SALIVARY EXOSOMES MEDIATED DIAGNOSTIC SIGNATURES AND PHYTOTHERAPEUTICS FOR BREAST CANCER

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“SALIVARY EXOSOMES MEDIATED DIAGNOSTIC SIGNATURES AND PHYTOTHERAPEUTICS FOR BREAST CANCER”

by

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Dedicated to Lord Radhavallabh…

My beloved grandparents, parents, brother and sister

For their unconditional love, care, support, and endless sacrifices

CERTIFICATE

This is to certify that the thesis titled "**SALIVARY EXOSOMES MEDIATED DIAGNOSTIC SIGNATURES AND PHYTOTHERAPEUTICS FOR BREAST CANCER**", submitted by **Mr. Sunny Gupta** to the Indian Institute of Technology Delhi for the award of the degree of "**Doctor of Philosophy**" is a record of the bonafide research carried out by him, which has been prepared under my supervision and guidance in conformity with the rules and regulations of the Indian Institute of Technology Delhi, India. The results presented in this document have not been submitted, in whole or in part, to any other university for the award of any degree or diploma.

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ABSTRACT

Breast cancer ranks as the second most common cause of mortality among women. Timely detection and suitable treatment, accompanied by diligent monitoring, are crucial in minimizing the impact of this fatal disease. In the present study, we aimed to identify biomarkers that could assist in early and non-invasive breast cancer diagnosis. We isolated the exosomes from the saliva of HER-2-positive breast cancer patients with grade II ductal carcinoma and performed comparative proteomics using label-free LC-MS.

Through comparative proteomics, we observed that most of the differentially expressed proteins (DEPs) were involved in the regulation of enzyme activity, binding with actin filament, regulation of various signalling pathways (VEGF and IL-17 signalling), and neutrophil extracellular trap formation. Among the DEPs, HSPB1, ANXA1, SERPINB3 and CSTB were upregulated; and FLNA, CAP1, and CST4 were downregulated. We further validated two upregulated and downregulated proteins through Western blotting. The obtained salivary biomarkers could be employed in the early diagnosis of HER2-positive breast cancer.

Exosomes are extracellular vesicles released by cells that mediate intercellular communication by shuttling their cargo of biomolecules, including proteins and nucleic acids. Besides having diagnostic roles, exosomes also play a role in cancer progression, angiogenesis, and metastasis. Inhibiting exosome release from cancer cells could be used as a therapeutic. To address this, we investigated the inhibitory effects of *Acorus calamus* extract on exosome secretion from breast cancer cells. Our approach involved targeting Rab27a and nSMase2 (neutral sphingomyelinase 2). We observed that treatment with *A. calamus* significantly downregulated the expression of Rab27a and nSMase2 in all tested breast cancer cells (MDA-MB-453, MCF-7, and MDA-MB-231). In addition, we observed that *A. calamus* enhanced the efficacy of tipifarnib and GW4869 in downregulating Rab27a and nSMase2. Immunofluorescence

staining revealed a decreased fluorescence intensity of Rab27a and nSMase2 in all treatment groups compared to the untreated control. We observed a reduced exosome secretion from breast cancer cells as revealed by NTA and decreased acetylcholine esterase activity. Next, we conducted metabolomic profiling of *A. calamus* extract to reveal the phytochemicals present and docked them on Rab27a and nSMase2 to decipher the compounds responsible for protein inhibition. Molecular dynamic simulations were conducted on lead compounds, and we observed that calcitriol lactone showed the most stable binding interactions with nSMase2. Treatment of breast cancer cells with calcitriol lactone significantly downregulated nSMase2 expression. These findings suggest that *A. calamus* could inhibit exosome secretion, potentially paving the way for cancer therapeutics.

Historically, medicinal plants have been used to manage breast cancer. However, the ability of these plants to specifically reduce HER2 protein expression lacked scientific backing. This study aimed to evaluate the anti-cancer properties of extracts from ten medicinal plants against HER2-positive breast cancer cells. Using the SRB assay, we observed that all extracts inhibited the proliferation of HER2-positive breast cancer cells. Notably, extracts from *Terminalia chebula*, *Berberis aristata*, and *Punica granatum* significantly downregulated HER2 expression in these cancer cells. In addition, we observed that treatment with plant extract induced apoptosis in breast cancer cells, as revealed by increased Bax/Bcl-2 ratio, AO/EB staining, and flow cytometry. A comparative proteomic analysis showed modulation in the proteome profile of breast cancer cells after treatment with *T. chebula*, *B. aristata*, *P. granatum*, *M. pruriens*, and *A. calamus*. Metabolic profiling of lead plants was done, and molecular docking was performed with the HER-2 receptor. The top hits obtained during docking were subjected to MD simulation, and we observed that Protopine, hypoxylone, Cyclonovobiocic acid and OSI-7904 showed stable binding with HER-2. Our study suggests that these plants hold promise as novel therapeutic approaches for HER2-positive breast cancer.

