Cairo University

Faculty of Veterinary Medicine

Evaluation of the Circulatory Mammary Cancer Stem Cells Enumeration in Monitoring and Prediction of Plasmonic Photothermal Therapy Antitumor Activity in Cats

A thesis submitted by

Rasha Hamdy Abdelaziz Elsabagh

(BVSc, Sadat University, 2007; MVSc, Zagazig University, 2014)

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Under Supervision of

Salah A. Selim

Prof. Emeritus of Microbiology, Immunology, and Mycology, Faculty of Veterinary Medicine, Cairo University.

Eman Ragab

Associate Prof. of Microbiology, Immunology, and Mycology, Faculty of Veterinary Medicine, Cairo University.

Haithem A. M. Farghali

Prof. of Surgery, Anesthesiology, and Radiology, Faculty of Veterinary Medicine, Cairo University.

Abdelfattah A. Nada

Chief researcher of Immunology, Animal Health Research Institute (AHRI), Agriculture Research Centre, Egypt.

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Name: Rasha Hamdy Abdelaziz Elsabagh
Nationality: Egyptian.
Date of Birth: 10/9/1985

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Abstract

Many studies have demonstrated the importance of Breast Cancer Stem Cells (BCSCs) with the phenotypes $CD44^{+}/CD24^{-}$ and/or $CD133^{+}$ in the initiation of invasive and metastatic mammary tumors. In felines, most of the queens with mammary carcinoma (MC) exhibited an aggressive metastatic tendency, that's why the developing of rapid, accurate and noninvasive technique for prediction and monitoring of metastasis in cats is important. Unlike conventional therapies, gold nanorods (AuNRs)-mediated plasmonic photothermal therapy (PPTT) selectively eliminates BCSCs. It is the first time in the current cohort study to: (1) detect and enumerate circulatory (BCSCs) in the feline peripheral blood against their specific cellular markers using flow cytometer analysis (FCA), (2) set up a cutoff value of diagnostic significance (>276 for $CD44^{+}/CD24^{-}$ populations and >12 for $CD133^{+}$ cells), and (3) investigate the anti-tumor activity of AuNRs PPTT on the circulatory BCSCs by FCA in cats with spontaneous metastatic and non-metastatic forms of MC. Our findings revealed statistically significant differences (p 0.0001) between healthy and diseased animal groups, as well as a statistically significant (p 0.046) increase in the $CD44^{+}/CD24^{-}$ and $CD133^{+}$ cell counts in cats with metastasis between baseline and last sample values. This supported the use of FCA as a sensitive, specific, and rapid diagnostic and monitoring tool for mammary carcinoma. As the PPTT was applied on each animal in this investigation either alone (G.AI &II) or in combination with mastectomy (GB), the treated exhibited a gradual decrease in the tumor sizes till complete ablation 6-8 weeks post treatment. The total survival rate was 53.8%, the metastatic animals (G.AI) had a lower Overall Survival (17. months) and Progression Free Survival (4.08 months) than the nonmetastatic cats (22.5 &19.3months)respectively.

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List of Abbreviations

ALDH	Aldehyde Dehydrogenase
AUC	Area Under the Curve
AuNRs	Gold Nano Rods
BCSCs	Breast Cancer Stem Cells
CBCSCs	Circulating Breast Cancer Stem Cells
CD	Cluster of Differentiation
CEA	Carcinoembryonic Antigen
CKs	Circulating Cytokeratins
CKs	Cytokeratins
CMT	Canine Mammary Tumor
CSTs	Cancer Stem Cells
CTCs	Circulating Tumor Cells
ctDNA	Circulating Tumor DNA
DTCs	Disseminated Tumor Cells
EGFR	Epidermal Growth Factor Receptor
EMT	Endothelia Mesenchymal Transition
EpCAM	Epithelial Cell Adhesion Molecule
ER	Estrogen Receptor
FACS	Fluorescence-activated cell sorting
FCA	Flow Cytometry Analysis
FMC	Feline Mammary Carcinoma
FMTs	Feline Mammary Tumors
FNAS	Fine Needle Aspiration Assay
FS	Forward Scatter
HBC	Human Breast Cancer
HER2	Human Epithelial Growth Factor Receptor 2
LR	Likehood Ratio
MET	Mesenchymal Endothelial Transition
miRs	microRNAs
NIR	Near Infra-Red

