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SUSCEPTIBILITY TO RENAL CANDIDIASIS DUE TO IMMUNOSUPPRESSION INDUCED BY BREAST CANCER CELL LINES

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ABSTRACT
Candidiasis is a fungal infection which is prone to occur in people with immunosuppression due to debilitating diseases and nosocomial causes. While few studies have shown evidence of this disease co-existing with malignancy-induced immunosuppression disease, there never were any exclusive animal studies demonstrating this relationship, especially renal candidiasis with breast cancer. This study aims to demonstrate the relationship between renal candidiasis and breast cancer by observing the histopathological changes of the kidneys harvested from female Balb/c mice experimentally induced with breast cancer and inoculated with candida. The mice were randomly assigned to 5 different groups (n=12). Group 1 was injected with phosphate buffer solution (PBS), Group 2 with candida, Group 3 with breast cancer and Groups 4 and 5 having co-existence of candidiasis and breast cancer at 2 different doses of candidiasis respectively. Inoculation of mice with candidiasis spores was done by intravenous injection of *Candida albicans* via the tail vein. Induction of mice with breast cancer was via injection of 4T1 cancer cells at the right axillary mammary fatpad. Stained slides of Haematoxylin and Eosin (H&E), Periodic Acidic Schiff (PAS) and Gomori Methenamine Silver (GMS) were preapred for histopathology analysis. Grading of primary tumour and identification of metastatic deposits were carried out. Scoring of inflammation and congestion in the kidney was also carried out. Results revealed that group 4 exhibited a highly significant increase in inflammation and congestion ($p<0.01$). The median severity of candidiasis was also increased in group 4 as compared to group 2. It is concluded that renal candidiasis was significantly increased in mice with breast cancer.

***Keywords*:** Renal candidiasis, breast cancer, *Candida albicans*, immunosuppression

INTRODUCTION
Candidiasis is a disease caused by *Candida* sp which are part of the normal flora found in the upper respiratory, gastrointestinal and female genital tract of the human body. Most cases of *Candida* infection result from *Candida albicans*, which is an opportunistic fungus as it does not

induce disease in immunocompetent individuals but only in those with impaired host immune defenses. Its infection is generally classified into superficial and deep. It commonly infects the nails, skin and mucous membranes, especially the oropharynx, vagina, oesophagus and gastrointestinal tract. Occasionally, the fungus invade the bloodstream and spread to other deep structure organs in the body such as kidneys, lungs, brain or other structures, causing systemic candidiasis (Levinson, 2006).

Even though bloodstream infection in on the decline, the number of risk factors which could eventually lead to candidiasis has been increasing steadily. The risk factors for candidiasis include immunosuppression due to chemotherapy or corticosteroid therapy, diabetes mellitus, low birth weight in neonates, broad spectrum antibiotics, long term catheterization, haemodialysis and parenteral nutrition. However, it has generally been observed that 3 main group of patients are associated with candidiasis, namely those with neutropenic cancer, organ or stem cell transplant patients and those undergoing intensive care procedures.

There is a marked geographical difference in the worldwide incidence of breast cancer, with a higher incidence in developed countries compared to developing countries (Contreras & Stoliar, 1988). It is the most common cancer among Malaysian women, with approximately 1 in 20 women in the country developing breast cancer in their lifetime (Yip *et al.,* 2006). In a survey done in 2 prominent hospitals in Malaysia, the age incidence was similar and it was discovered that on average, half of the cases are delayed in presentation. This was possibly attributed to a strong belief in traditional medicine, the negative perception of the disease, poverty and poor education, coupled with fear and denial (Hisham & Yip, 2004).

While the exact mechanism leading to candidiasis is not known, the initiation and progression of candidiasis can be viewed as an imbalance in the host-pathogen relationship in favour of infecting fungus. Recent studies have shown that invasive candidiasis is a common and serious complication of cancer and its therapy (DiNubile *et al.,* 2005). In cancer patients, it has been hypothesized that it develops from initial gastrointestinal colonization with subsequent translocation into the bloodstream. It is unclear what components of the innate immune system are necessary for preventing *C. albicans* dissemination from the GI tract, but it is hypothesized that both neutropenia and GI mucosal damage are critical for allowing widespread invasive *C. albicans* disease (Koh *et al.,* 2008).

