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Original Article

Histopathological Assessment of Testicular Tissues from Mice with Xenografted Human Triple-Negative Breast Cancer

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HIGHLIGHTS

- Mammary tumors are a common cancer in human but little is known about their histopathological characteristics and effects on other organs.
- Understanding tumor effects on testicular tissue has implications for developing mouse models of human breast cancer.

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ABSTRACT

Introduction
Mammary tumors are a common cancer in human but little is known about their histopathological characteristics and effects on other organs. Understanding tumor effects on testicular tissue has implications for developing mouse models of human breast cancer.

Methods
Mammary tumors are a common cancer in human but little is known about their histopathological characteristics and effects on other organs. Understanding tumor effects on testicular tissue has implications for developing mouse models of human breast cancer.

Results
Tumors from experimental mice showed significantly higher mitotic rate, necrosis, angiogenesis, inflammation, and atrophy compared to controls. Multiple differences were observed in testis pathology compared to control group. Experimental tumors displayed more aggressive qualities.

Conclusions
This study demonstrates that mammary tumors generated in nude mice exhibit clearly elevated pathological characteristics tied to aggressiveness, including proliferation and cell death, compared to control mice. Notably, tumor growth affected testis histology including atrophy and hyperemia. These results provide insights into using this mice model of breast cancer and establish a baseline for pathological features of experimental mammary tumors versus healthy tissues.

Keywords: Breast Cancer; MDA-MB-231 Xenograft Model; Nude Mice

Introduction

The testis is a vital organ that plays a significant role in male reproductive health and fertility. Within the testis, spermatogenesis is the multi-step process of sperm production that takes place within the seminiferous tubules (1). Spermatogenesis is a tightly regulated process that requires precise coordination of numerous intrinsic and extrinsic factors. Any disruption to the normal structure and function of the testis could potentially impact spermatogenesis and compromise male fertility (2).

One source of disruption could come from systemic diseases originating from distant sites in the body. For example, cancers developing at non-testicular sites may be able to directly invade the testis through metastasis, or indirectly affect testicular function through systemic factors released into circulation (3). Breast cancer is a disease that frequently metastasizes to distant organs, but whether it involves the testes directly or indirectly is still not well understood. Characterizing the effects of breast cancer on testicular morphology and pathology could provide important insights into male reproductive health implications (4).

This study aimed to investigate the potential impacts of breast cancer on the testes using an established mouse model. A total of 10 mice were divided into two equal groups - a control group and an experimental group implanted with human breast cancer cells. Careful histopathological analysis was then performed on the resulting mammary tumors as well as on testicular tissue from both groups of mice (5).

The results revealed several important findings. First, analysis of mammary tumor slides found characteristic features of aggressive cancer in the experimental group, including elevated mitotic activity, necrosis, angiogenesis, inflammation, and cellular atrophy compared to controls. These hallmarks indicate the tumors successfully recapitulated late-stage breast cancer behaviors as intended in this model.

Histopathological evaluation of testicular tissues, on the other hand, found multiple differences between control and experimental groups according to quantitative scoring (6).

To better understand these results, it is important to review our current knowledge of testicular structure, function, and susceptibility in the context of cancer. The testis is composed of seminiferous tubules surrounded by interstitial tissue and wrapped within the tunica albuginea. Spermatogenesis occurs inside the tubules under tight regulation by somatic cell types including Sertoli and Leydig cells (7) Specialized blood-testis barriers formed by tight junctions between Sertoli cells help maintain the specialized microenvironment required for germ cell development within tubules (8). At the same time, certain attributes of testicular anatomy and physiology could hypothetically influence susceptibility to effects from non-testicular diseases like cancer. For example, the extensive blood flow through the pampiniform plexus could facilitate the delivery of tumor-derived factors to the interstitium. In addition, endocrine signaling pathways important for spermatogenesis involve the hypothalamic-pituitary-gonadal axis, which could be disrupted indirectly by systemic cancer influences (9). While traditionally thought to have protected functions, the possibility remains that certain environmental toxicants, chemical exposures, or disease processes may still impair testicular dynamics (10, 11, 12).