OR	Odds Ratio
PB	Peripheral Blood
PPTT	Plasmonic Photo Thermal Therapy
PR	Progesterone Receptor
ROC	Receiver Operating Characteristic
SS	Side Scatter
TAAs	Tumor Associated Antigens
TICs	Tumor-Initiating Cells
WHO	World Health Organization

6. Summary

Feline mammary carcinoma (FMC) represents the third common type of tumors in cats. Almost 90% of these tumors are malicious, aggressive and have metastatic capacities. Cats consider the compatible animal model for human breast cancer (HBC) researches. Many of studies have proved the role of Tumor Initiating Cells (TIC) or Cancer Stem Cells (CSC) in breast cancer recurrence and metastasis. Breast Cancer Stem cells (BCSCs) that have $CD44^{+}/CD24^{-}$ and /or $CD133^{+}$ phenotype have been associated with enhanced invasive and metastatic potentials in human and companion animals' cancers.

By Endothelial- Mesenchymal Transition (EMT) process, some tumor cells (known by Circulating Tumor Cells, CTCs) can intravasate to the surrounding blood or lymphatic vessels, survive in the circulation or lymphatic system and finally extravasate to the remote sites. The serial analysis of CTCs and cCSCs in the Peripheral Blood (PB) considered an efficient non-invasive easily accessible approach for the prediction, diagnosis, and prognosis of metastatic breast cancer in both human and veterinary oncology researches.

As TICs showed high resistance against the regular chemo and radiotherapies, here appears the importance of the gold-based nanoparticles (AuNRs) in association with thermal therapy as a promising targeting therapy of BCSCs.

In pets, Ali et al. were the first to apply the Plasmonic PTT in association with AuNRs for the treatment of feline and canine mammary carcinoma. Even one year after treatment, no relapse or toxicity effect was observed in any of the animals exposed to AuNRs-PPTT. Herein, we investigated and for the first time in cats the Flow Cytometry analysis (FCA) of feline peripheral blood to detect and enumerate cBCSCs against their specific cellular markers: $CD44^{+}/CD24^{-}$ combination and $CD133^{+}$ individually. We evaluated the sensitivity and specificity of this technique for the early diagnosis of FMC and for the prognosis and monitoring of animals that subjected to AuNRs-PPTT by means of the receiver operating characteristic (ROC) analysis and the Mann Whitney U test, the Wilcoxon Sign Rank test respectively. Also, we set up a cutoff point of diagnostic value for each used phenotypic marker/ combination.

We examined the relationship between cancer occurrence and cBCSC counts using binary logistic regression analysis. In addition, for the first time, we investigated the effects of AuNRs PPTT in cats with spontaneous metastatic and non-metastatic MC using Flow Cytometry Analysis of the cBCSCs. We used the Spearman's rank correlation coefficient test to examine the relationship between $CD44^{+}/CD24^{-}$ cells and $CD133^{+}$ levels before and after the diagnosis of metastasis in cases treated only with AuNRs-PPTT.

The results revealed that that BCSCs could be detected and counted in the PB of feline depending on their definite cellular markers ((CD44^{+}/CD24^{-}) and CD133^{+}); FCA is a sensitive, specific, and rapid diagnostic tool for mammary carcinoma and an effective prognostic and monitoring approach for the metastatic form of breast cancer in cats. Also, we established a cutoff point of diagnostic values for both (CD44^{+}/CD24$^{-/low}$) and CD133^{+} cell populations (>276 and >12), respectively. The odds of having a mammary tumor were 1.004 times as likely to have one CD44^{+}/CD24^{-}cell and 2.240 times as likely to have one CD133^{+} cell. In addition, this research specified the localized effect of AuNRs PPTT in the treatment of metastatic and nonmetastatic forms of FMC in queens either alone or in combination with surgery (tumors>3cm) as none of the treated tumors revealed any recurrence or relapse over the long follow-up period of (28 months). The Spearman's rank correlation coefficient test confirmed a strong statistically significant (p=0.005) correlation between values of CD44^{+}/CD24^{-} cells before and after diagnosis of metastasis unlike those of CD133^{+} population.

The queens that were subjected to PPTT showed a 53.8% total survival rate. Metastatic Animals posed an overall survival (OS) (17.3 months) and progression-free survival (PFS) (4.08 months), lower than those without metastasis (OS of 22.5 months and a PFS of 19.3 months) respectively. At cats that were subjected to PPTT in combination with

surgery, the queens demonstrated an OS of mean 8.3 months and a mean PFS of 6.7 months.