Very few studies have documented the co-existence and plausible relationships between breast cancer and candidiasis (Anderson *et al.,* 2000; Safdar *et al.,* 2001; Gottfredson *et al.,* 2003; Ghoneum & Gollapudi, 2004). This study hopes to establish a hypothetical relationship between the most common cancer in women in Malaysia and renal candidiasis by using a mouse breast cancer model with *Candida* inoculation. Results from this study will provide a groundwork from which further immunological studies can be carried out to better understand the pathogenesis of *Candida* in cancer patients. It may also help bring better insight into the current treatment and pathophysiology of cancer which has itself been shown to be a risk factor to the predisposition of candidiasis.

MATERIALS AND METHODS
Experimental Animals: A total of 60 female Balb/c mice were used for the research, after prior approval from the Ethical committee housed in groups of 6 per metal cage located within the Animal Housing Facility in International Medical University (IMU). The 10 cages were divided into 5 groups (Table 1). Dosing began when the mice were 10 weeks old and weighing between 15-25 g. The animals were fed with standard mice chow and given free access to water. The weight of each mouse and the mean was recorded at the start, once every week thereafter and finally at the end of the experiment.

TABLE 1. GROUPS USED IN THIS RESEARCH WITH THEIR RESPECTIVE CHARACTERISTICS

Group No	Group Description	Concentration per dose of 0.1mL	Duration before dissection
1	Control Group (injected with PBS only)	-	2 wks
2	Mice inoculated with *Candida albicans*	$5x10^6$ cells/mL	2 wks
3	Mice induced with breast cancer	$1x10^5$ cells/mL	4 wks
4	Mice induced with breast cancer and subsequently inoculated with *Candida albicans*	$1x10^5$cells/mL of 4T1 breast cancer cells and $5x10^6$ cells/mL for *Candida albicans*	3 wks + 1 wk
5	Mice induced with breast cancer and subsequently inoculated with *Candida albicans*	$1x10^5$cells/mL of 4T1 breast cancer cells and $5x10^8$ cells/mL for *Candida albicans*	3 wks + 1 wk

Culture of *Candida* yeast cells: The *Candida* yeast cells were obtained from patient clinical isolates in IMU research laboratory. Usage of sample was done with prior permission from the researcher. The cells were subcultured onto a solid media of Sabouraud agar by streaking methods (Ref) and stored in an incubator at 37 ^oC. Before harvesting the colonies for inoculation, one of the *Candida* colonies was subcultured into the YPD broth and left for 72 hrs in a shaking incubator (*Certomat S11*) fixed at 100 rpm at a controlled temperature of 37 ^oC. After 3 days (day of inoculation), serum was added to the broth to allow for germ tubes formation to occur and left in the shaking incubator for additional 3 hrs with similar settings. The colonies were then harvested by means of centrifugation. The volume and concentration needed for inoculation was prepared by dilutions and calculated using a haemocytometer.

Inoculation of mice with Candida spores: A 27G needle syringe was used to inject 0.1 mL of candida blastospores suspended in phosphate buffer solution (PBS) with a concentration of 5 x 10^6 cells/mL via the tail vein made dilated by ethanol swap. This step was repeated with another group of mice at a concentration of 5 x 10^8 cells/mL.

Culture of 4T1 breast cancer cells: The breast cancer cells (4T1 cell line, IMU research lab) were maintained and sub cultured into a 25 cm^3 culture flask until they were healthy and had achieved a steady replicative rate. They were then harvested by means of centrifugation and kept suspended in the culturing medium. The volume and concentration needed for inoculation was prepared by dilutions and calculated using a haemocytometer.

Inducing mice with 4T1 cancer cells: The mice were first anesthesized with diethyl ether before injection of 0.1mL of 1 x 10^5cells/mL subcutaneously into the mammary fatpad at the axilla of the right arm.

Sample Collection: The mice were weighed at the end of the experiment before being sacrificed with diethyl ether in a desiccator. The kidneys from all groups, primary breast tumours and spleens, livers, lungs, heart and brain were harvested. They were subsequently fixed in 10% formalin for at least 2 days.

Tissue Processing: The fixed organs were sectioned and processed to paraffin blocks. Sections of 4 µm were taken on glass slides, stained with Haematoxylin & Eosin (H&E), Periodic Acid Schiff (PAS) and Gomori Methenamine Silver (GMS) and dehydrated, cleared and mounted with cover slips using DPX mountant media.