Turning attention now to metastatic behavior, breast cancer has a well-defined pattern of spreading to preferential organ sites like bones, lungs, liver, and brain. Less frequent locations reported include adrenal glands and even ovary in post-menopausal patients (13, 14). The testis, being an end organ with unique anatomical properties, could conceivably host have disseminated tumor cells under permissive conditions. However, rates of testicular metastases from breast or other common cancer primaries have historically been considered exceedingly rare based on clinical autopsy studies (15).

Experimental research specifically modeling testicular involvement is also limited. Previous rodent investigations have primarily focused on toxicity and examined various chemical or environmental exposures rather than evaluating testicular metastatic susceptibility. The possibility remains that despite the appearance of normal histology as seen here, factors produced systemically by cancers may still subtly impact testicular microenvironments or regulatory pathways over longer timeframes not captured. A more comprehensive characterization is prudent considering the clinical importance of preserving male fertility and reproductive health (16).

There are several key ways the results could be strengthened. The current short-term, single-time point analysis precludes evaluating temporal or dose-dependent effects that cancer progression may impart. Incorporating multiple analysis windows out to 12 weeks or longer mimicking clinical disease courses better could reveal subtler dysfunctions not yet evident. Quantifying intratesticular factors like testosterone production, inflammation markers and hormones regulating spermatogenesis would complement traditional histopathology. Assessing functional readouts such as daily sperm counts, motility and DNA integrity offers more sensitive metrics of indirect disruption.

Analyzing a panel of breast cancer subtypes encompassing luminal, HER2-positive, and triple-negative molecular profiles modeled in both estrogen-replete and castrate settings may also provide clinically applicable insights. Incorporating genetic or immunocompetent models enabling endogenous immune influences could impact testicular involvement by modifying tumor-host

interactions. Additional controls manipulating endocrine or inflammatory pathways potentially linking cancers to testicular perturbations would help establish causality over association alone. Finally, characterizing metastatic patterns and directly quantifying disseminated tumor cells within testes could clarify the potential for direct colonization beyond indirect systemic factors (17).

In summary, further investigating the potential impacts of breast cancer on male reproductive health using optimized experimental designs holds value. Findings would advance understanding of testicular susceptibility and inform monitoring of reproductive function for cancer survivors. While no direct involvement was observed presently, subtler systemic dysfunctions remain possibilities worth rigorous exploration. Elucidating testicular consequences could ultimately improve prevention and management strategies aiming to preserve fertility for patients diagnosed with hormone-sensitive malignancies at a young age.

Methods

Experimental Animals

Ten 4–5-week-old male BALB/c nude mice weighing approximately 25 grams were used in this study. Nude mice were selected to enable xenograft tumor establishment without rejection by the recipient's immune system. The animals were obtained from Pasteur Institute's centralized breeding facility and housed in sterile micro-isolator cages with ventilated tops under specific pathogen-free conditions. Mice were provided food and water ad libitum and maintained on a 12-hour light/dark cycle in a temperature (20-22°C) and humidity (40-60%) controlled environment. All procedures involving animals were carried out according to protocols approved by Pasteur Institute's Institutional Animal Care and Use Committee (IACUC). This study was approved by ethics committee of Golestan University of Medical Sciences, Gorgan, Iran (GOUMS.REC.1398.350).

The health and comfort of experimental mice were strictly monitored throughout the study duration. Animals were inspected at least twice daily, with careful attention paid to distress signs such as decreased activity levels, rough coat appearance, crying, or aggressive behaviors that may indicate pain or suffering. Body weights were measured and recorded every other day using an electronic scale. Any animal exhibiting greater than 20% weight loss from baseline or persisting signs of severe discomfort was evaluated for early humane euthanasia based on IACUC guidelines to prevent unnecessary suffering. Cage bedding was changed at a minimum of twice weekly to ensure optimal hygiene. Overall animal usage was minimized as far as possible through strict experimental planning.

