcinoma of pregnant aged BALB/c female mouse (figure 1) (11).

Figure 1: pregnant aged BALB/c female mouse with spontaneous mammary adenocarcinoma

Implantation of tumors:

For the first transplantation of the tumor tissue, immunosuppressed mice were anesthetized by intraperitoneal (I.P) injections of zilazine (40mg/kg) (laboratories Calier, Barcelona, Spain) and fragments of tumor tissue (3-5mm) were implanted S.C. into the axillary region with incisions in inguinal region the wound was sutured with 5-0 silk (astra sutures, Turkey).

Implantations of tumor tissue in the following passages were carried as followings:

1- Mice were anesthetized by intraperitonial (I.P) injections of zylazine (40mg/kg) (laboratories Calier, Barcelona, Spain).

2- The tumor mass region was well disinfected with 70% ethanol.

3- Implantations of tumor tissue were carried out by aseptically aspirating the subcutaneous tumors using needle gage 18.

4- The tissue fragments were placed immediately in sterile PBS and the tumor cells were allowed to settle down and the supernatant was discarded, and then the tumor fragments were resuspended in PBS at appropriate volume.
5- Single cell suspension was made through mechanical disaggregation of the cells by vigorous pipetting.
6- Tumor suspension aspirated by syringe with needle gage 18 and inoculated with subcutaneous injection of 10 x 106 viable cells in 0.1ml cell suspension into shoulder region with puncture in thigh region.

Tumor growth rate:

The tumors were measured from the time that they become palpable until the death of the animal. The tumor volume was measured twice weekly with vernier caliper and estimated from the formula: tumor volume = a.b/2

Where a: the length, b: the width (12).

Histopathology:

Tissues were fixed in formalin 10% and stained by Harris hematoxyline and eosin, Special stains were also used; Mallory triple stain and PAS (periodic acid shif).

Electron microscopy:

For electron microscopy examination, tumor tissue was fixed in 2.5% glutaraldehyde for 24 h at 4 ˚C and post fixed in 1% osmium tetraoxid; specimens were then dehydrated with ethanol and embedded in araldite. Thin sections were then stained with uranil acetate and lead citrate and examined with Philips CM10 transmission electron microscope.

Chromosome analysis:

Single cell suspensions from each tumor passage were prepared by mechanical disaggregation. These were incubated with colcemid for 30 min, cultures were harvested, slide prepared and chromosomes banded as reported in (13).

Protein quantification for the AM3 mammary adenocarcinoma transplantable line secretions:

Several dilutions of protein standard bovine serum albumin (BSA) were prepared in normal saline; 4–40mg/0.1ml were prepared in LP3 tubes. This was mixed thoroughly and left to stand for 10 minutes at room temperature. The optical density of the contents of the tubes was measured at 280 nm Double samples for each, the standard one and the unknown, were used. A standard curve was plotted on the concentration against optical density of the protein standards. The protein concentration of unknown samples was calculated from the standard curve according to Bradford method (14).

Results:

Take rates:

Tumor was developed in one out of ten immunosuppressed mice (10%) at the first passage after 2 months. In the second passage, 50 immunosuppressed mice were inoculated with tumor cells aspirated from the tumor of first passage. After 18-20 days of inoculation the tumor grew in 37 mice (74%). No tumor growth was recorded in immunocompetent mice. At the third passage 16 out of 20 immunosuppressed mice showed tumor growth (80%). The tumor grew in all of the thymectomized mice (100%), and 2 out of 5 (40%) immunocompetent mice showed tumor growth. Tumor growth was recorded 10-12 days after tumor cell inoculation in all groups.

The tumor that was grown in immunocompetent mice was selected to be used in the fourth passage, in which 20 immunocompetent mice were inoculated and only 15 mice showed tumor growth 7-8 days after inoculation (75%). In the fifth passage 100% of the inoculated animals showed tumor growth after 7-8 days and this continues in the following passages until the tenth passage was reached, in which the take rates was 100%. The take rate in the following passages ranged from 70-90% according to transplanted mice breed used.