Observation of prepared slides: The slides were observed under the light microscope for grading of the primary tumour, presence of metastatic deposits, and extent of candidiasis in the kidneys for organ inflammation and congestion by comparatively examining the slides stained in H&E, PAS and GMS. A correlation was made between the pathological lesions observed in the groups with that of the groups' mean gross weight changes.

The presence of candidiasis and histopathological scoring of inflammation and congestion changes in the kidneys was based on standard techniques used in previous studies (Black *et al.,* 1999; Lee *et al.,* 2008) (Figs 1 & 2).

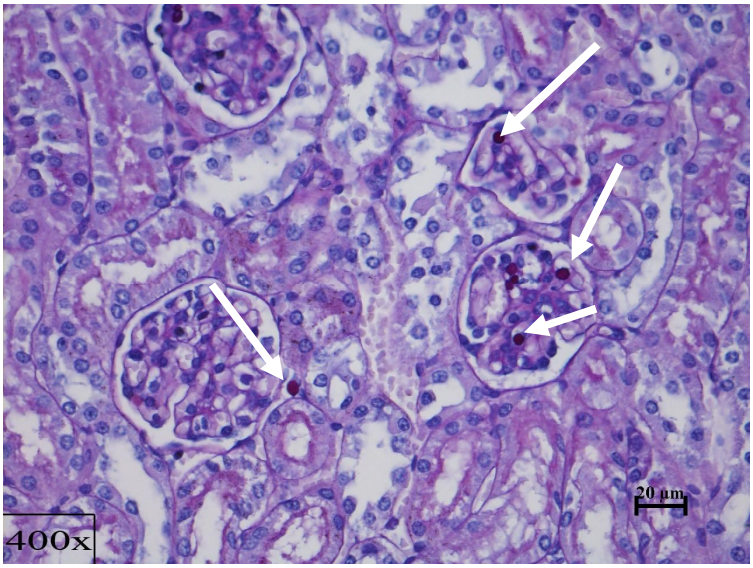

FIG1. PHOTOMICROGRAPH WITH *CANDIDA* YEAST FORMS ATTACHED TO THE GLOMERULUS OF THE KIDNEY (ARROW) (400X, PAS)

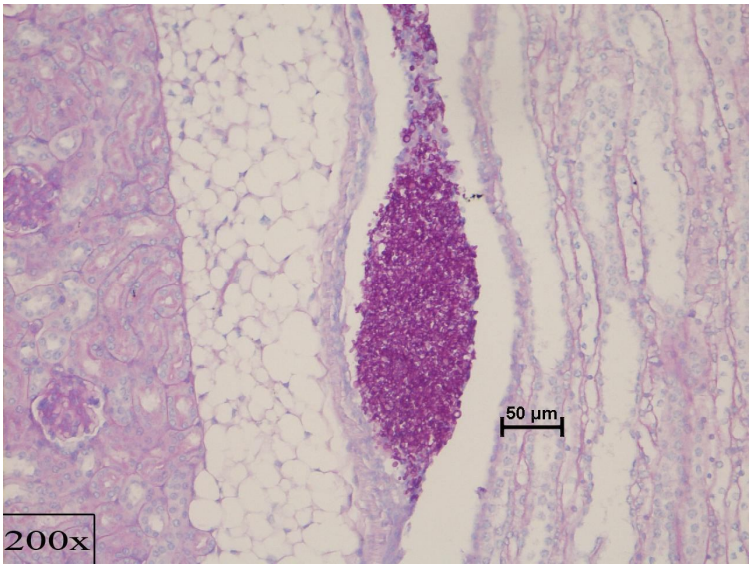

FIG 2. PHOTOMICROGRAPH OF THE RENAL PARENCHYMA SHOWING A COLONY OF *CANDIDA* YEAST CELLS AND HYPHAE AMIDST THE GLOMERULI AND TUBULES. (200X)

Scoring and grading of tumour: Scoring of candidiasis (Balish, 2009) and grading of the primary tumour was done using the conventional method of analyzing the similarity of the cells to its tissue of origin as poorly differentiated, moderately differentiated and severely differentiated (Vinay Kumar *et al.,* 2003).