Experimental Design

Ten mice were randomly assigned to two experimental groups of 5 animals each: Non-tumor bearing control group (Group 1): Mice did not receive any tumor xenograft inoculation and served as negative disease controls. The tumor-bearing experimental group (Group 2): Mice received subcutaneous flank injections of human MDA-MB-231 breast adenocarcinoma cells to induce solid tumor formation. The MDA-MB-231 cell line was selected based on its reproducible tumorigenicity and aggressive metastatic phenotypes in preclinical models that realistically mimic important aspects of late-stage human breast cancer (18). Furthermore, MDA-MB-231 cells lack expression of estrogen and progesterone receptors as well as HER2 status, classifying them biologically as triple-negative/basal-like - an inherently difficult-to-treat molecular subtype.

Tumor Induction and Measurements

MDA-MB-231 cells were purchased from the American Type Culture Collection (ATCC) and expanded under standard conditions in Minimum Essential Medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin antibiotic mixture. Exponentially growing cells were harvested from flasks using 0.25% trypsin/EDTA, pelleted by centrifugation, and resuspended to a final concentration of 5 x 10^6 viable cells/mL in phosphate-buffered saline.

On the day of tumor inoculation, Group 2 mice were anesthetized with an intraperitoneal injection of ketamine (80mg/kg) plus xylazine (10mg/kg). Once deeply sedated, 150µL of the cell suspension (equivalent to 7.5 x 10^5 cells) mixed 1:1 with growth factor-reduced Matrigel basement membrane matrix was slowly injected subcutaneously into the right upper flank region using a 26-gauge needle. Matrigel was included to aid engraftment through improved cell retention at the inoculation site. Tumor growth was monitored every other day by measuring perpendicular tumor diameters with electronic calipers. Based on previous experiments, tumors were allowed to establish and reach an average size of 20mm (approximately 3 weeks post-inoculation) before initiation of designated treatment interventions. The target volume ensured sufficient numbers of proliferating cancer cells were present to enable analysis of treatment response parameters.

Tissue Collection

At the experimental endpoint after 6 weeks, all mice were humanely euthanized by carbon dioxide narcosis followed by cervical dislocation as per IACUC standard operating procedures. Primary tumors as well as organs likely to incur metastatic spread based on clinical experience, including lungs, liver, kidneys, and testes, were extracted. Testes were the organs of primary interest in this study to evaluate for potential direct involvement

or indirect effects from established mammary tumors. Fresh tissue weights were promptly recorded on an analytical balance.

One testis from each animal was immediately frozen in liquid nitrogen and stored at -80°C for future molecular analyses such as protein and gene expression assays if needed. The contralateral testis together with tumor samples underwent fixation in 10% neutral buffered formalin for 24-48 hours. This process cross-links and stabilizes tissue cellular and extracellular components to withstand subsequent processing and long-term storage. All tissue samples were then embedded in paraffin wax blocks using standard procedures. Multiple 5-µm sections were prepared from representative regions of each block and mounted on positively charged microscope slides for histological analysis.

Histopathological Analysis

Hematoxylin and eosin (H&E) staining was performed on prepared tissue sections using a Shandon Linistain GLX automated slide Stainer (19). This common staining protocol utilizes hematoxylin's affinity for acidic tissue components like nucleic acids to produce a purple hue, while eosin counterstains basic structures such as collagen fibers and cytoplasm in varying shades of pink. Stained sections were qualitatively evaluated under brightfield microscopy by a board-certified pathologist blinded to sample identities.

Specifically for tumor tissues, the mitotic rate was determined by surveying 5 independent high-power fields (40x objective lens) and counting the number of cells in metaphase. Necrotic areas characterized by loss of nuclei and cytoplasmic eosinophilia were also graded on a scale of 0-3 relative to the total lesion area. Features indicative of angiogenesis including micro vessel density, presence of thin-walled endothelial cell-lined lumens, and perivascular cell clustering were recorded. Additional assessments encompassed inflammatory cell infiltration, nuclear abnormalities, and the proportion of apoptotic versus viable cells.