Growth rates:

The measurements were usually made on tumors in the diameter ranged (8-13mm). The growth rate often decreased with increasing tumor size in the subsequent passages. An increase in growth rate was found in the first three passages when the immunosuppressed mice were employed. The overall average doubling time fell from 15 days in passage 1 to 6 days in passage 2 to reach 4.5 days in passage 3, while an increase was occurred in tumor doubling time when the immunocompetent mice were used, in which tumor doubling time (in passage 4) was 12 days. The doubling time returned again to decrease in the following passages from 12 to 9 days in passage 5. Further acceleration sometimes occurred beyond passage 5, (figure 2). At passage 50 after 6 years of transplantation in immunocompetence mice the mean doubling time was 3-6 days.

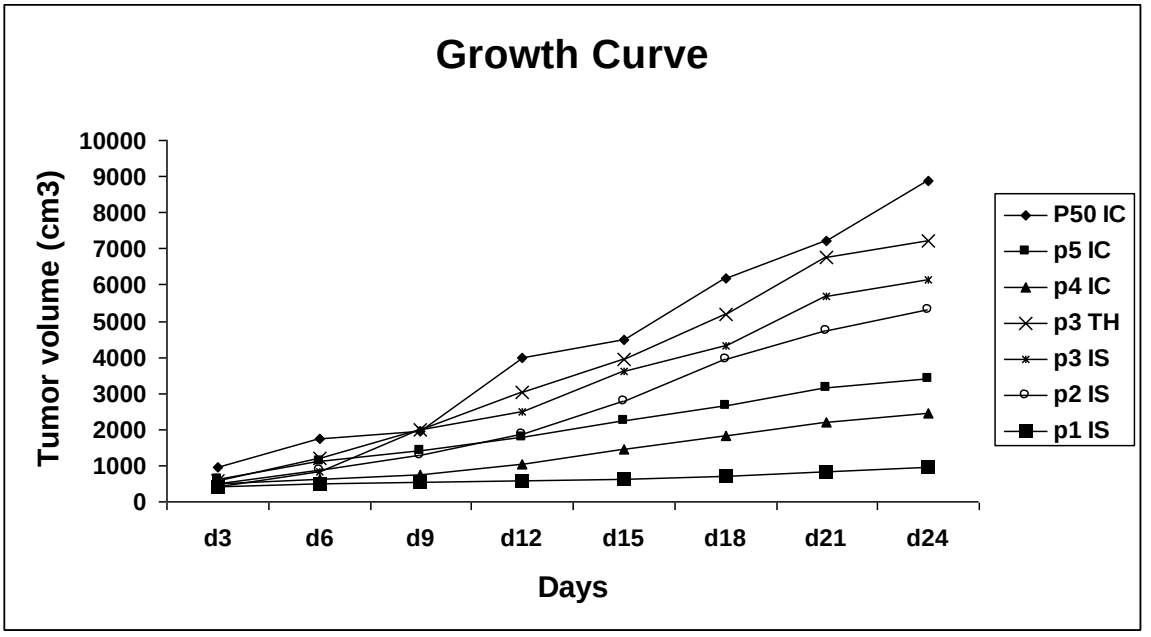

Figure 2: Growth curve of the implanted tumors

P1: first passage in immunosuppressed mice

P2: second passage in immunosuppressed mice

P3 IS: third passage in immunosuppressed mice

P3 TH: third passage in Thymectomized mice

P4 IC: forth passage in immunocompetent mice

P5 IC: fifth passage in immunocompetent mice

P 50 IC: Passage fifty in immunocompetent mice

Gross Examination:

Small nodules generally became palpable in the implant area and propagated to big tumor mass which caused cachexia and death of the animal when the diameters reach 30-40mm (figure 3). Autopsy showed no invasion or infiltration of the tumor to the muscular layer, (figure 4), except some individual cases in passage 6 and 7. The tumor was characterized with highly angiogenesis activity in all studied passages, and milky like secretions were noticed in all passages which contain 104 mg/ml protein in passage five and 120 mg/ml in passage seven.