Statistical Analysis: All analytical data were expressed as means with standard deviation and with a 95% confidence interval. The level of significance was set at 0.05. Paired t-test was used for comparison of initial and final mean weight of mice in each group. Kruskal-Wallis test was used for global comparison of groups for all the parameters. Non-parametric Mann-Whitney-U test was used for comparison between 2 groups for each parameter while Spearman's rho Test was used for correlation of candidiasis, cancer metastases, inflammation and congestion.

The statistical tests were conducted with the aid of SPSS Statistical software version 16. For all the individual tests, a p-value of less than 0.05 ($p<0.05$) was taken and considered as significant. Paired T test is a parametric method to test for any significant difference between the means on the same or related subject over time or in differing circumstances.

RESULTS

A total of 60 samples were analysed. The result shows a significant difference ($p<0.05$) in the weight of the mice in all the groups at the initial and end of experiment (Table 2).

TABLE 2. RESULTS OF PAIRED T-TEST FOR GROSS WEIGHT OF MICE AT INITIAL AND END OF EXPERIMENT.

Group	Mean Initial Weight (g)	Mean Final Weight (g)	Asymptote significance (p<0.05)
1	17.71	19.09	0.001**
2	18.36	16.85	0.009**
3	19.00	20.00	0.000**
4	19.40	18.01	0.039*
5	20.25	18.04	0.032*

The mean gross weight in grams at initial and end was included.
*Significant difference at *p* value < 0.05;
**Significant difference at *p* value <0.01

Renal Candidiasis: In group 2, the experimental mice were solely inoculated with Candida *albicans* by intravenous injection via the tail vein for 2 weeks. During the course of the experiment, signs of the disease in these mice included protruded eye balls, roughened fur and general reduction in activity compared to the normal group with increased tendency for mice to huddle together as a group. They also appear very weak and lean with the curvatures of the bony structures beneath the mice visible to the naked eye. In addition, the weight taken at the beginning and end of the experiment showed a significant reduction in their mean weight. This could be attributed to the possible loss of appetite and general cachexic state of the mice.

Histopathologically, good growth of *Candida* colonies in the form of hyphae, yeast cells and pseudohyphae were discovered in the renal glomeruli, tubules and interstitium near the pelvis. This was attributed to the mild dose of 5 x 10^6 cells/mL candida cells injected and the short duration of the experiment as shown in few earlier studies (Safdar et al, 2001; Wong et al, 2008).

By comparing the median severity of renal candidiasis between group 2 and 4, it was observed that there was significant difference ($p<0.01$) in its severity. In group 2, the severity of candidiasis was mild while that in group 4 was moderate (Table 4). These observations were also observed in slides stained in Periodic Acidic Schiff (PAS) and Gomori Methenamine Silver (GMS) stains.

Breast Cancer Study: Group 3 mice were injected at the mammary fatpad with 4T1 cancer cells in the right axilla region with a concentration of 1 x 10^6cells/mL and sacrified after 4 weeks of growth and metastases. During the course of experiment, the weights of the mice reduced during the first week before gradually increasing in the 3rd week. The growth of the primary tumour was detected as a palpable mass as early as the 10th day. The mice were generally active for the first 2 weeks with no apparent deviations from that usually seen in the normal control group. However, by the 3rd week, they began to exhibit signs of lethargy and were not that active. The tumour masses showed significant ($p<005$) gross enlargement by the middle of the 3rd week. Their general appetite was good. There were no distinct changes to the fur, eyes or evidence of loss of weight.

Grading for primary tumour showed it to be moderate to poorly differentiated with the majority presenting as poorly differentiated. Metastatic deposits were discovered in the lungs, liver and spleen

of the mice with varying frequencies. Scoring for inflammation showed that the median of severity of the entire group was moderate in the kidneys. The microabscesses observed in group 2 were not seen in this group. Therefore, in the group with infected breast cancer, the severity of inflammation and congestion seen in the kidney are mostly mild in severity with metastatic deposits found in the lungs, liver and spleen.

Based on the overall comparison done between the various groups with breast cancer for metastasis in each of the organs for all the groups, Mann-Whitney test for comparison between groups 3 and 4 showed a significant difference in all the organs except the brain ($p<0.01$) (Table 3). The kidneys showed a greater level of significance ($p<0.01$) as compared to the other organs. This shows that the presence of renal candidiasis as in group 4 has an effect on the extent of the metastatic growth in these organs.