Testicular sections underwent detailed examination of seminiferous tubule morphology and quantification of pathologic findings. Parameters documented included germ cell disposition, presence of sloughing cells within tubule lumens, the thickness of basement membranes, Sertoli cell shape/vacuolation, and spermatid density. Any foci of inflammation, edema, tubule deformations, or atrophic changes altering the normal concentric testicular architecture were noted.

Statistical Analysis

All quantitative data are reported as mean ± standard deviation. Statistical analyses were performed using GraphPad Prism version 7 statistical software (20). The unpaired two-tailed Student's t-test was applied to assess differences in tissue weights between groups. One-way analysis of variance followed by Tukey's multiple comparisons tests evaluated variations in tumor growth and histopathological scoring. The correlation between pathological features and treatment regimen was analyzed using Fisher's exact test. Differences were considered statistically significant at P-value<0.05.

Results

A total of 10 mice were included in this study, divided into two equal groups. Group 1 was the control group with 5 mice, and Group 2 was the experimental group with 5 mice implanted with mammary tumor cells. Histopathology analysis of tumor slides revealed Round to oval nuclei with prominent nucleoli in mammary tissues. Thick arrows indicate the metaphase of mitosis and the thin ones show the telophase of the mitotic cycle. Stars demonstrate the necrotic foci (Figure 1).

Picture A-E is related to the first group and the others are from group two. Images from group one shows the normal structure. Stars demonstrate the atrophic seminiferous tubules (Figure 2).

The histopathological characteristics of the tumors and testis were evaluated according to Table 1 and quantitatively scored for various pathological features in 5 high power fields for each sample. The tumors from Group 2 mice showed a significantly higher mitotic count (++++) compared to Group 1 mice (+). Necrosis (cell death) was also markedly elevated in Group 2 tumors (++) versus control (+). Features of angiogenesis and inflammation were visible in Group 2 tumors (+) but not detected in Group 1 tumors. Hyperemia was present in Group 2 tumors (+++) but absent in Group 1. Atrophy of tumor cells was seen to a higher degree in Group 2 (++++) compared to Group 1 (+). In the testis samples, there was a significant difference observed between the two groups for atrophy. Moreover, hyperemia and inflammation were

Table 1. Qualitative average of the intensity of pathological observations (5 hpf)

Tissues	**Tumor**			**Testis**		
Groups	Mitosis	Necrosis	Angiogenesis	Inflammation	Hyperemia	Atrophy
Group 1	+	-	-	-	-	+
Group 2	++++	++	+	+	+	++++

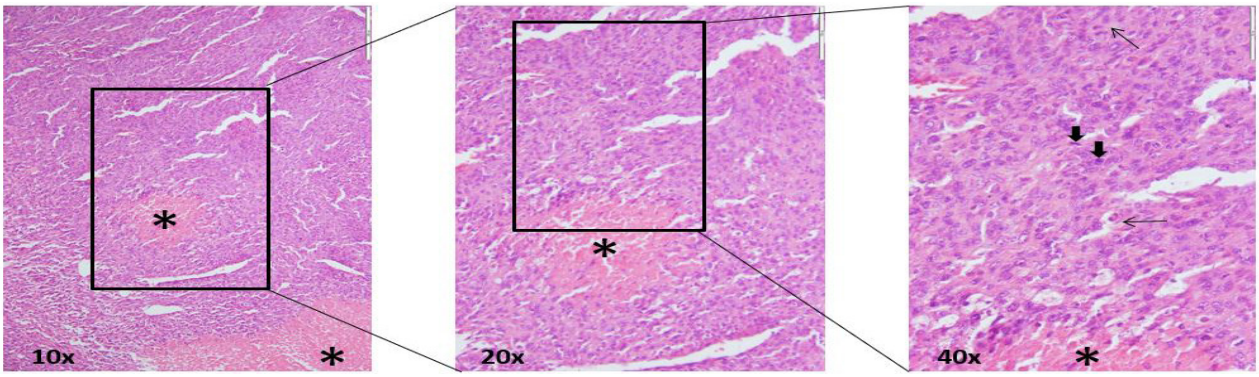

Figure 1. Histopathological sections of mammary gland tumor xenografts, male nude mice, X100, X200 & X400 magnification, Hematoxylin, and Eosin staining