Histopathological examination:

The histological appearance of all transplanted tumors was in general similar to the original biopsy material. Histological examination showed acinar structure, and the epithelial cells were cuboidal, and they were arranged to form cavities or tubules. This histological structure appear well-differentiated sometimes, and in other sections undifferentiated area with presence of intracystic papillary projections, sheets and nests were observed (figure 5). The glandular Lumina might have been distended by secretion (figure 6).

The amount of stroma vary, but in general there was a good amount of stroma rich with blood vessels as showed by Mallory triple stain (figure 7), with presence of mucous secretions positive for PAS stain (figure 8). At later passages (25-50) there was marked liver metastasis which showed extensively in 90% of transplanted animals (figure9).

Figure 3: Tumor bearing mouse passage 2 of AN3 tumor line

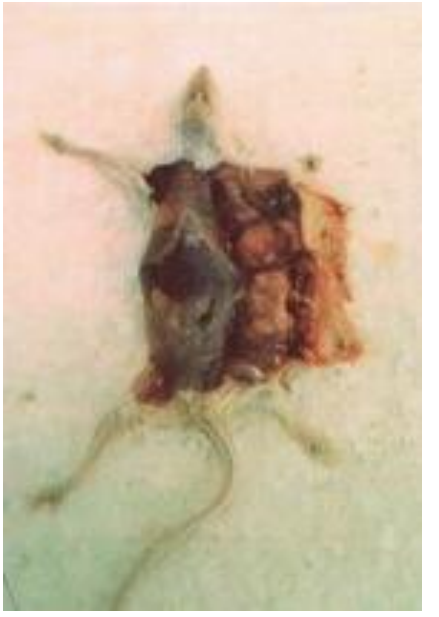

Figure 4: Autopsy shows no invasion or infiltration of the tumor to the muscular layer

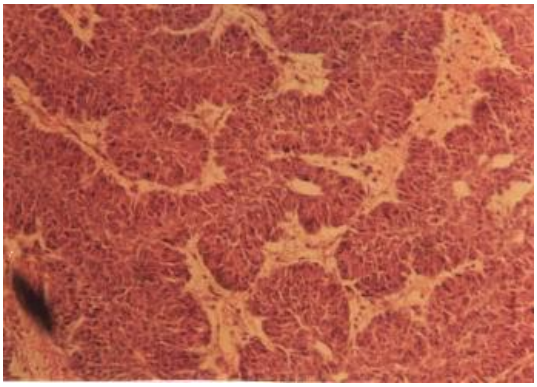

Figure 5: The glandular structure, papillary projections the characteristic features of histological picture of mammary adenocarcinoma. 40x H&E stain

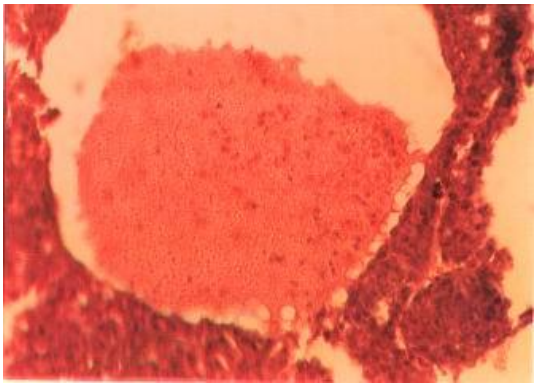

Figure 6: The glandular Lumina distended by secretion (white arrow) 40x. H&E stain