In conclusion, we identified salivary exosome proteins as potential biomarkers for early detection of HER2-positive breast cancer. We also found that the aqueous extract of *Acorus calamus* downregulated Rab27a and nSMase2 in breast cancer cells, leading to reduced exosome secretion compared to the control group.

सारांश

स्तन कैंसर महिलाओं में मृत्यु का दूसरा सबसे बड़ा कारण है। इस घातक बीमारी के प्रभाव को कम करने में समय पर पता लगाना और उचित उपचार के साथ-साथ लगन से निगरानी करना महत्वपूर्ण है। वर्तमान अध्ययन में, हमने बायोमार्कर की पहचान करने का लक्ष्य रखा है जो स्तन कैंसर के शुरुआती और गैर-आक्रामक निदान में सहायता कर सकते हैं। हमने ग्रेड II डक्टल कार्सिनोमा वाले एचईआर-2 पॉजिटिव स्तन कैंसर के रोगियों की लार से एक्सोसोम को अलग किया और लेबल-फ्री एलसी-एमएस का उपयोग करके तुलनात्मक प्रोटिओमिक्स का प्रदर्शन किया।

तुलनात्मक प्रोटिओमिक्स के माध्यम से, हमने देखा कि अधिकांश विभेदित रूप से व्यक्त प्रोटीन (DEPs) एंजाइम गतिविधि के विनियमन, एक्टिन फिलामेंट के साथ बंधन, विभिन्न सिग्नलिंग मार्गों (वी. ई. जी. एफ. और आई. एल.-17 सिग्नलिंग) के विनियमन और न्यूट्रोफिल बाह्य कोशिकीय जाल निर्माण में शामिल पाए गए। डी. ई. पी. में HSPB1, ANXA1, SERPINB3 और CSTB को अपग्रेडित किया गया था और FLNA, CAP1 और CST4 को डाउनरेग्यूलेट किया गया था। हमने आगे वेस्टर्न ब्लॉटिंग के माध्यम से दो अप्रेगुलेटेड और डाउनरेगुलेटेड प्रोटीन को मान्य किया। प्राप्त लार बायोमार्कर को एचईआर2-पॉजिटिव स्तन कैंसर के प्रारंभिक निदान में नियोजित किया जा सकता है।

एक्सोसोम कोशिकाओं द्वारा जारी बाह्य कोशिकीय पुटिकाएं हैं जो प्रोटीन और न्यूक्लिक एसिड सहित जैव अणुओं के अपने कार्गो को बंद करके अंतरकोशिकीय संचार में मध्यस्थता करती हैं। नैदानिक भूमिकाओं के अलावा, एक्सोसोम कैंसर की प्रगति, एंजियोजेनेसिस और मेटास्टेसिस में भी भूमिका निभाते हैं। कैंसर कोशिकाओं से एक्सोसोम रिलीज को रोकने का उपयोग एक चिकित्सीय के रूप में किया जा सकता है। इसका समाधान निकालने के लिए, हमने स्तन कैंसर कोशिकाओं से एक्सोसोम स्राव पर एकोरस कैलमस के अर्क के निरोधात्मक प्रभावों की जांच की। हमारे दृष्टिकोण में Rab27a और nSMase2

(न्यूट्रल स्फिंगोमाइलिनेज 2) को लक्षित करना शामिल था।हमने देखा कि ए. कैलमस के साथ उपचार ने सभी परीक्षण किए गए स्तन कैंसर कोशिकाओं में Rab27a और nSMase2 की अभिव्यक्ति को काफी कम कर दिया. इसके अलावा, हमने देखा कि ए. कैलमस ने Rab27a और nSMase2 को डाउनरेगुलेट करने में टिपिफार्निब और जीडब्ल्यू4869 की प्रभावकारिता को बढ़ाया। इम्यूनोफ्लोरेसेंस स्टेनिंग से पता चला कि अनुपचारित नियंत्रण की तुलना में सभी उपचार समूहों में Rab27a और nSMase2 की प्रतिदीप्ति तीव्रता में कमी आई है। हमने एन. टी. ए. द्वारा प्रकट स्तन कैंसर कोशिकाओं से एक कम एक्सोसोम स्राव देखा और एसिटाइलकोलाइन एस्टेरेस गतिविधि में कमी आई। इसके बाद, हमने उपस्थित फाइटोकेमिकल्स को प्रकट करने के लिए ए. कैलमस एक्सट्रैक्ट की मेटाबोलोमिक प्रोफाइलिंग का संचालन किया और प्रोटीन अवरोध के लिए जिम्मेदार यौगिकों को समझने के लिए उन्हें Rab27a और nSMase2 पर डॉक किया। आण्विक गतिशील सिमुलेशन लीड यौगिकों पर आयोजित किए गए थे, और हमने देखा कि कैल्सीट्रियोल लैक्टोन ने nSMase2 के साथ सबसे स्थिर बाइंडिंग इंटरैक्शन दिखाया। कैल्सीट्रियोल लैक्टोन के साथ स्तन कैंसर कोशिकाओं का उपचार काफी हद तक nSMase2 अभिव्यक्ति को कम करता है। इन निष्कर्षों से पता चलता है कि ए. कैलमस एक्सोसोम स्राव को रोक सकता है, जिससे संभावित रूप से कैंसर चिकित्सा का मार्ग प्रशस्त हो सकता है।