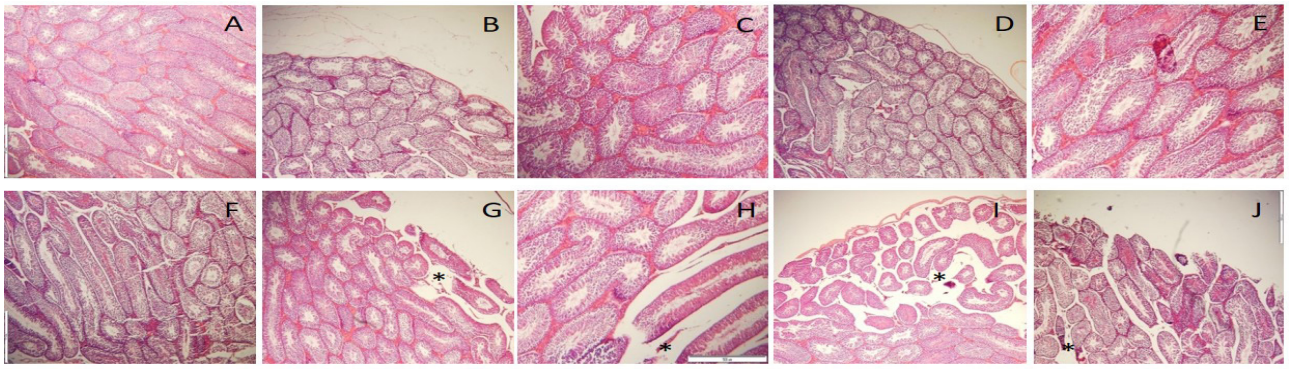

Figure 2. Histopathological sections of testes, male nude mice, X 400, Hematoxylin and Eosin staining

seen, unlike the control group. In summary, the mammary tumors generated in nude mice (Group 2) showed clearly more aggressive pathological characteristics compared to controls, with higher mitotic rate, more necrosis, angiogenesis, inflammation, and atrophy. In addition, multiple pathological changes were detected in the testis of tumor-bearing mice Table 1.

Discussion

While the testicular morphology was unchanged in tumor-bearing mice, it is important to consider the potential indirect impacts of breast tumors on testicular physiology and function. The process of spermatogenesis is energetically demanding and highly dependent on endocrine signaling (21, 22). Advanced breast cancer has been associated with adverse consequences such as hypogonadism, metabolic abnormalities, and cachexia that could theoretically compromise testicular function (23). Several endocrine alterations have been reported in patients with metastatic breast cancer that may disrupt the hypothalamic-pituitary-gonadal (HPG) axis regulation of spermatogenesis. Elevated levels of prolactin, estrogen, and cortisol as well as decreased testosterone levels often accompany progressive disease and are postulated to suppress the reproductive axis (24-27). Pro-inflammatory cytokines like interleukin-6 (IL-6) that are overproduced in cancer cachexia may directly inhibit Leydig cell testosterone production or act on the hypothalamus/pituitary to decrease gonadotropin secretion(28, 29). Malnutrition and weight loss induced by the cachectic state could deplete resources needed for normal testicular metabolism and gamete production as well (30).

While the mice in this study were not assessed for specific endocrine or metabolic changes induced by MDA-MB-231 tumor growth, a few other investigations provide insight into potential indirect effects on the testes. Odorico et al., analyzed serum hormone levels in tumor-bearing nude mice and found elevated estradiol and progesterone relative to controls, implicating aromatase activity in breast tumors (31). Increases in inflammatory factors including IL-6 have also been documented systemically with MDA-MB-231 xenografts(32, 33). Cachexia develops similarly to human patients, with weight loss correlating to tumor burden. Thus, hormonal and cytokine alterations as well as cachexia are plausible consequences that may interfere with gonadal function despite normal histology.