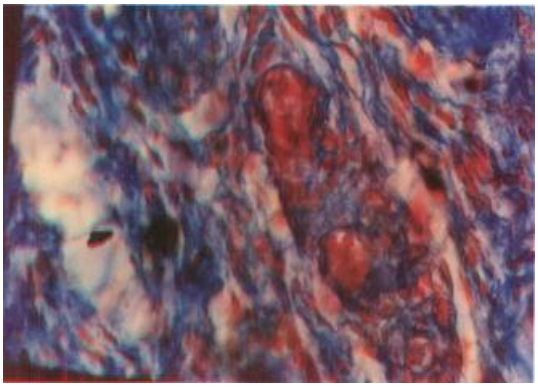

Figure 7: stroma rich with blood vessels (Mallory triple stain) 40x

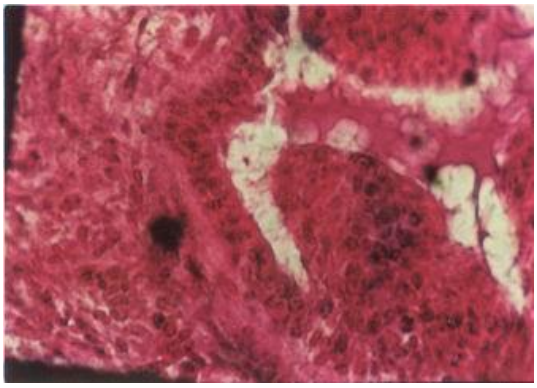

Figure 8: Mucous secretions positive for PAS stain. 40x

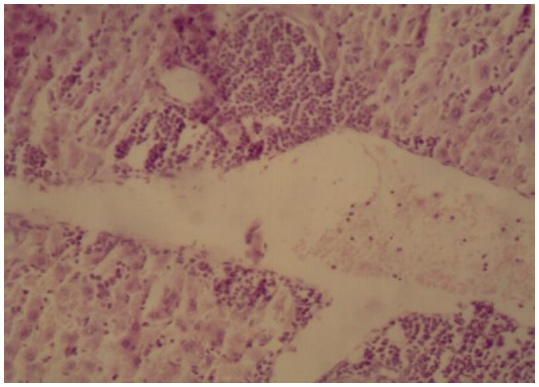

Figure 9: metastasis of mammary adenocarcinoma into the liver tissue at passage 50.

Electron microscopy:

Ultrastructural study of AM3 tumor cells showed vacuoles of secretions in different size (figure 10). Also it featured the malignant nature of the tumor in presence of active condensed chromatin, presence of more than one nucleus and differences in size and shape of cells and nuclei (figure 10, 11).

Chromosome analysis:

The most characteristic feature of chromosomal analysis was polyploidy in the tumor cells (90-120 chromosomes) (figure 12).

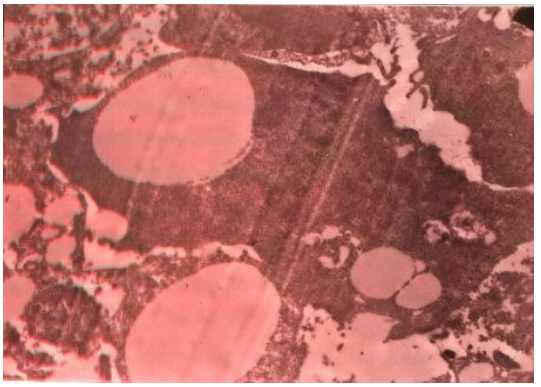

Figure 10: different sizes of vacuoles of secretion in AM3 tumor cells. 45000x

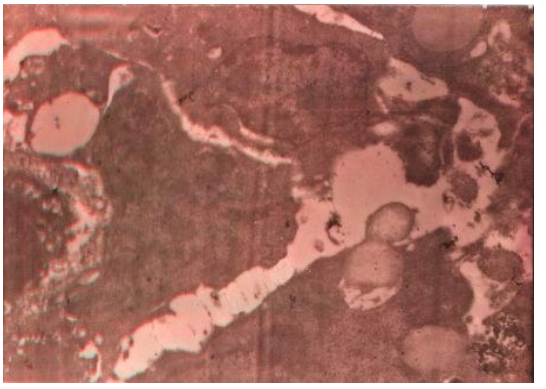

Figure 11: Presence of more than one nucleus and differences in size and shape of cells and nuclei. 35000x