TABLE 3. RESULTS OF MANN-WHITNEY TEST FOR COMPARISON BETWEEN GROUPS 3 AND 4 FOR EXTENT OF ORGAN METASTASES

Organs	Kruskal-Wallis Test for global comparison of organ metastases among groups	Mann-Whitney Test for comparison between group 3 and 4 for extent of organ metastases
	Asymptote Significance ($p<0.05$)	Asymptote Significance ($p<0.05$)
Brain	1.000	-
Kidneys	0.001**	0.001**
Lungs	0.000**	0.016*
Liver	0.001**	0.015*
Spleen	0.001**	0.016*

*Significant difference at *p* value < 0.05
** Significant difference at *p* value <0.01

TABLE 4. HISTOPATHOLOGICAL SCORING OF CANDIDIASIS IN H&E

Experimental Group	Median of Severity of Candidiasis
	Kidneys
Group 2- Mice + *Candida* ($5x10^6$cells/mL)	+
Group 4- Mice + Breast Cancer + *Candida* ($5x10^6$ cells/mL)	+++

absent (-)
mild (+),
moderate (++)
severe (+++).

Kruskal-Wallis test for global comparison between the groups for inflammation and congestion showed a significant difference ($p<0.01$) between these groups in all the kidneys (Table 5)**.** Mann-Whitney test for comparison between group 2 and 4 for inflammation response showed a significant difference ($p<0.005$) in all the kidneys. This shows that the co-existence of both candidiasis and cancer in the mice had a heightened effect on the severity of inflammation as compared to mice with candidiasis alone.

Mann-Whitney test for comparison between groups 4 and 5 for extent of candidiasis showed that the increase in *Candida* dosage from concentration of 5 x 10^8 cells/mL in group 5 compared to 5 x 10^6 cells/mL in group 4 exhibited statistical significant difference. This shows that the higher increased dose in group 5 had a statistically significant effect ($p<0.001$) on the inflammatory response seen in the kidneys. The correlation made between renal candidiasis and cancer metastases was significant.

TABLE 5. RESULTS OF ANALYSES COMPARISON BETWEEN GROUPS FOR RENAL PARAMETERS

Liver	Kruskal-Wallis Test of Global Comparison	Mann-Whitney Test for comparison between group 2 & 4
	Asymptote Significance ($p<0.05$)	Asymptote Significance ($p<0.05$)
Inflammation	0.000**	0.001**
Congestion	0.001**	0.005**
Candidiasis	0.000**	0.000**
Cancer metastasis	0.001**	0.001**

*Significant difference at p< 0.05
**Significant difference at p<0.01

Correlation between Renal Candidiasis and Breast Cancer: In group 4, the mice were first induced with breast cancer for 3 weeks and subsequently inoculated with *Candida* at a concentration of 5 x 10^6 cells/mL for 1 week. The time of induction with breast cancer was set at 3 weeks based on previous studies demonstrating that by this period, adequate metastases must have occurred in all the organs (Tao *et al.,* 2008). The initial stages of tumour growth and changes in the mice were similar to that seen in group 3 but subsequently when *Candida* was injected, changes seen in group 2 were exhibited within days instead of the 2nd week. These changes included protruded eyes and roughened fur and the animals were generally less active with increased huddle and sleep. Also, in the final stages of the experiment, a surge in the growth of tumour size was observed.

Grading carried out for the primary tumour exhibited poorly differentiated tissue with atypical cells and high number of mitotic figures. Metastatic deposits were also discovered in the lungs, liver, spleen and even in the kidneys at a higher frequency as compared to that seen in group 3. These differences were significant ($p<0.05$). This shows an increased frequency of metastatic deposits in these organs in group 4 as compared to that in group 3, suggesting a possible role of *Candida* in causing immunosuppression which by itself attributed to the increased metastatic deposits of the cancer seen in these organs. It also explains the late surge in tumour growth seen late in the experiment.

Notable changes in the kidneys include candidiasis involvement in the renal parenchyma, renal tubules and pelvis. Within the liver parenchyma and vasculature, distinct changes like microabcesses, chronic inflammation and congestion were observed at a greater level in group 4 compared to that seen in group 2. This group also exhibited increased group median of severity in *Candida* infection in the kidneys and liver. The kidneys demonstrated moderate severity compared to mild in group 2 while the liver showed moderate severity of candidiasis compared to absence of candidiasis seen in group 2. Group 4 therefore showed an extra involvement of liver compared to only kidneys as seen in group 2. This observation holds true in scoring both PAS and GMS.