A limitation of the current study is the lack of functional assessment of spermatogenesis or fertility potential in tumor-bearing mice. Several methods could be employed to evaluate testicular function indirectly. Daily or weekly sperm counts from the cauda epididymis

would provide information on quantitative and qualitative changes in spermatogenesis throughout tumor progression (34). Spermatozoa quality parameters like motility, viability and DNA integrity assessed by methods such as fluorescence microscopy or TUNEL staining may reveal subclinical effects not evident by histology alone (35). Isolation of testicular cells followed by flow cytometric analysis of germ cell populations at different stages could detect disrupted kinetics of spermatogenesis (36). Measurement of intratesticular testosterone levels through enzymatic conversion or ELISA would indicate if decreased production occurs despite normal histology (37).

Additional experiments manipulating particular hormonal or inflammatory factors may help establish their relevance in mediating potential testicular dysfunction in this model. For example, neutralizing antibodies against estradiol, IL-6 or TNF-α could counteract their effects if responsible for impairing spermatogenesis (30, 38). Administration of testosterone, leutinizing hormone, or follicle-stimulating hormone to tumor-bearing mice may restore spermatogenic parameters if hypothalamic-pituitary suppression underlies any defects observed (1). Further studies incorporating functional assessments and targeted manipulation of endocrine/paracrine factors are warranted to fully characterize indirect testicular consequences of breast cancer in this xenograft model.

Another limitation is that only a single highly metastatic breast cancer cell line, MDA-MB-231, was investigated. While useful as a reproducible model of advanced disease, results may not extrapolate to all breast cancer subtypes or stages. The tumor microenvironment consists of diverse cellular components including cancer-associated fibroblasts, endothelial cells, and immune cells that crosstalk dynamically to influence disease progression (39, 40). Hormone receptor status, particular genetic/epigenetic aberrations, and various stromal cell influences vary greatly between individual tumors and impact pathogenesis (37). Future investigations incorporating cell lines representative of luminal, HER2-positive, and triple-negative subtypes as well as tumor organoid and patient-derived xenograft models would enhance clinical relevance. Delivery of co-inoculated stromal cells or immune cells may better recapitulate the native tumor ecosystem.

It may also be informative to study testicular effects in mouse models induced to metastasize to simulate advanced disease more closely. For instance, orthotopic implantation of primary mammary tumors that undergo spontaneous metastasis could create a more physiologically relevant microenvironment compared to subcutaneous xenografts (41). Genetically engineered mouse models integrating genetic drivers of breast cancer initiation and progression such as loss of p53 and activation of Wnt/β-catenin signaling induce primary tumors that metastasize to multiple organ sites including testis (38). This would allow examination of the consequences of metastatic infiltration versus indirect systemic impacts alone.

A major caveat of preclinical cancer studies is the lack of an intact immune system in commonly used nude/SCID mouse strains. While advantageous for consistent tumor engraftment, the immunodeficient background fails to account for immune surveillance and inflammation-sculpting tumor-host interactions in immunocompetent individuals (1, 42). Syngeneic mouse models transplanting carcinogen-induced autochthonous mammary tumors or transplanting mouse breast cancer cell lines into immunocompetent recipients better represent interactions between the innate and adaptive immune systems during cancer progression (39). Recent evidence highlights the importance of the testicular immune microenvironment in spermatogenesis, so the effects of mammary tumors on immune cell populations within the testes warrant investigation (43, 44).

Stromal cells present in the testicular interstitium called macrophages, lymphocytes and mast cells play regulatory roles through secreted factors including cytokines, growth factors, and hormones (44). Infiltrating or activated immune cells could influence spermatogenesis directly through cell-cell contact and soluble mediators or indirectly by disrupting Sertoli or Leydig cell function integral to sustaining spermatogenesis. The consequence of breast cancer on the localization and activation state of immune subsets residing within testicular tissue remains unknown ((45, 46). Characterizing changes to the intratesticular immune microenvironment in response to tumor growth may provide new insights into the potential susceptibility of the testes to cancers.