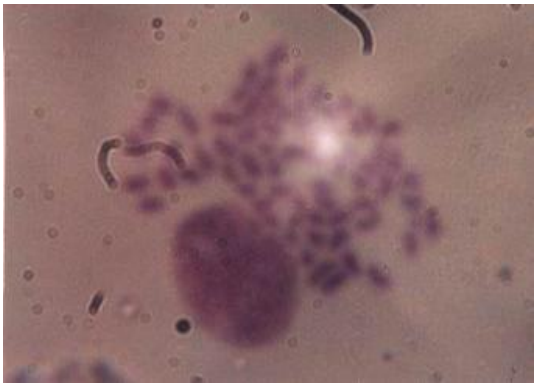

Figure 12: Metaphase of a tumor cell shows polyploidy (1000x)

Discussion:

The AN3 transplantable tumor line established as the first transplantable tumor line adapted to grew in immunocompetent mice which provide useful animal model for assessing anticancer agents. Mouse models of diseases provide the next step in understanding the disease by allowing exploration in a live animal, where microenvironments and other nebulous factors are more representative of the situation in humans. For the same reason, novel therapies can be developed in mouse models to evaluate both efficacy and undesirable side effects, thereby accelerating the pace of drug development without endangering people. One of these promising models is murine mammary adenocarcinoma as a novel mouse model of breast cancer (15,16). Furthermore, transplantable tumors have contributed much to our understanding of tumor biology and pathology as the study of metastasis of malignant tumors, such as melanoma S91 transplantable tumor line (17).

Murine mammary adenocarcinomas reproduce many features of human mammary carcinoma (18) so it can be used to simulate the situation in human. Some researcher (19) used murine mammary adenocarcinoma transplantable tumor line (TSA) as a model in cancer immunotherapy studies. The TSA tumor line was established from moderately differentiated mammary adenocarcinoma that spontaneously arose in aged multiparous BALB/C mouse, which resemble the origin of our line (AN3) that spontaneously arose in aged multiparous BALB/C mouse.

Spontaneous mammary tumors have a variable frequency in different mouse strain, depending on differences in genetic susceptibility, the presence or absence of milk –transmitted virus (MTV) and according o the hormonal condition, normal breeders or forced breeders, increase the occurrence of mammary tumors (11). The same researcher referred to BALB/C strain that it is free from milk –transmitted virus (MTV) and the mammary tumor incidence in breeding females ranges from low to 22%.

The first passage take longer time to appear than other passages due to the need for adaptation to the new environment of the host and initiation of blood supply and we notice the presence of marked decrease in the time of appearance , take rate and an increase in growth rate . similar result described by (6) in growth of C3HBA transplantable tumor line and he found the slow growth rate related to development of blood supply , gross examination of our tumor line (AN3) characterized by heavy blood supply which refer to the highly angiogenesis activity which lead to rapid tumor grew, (20) mentioned that the establishment and progressive expansion of malignant tumors following the a vascular growth period are possible only if convective nutrient supply and waste removal are initiated through nutritive blood flow. That explain the marked decrease in time of appearance (2 months – 20 day – 12 day to end with 7-8 day) also in take rate (10% - 74% - 80% - to end with 100%) and marked increase in growth rate (tumor doubling time 15 – 6 – 4.5 days) in AM3 tumor line .