Scoring of inflammation showed moderate severity in the brain, liver and lungs while the kidneys showed severe changes compared to mild seen in all the organs in group 2. Comparison of inflammation severity between these 2 groups was statistically significant ($p<0.01$).

As for congestion, group 4 exhibited moderate congestion in the brain and kidneys compared to mild in group 2. While congestion

in the lungs was not seen in group 2, this group (4) showed mild congestion. Also, the kidneys showed severe congestion as compared to just moderate congestion seen in group 2. Comparison between group 2 and 4 for congestion were significant ($p<0.05$). It is recommended that the severity of candidiasis, inflammation and congestion were seen at greater levels in breast cancer induced mice with candidiasis as compared to mice with only candidiasis.

Dose Dependent Study: In group 5, the mice were first induced with breast cancer and subsequently with Candida *albicans* at a higher dose of 5 x 10^8 cells/mL. They were similar to group 3 at the initial stages of the cancer growth but subsequently when candidiasis was injected, the mice died within the first week of inoculation at varied timings compared to group 4 where the time of inoculation with candidiasis was 1 week and mice living till the end of experiment. The sudden immediate death could be attributed to septicaemia.

Grading done on the primary tumour showed them all to be poorly differentiated. Metastatic deposits were found in the kidneys, lungs liver and spleen. Scoring of candidiasis done showed moderate to intense severity, which was highly significant. This means that with an increased dose, the kidneys exhibited candidiasis with increased levels of severity. Perhaps with a higher dose, the higher reaches of the body are much better accessible as the proportion eliminated by the liver or spleen is less.

In the scoring for inflammation, the kidneys showed a statistical significant difference ($p<0.001$) when compared to group 4. The inflammation is much less in severity compared to that in group 4 which could be attributed to the short period of inoculation time before the demise of the mice resulting in inadequate time for chronic inflammation to take place.
In the scoring for congestion, group 5 showed significant severe congestion in the kidneys, and this could be attributed to the acute changes seen in host response to a foreign pathogen.

DISCUSSION

Few studies have been conducted on experimental candidiasis in mice. This provided our research team the necessary information on the dosages and ealier observations on the subject which this experiment used to make comparison (Ashman & Papadimitriou, 1987; de Repentigny, 2004; Wong *et al.,* 2008). Some of these studies were conducted to observe the correlation between candidiasis and other forms of immunosuppression such as chemotherapy, steroid therapy, antibiotic therapy and some other form of malignancies such as leukemia and oesophageal cancer.

Even though few epidemiological studies have shown a co-existence of breast cancer and systemic candidiasis in humans (Safdar *et al.,* 2001; Gottfredson *et al.,* 2003; Talarmin *et al.,* 2009), there has never been an exclusive study on renal candidiasis and its relationship with breast cancer. This study thereforefocused on the relationship between renal candidiasis and breast cancer when the body is subjected to a chronic disease state. The study was done, bearing in mind that breast cancer was not only chosen as an ideal representation of a chronic illness but also one that is capable of suppressing the host immune system (Semiglazov *et al.,* 1978; Mandeville *et al.,* 1982; Das *et al.,* 1985; Contreras & Stoliar, 1988).

Correlation between Renal Candidiasis, Cancer Metastases, Inflammation and Congestion: The significant correlation between renal candidiasis and cancer metastases indicates that an increase in cancer metastatic deposits was accompanied by an increase in candidiasis severity. The statistically significant correlation of renal candidiasis with inflammation and congestion shows that increased levels of candidiasis is accompanied by increased levels of inflammation and congestion in the respective organs studied.

Based on the results from this study, renal candidiasis appears more severe in experimentally induced mice with breast cancer than in mice without cancer cells. This study also opens more lines of thought into various aspects of breast cancer and immunosuppression as a whole. Immunosuppression from effect of cancer increases susceptibility to systemic candidiasis. The development of this form of systemic candidiasis further breaks down the host defence and permit severe and early metastases. This study also throws a light to encourage autopsy in humans who die of immunosuppression to establish opportunistic infection (tissue diagnosis). This may indicate the need for early screening of breast cancer patients for candida infection and prompt treatment where that is established.

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