Future studies would benefit from utilizing newer immunocompetent genetic engineering approaches like CRISPR/Cas9 that induce autochthonous mammary tumors without engraftment into immune-deficient hosts (47). This allows intrinsic immune surveillance and inflammation to naturally participate in tumor initiation and progression more representative of human disease. Transgenic mouse models driven by tissue-specific expression of oncogenes commonly altered in breast cancer like those targeting the estrogen and progesterone receptors can induce primary mammary tumors that metastasize in an immunocompetent environment (48). Correlating testicular phenotypes to metastatic dissemination patterns in these models may help discern whether direct organ invasion poses greater risk versus indirect hormonal/cytokine influences alone (49).

Studying later endpoints beyond the 6-8 weeks' timeframe of this initial investigation is warranted as well. In clinical scenarios, adrenal and gonadal metastases are generally late-stage complications arising months to years after primary diagnosis, suggesting the need for long-term follow-up in preclinical models (48, 50). Later phases of

disease may unveil subtler functional or morphological aberrations in the testes beyond what immediate analyses revealed. Time course experiments mapping out tumor progression, onset of metastases, changes in endocrine/immune profiles, and spermatogenic parameters would provide a more complete picture of testicular vulnerability over an entire disease trajectory emulating realistic patient cases. Insights gained could guide the development of mitigation strategies when cancer involves critical reproductive organs (51).

Characterization of molecular pathways disrupted in the testes directly or indirectly by mammary tumors could offer new therapeutic targets. Global gene and protein expression profiling of testicular tissue from tumor-bearing versus control mice through techniques like microarray, RNAseq, and proteomics may reveal deregulated signaling cascades and metabolic pathways (52, 53). Interestingly, the expression of estrogen and progesterone receptors has been documented in rodent testicular tissue, suggesting potential sites of endocrine disruption (54) . Estrogen receptor beta regulates the hypothalamic-pituitary axis in part via gonadotropin inhibitory hormone, so its aberrant activation within testes by mammary-derived estradiol could mediate dysfunction (55).

Proteomic analysis mapping post-translational modifications like phosphorylation may provide clues into altered signal transduction cascades influencing spermatogenesis as well. Phosphoproteomics identified dysregulated MAPK pathways in the testes of mice exposed to environmental toxicants with anti-spermatogenic effects (56). Similar approaches applied to the current model may uncover tumorigenesis-associated perturbations in testicular kinase signaling cascades integral to germ cell development, self-renewal, and survival. Metabolomic profiling comparing metabolic fluxes in control versus diseased testes may point to disrupted nutrient availability, energy production, or antioxidant defenses as underlying predispositions to disease indirectly conveyed by mammary tumors (57). Multi-omics data integration holds promise for generating new hypotheses on mediators of indirect testicular pathology suitable for validating and targeting therapeutically.

Conclusions

This study demonstrates that mammary tumors generated in nude mice exhibit elevated pathological characteristics tied to aggressiveness, including proliferation and cell death, compared to control mice. Notably, tumor growth affected testis histology including atrophy and hyperemia. These results provide insights into using this mice model of breast cancer and establish a baseline for pathological features of experimental mammary tumors versus healthy tissues

Authors' contributions

GM initiated and supervised the project. FK was involved in the lab experiment and writing of the ultimate version of the manuscript. RO analyzed the data and wrote the manuscript. AD performed all laboratory experiments, carried out animal modeling and data analysis, and drafted the manuscript. SMD, AI, KK, and JE helped with animal xenografting. GM, MC, TSC, and RA confirmed the pathology exams. GM and AD edited and approved the final paper. All authors contributed to the article and approved the submitted version.

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Conflict of interest

All authors declare that there is no conflict of interest.

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Ethics statement

This study was approved by Ethics committe of Golestan University of Medical Sciences, Gorgan, Iran (GOUMS.REC.1398.350).

Data availability

Data will be provided on request.

Abbreviations

ATCC	American Type Culture Collection
H&E	Hematoxylin and eosin
HPG	Hypothalamic-pituitary-gonadal
IACUC	Institutional Animal Care and Use Committee
IL-6	Interleukin-6

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