Cyclosporine A used in first three passages facilitate the growth of tumors by inhibiting any immune response may lead to graft rejection, Recent report (9) demonstrates the safety use of cyclosporine A in tumor cell implantation because it has no toxic effect on tumor cells and it inhibit T lymphocyte response against implant cells .Thymectomy and mouse whole body irradiation found very useful in tumor transplantation studies because it has no T cell immune response (2).

The result recorded the grew of tumor cells in immunocompetent mice from 3rd passage with take rate about (40%) which developed to reach (75%) in the 4th passage to end with 100% in followed passages, tumor doubling time decreased from 12 to 9, this development explained by what mentioned earlier of adaptation with new environment and rapid initiation of blood supply in combination with immunological escape mechanisms developed by tumor cells. Here it could be occurrence at least one of the following : 1-Selective outgrowth of antigen –negative variant during tumor progression ,strongly immunogenic sub clones may be eliminated (19), another author (19) reported presence of poorly immunogenic murine mammary adenocarcinoma tumor line (TSA) which may resemble our tumor line (AN3). 2-Loss or reduced expression of histocompatibility antigens (22). 3-Lack of costimulatory molecules (23). 4-Tumor products may be immunosuppressive, for example transforming growth factor –β (TGF- β) (24), IL-10 (25). The Histopathological examination showed the general histological structure of mammary adenocarcinoma which described by other researchers (6,11,26). Tumor cells exhibited the presence of cysts filled with milky like secretion, secretion in mammary tumors deserves mention since it means that these tumor cells are to some extent still dependent on hormones and these milky secretion may be responsible for reversible increases of growth in mammary tumor during lactation of the host animals (11). This author also reported the presence of mucous secretion which is PAS-positive, it is the same result we found in tumor section stained with PAS stain .Ultrastructural AN3 tumor cells have round and polygonal shape that resemble other mammary adenocarcinoma tumor line TSA, But this tumor line lack to cytoplasmic secretory vacuoles which

is present in our tumor line AN3, numerical chromosomal abnormalities reported in this cell line (polyploidy) represent one of important abnormal changes in cancer cell chromosomes (27).

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استحداث وتمايز خط AN3 ، أول خط قابل للغرس لسرطانة الغدة اللبنية في العراق

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الخلاصة:

تم غرس سرطانه الغدة اللبنية لفأرة أنثى مسنة في فئران مخفضة مناعيا وتم تطويعها للغرس والنمو في الفئران الطبيعية المناعة لأكثر من 50 تمريره في الكائن الحي. تم تقييم المعايير التالي: نسبة قبول الغرس، معدل زمن تضاعف الورم، ونسبة نمو الورم. الفحص العياني التشريحي لم يظهر وجود غزو أو ارتشاح للنسيج المجاور وان الورم ذا طبيعة مكونه للأوعية الدموية بشكل عالي مع وجود إفرازات تشبه الحليب.

أنجز الفحص النسيجي المرضي لكل تمريره واظهر الفحص صورة مميزة لسرطانه الغدة اللبنية والتي تتميز بوجود تراكيب غدية, صبغت المقاطع النسيجية بالصبغات الخاصة مثل صبغة مالوري الثلاثية وصبغة باس لدراسة التركيب العام للورم. اضهرت التمريرات المتأخرة (بعد التمرير 25) وجود نقيلات ورمية بكثرة وخصوصا في الكبد. أثبتت الدراسة المستدقة ان الورم ذو طبيعة إفرازية مع وجود الحويصلات الإفرازية.

اظهر التحليل للصبغيات إن اغلب الخلايا تحتوي على أضعاف العدد الطبيعي.

يستخدم هذا الخط الورمي القابل للغرس في الفئران والمسمى (AN3) كنموذج حيواني للأورام البشرية لتطوير وفحص العقارات الجديدة المضادة للأورام (العلاج الكيميائي, الإشعاعي والحيوي). كما يمكن ان يكون مفيداً للدراسات المرضية والحيوية عن الأورام.