ऐतिहासिक रूप से, स्तन कैंसर के प्रबंधन के लिए औषधीय पौधों का उपयोग किया गया है। हालांकि, इन पौधों की विशेष रूप से एचईआर 2 प्रोटीन अभिव्यक्ति को कम करने की क्षमता में वैज्ञानिक समर्थन का अभाव था। इस अध्ययन का उद्देश्य एचईआर 2-पॉजिटिव स्तन कैंसर कोशिकाओं के खिलाफ दस औषधीय पौधों से अर्क के कैंसर विरोधी कैंसर गुणों का मूल्यांकन करना है। एसआरबी परख का उपयोग करते हुए, हमने देखा कि सभी अर्क एचईआर 2-पॉजिटिव स्तन कैंसर कोशिकाओं के प्रसार को रोकते हैं। विशेष रूप से, टर्मिनलिया चेबुला, बर्बेरिस अरिस्टाटा, और पुनिका ग्रैनाटम के अर्क ने इन कैंसर कोशिकाओं में एचईआर 2 अभिव्यक्ति को काफी कम कर दिया।इसके अलावा, हमने देखा कि स्तन कैंसर कोशिकाओं में पौधे के अर्क से प्रेरित एपोप्टोसिस के साथ उपचार, जैसा कि बढ़े हुए बैक्स/बी. सी.

एल.-2 अनुपात, ए. ओ./ई. बी. धुंधलापन और फ्लो साइटोमेट्री से पता चलता है। एक तुलनात्मक प्रोटिओमिक विश्लेषण ने टी. चेबुला, बी. अरिस्टाटा, पी. ग्रेनाटम, एम. प्ररियन्स और ए. कैलमस के साथ उपचार के बाद स्तन कैंसर कोशिकाओं के प्रोटिओम प्रोफाइल में मॉड्यूलेशन दिखाया। लीड प्लांट्स की मेटाबोलिक प्रोफाइलिंग की गई और HER-2 रिसेप्टर के साथ मॉलिक्यूलर डॉकिंग की गई। डॉकिंग के दौरान प्राप्त शीर्ष हिट एमडी सिमुलेशन के अधीन थे, और हमने देखा कि प्रोटोपीन, हाइपोक्सिलोन, साइक्लोनोवोबायोसिक एसिड और ओएसआई-7904 ने एचईआर-2 के साथ स्थिर बंधन दिखाया। हमारा अध्ययन बताता है कि ये पौधे HER2-पॉजिटिव स्तन कैंसर के लिए उपन्यास उपचारात्मक तरीकों के रूप में आशाजनक हैं।

अंत में, हमने एचईआर2-पॉजिटिव स्तन कैंसर का जल्द पता लगाने के लिए संभावित बायोमार्कर के रूप में लार एक्सोजोम प्रोटीन की पहचान की। हमने यह भी पाया कि एकोरस कैलमस के जलीय अर्क ने स्तन कैंसर कोशिकाओं में Rab27a और nSMase2 को कम कर दिया, जिससे नियंत्रण समूह की तुलना में एक्सोसोम स्राव कम हो गया।

HIGHLIGHTS

The following key findings emerged from this study:

- CD63 and CD81 were upregulated in breast cancer patients compared to healthy individuals.
- HSPB1 and ANXA1 were significantly upregulated in the patient cohort, while CAP1 and FLNA were downregulated in the patient cohort compared to the control group.
- Treatment with *Acorus calamus* significantly reduced exosome secretion from breast cancer cells via downregulating the expression of Rab27a and nSMase2.
- An in-silico study identified calcitriol lactone as having the most stable binding interactions with nSMase2.
- Treatment with calcitriol lactone downregulated nSMase2 expression, but no changes in the expression of Rab27a were observed.
- *Berberis aristata, Terminalia chebula*, and *Mucuna pruriens* reduced HER-2 expression, cell migration, plating efficiency, and triggered apoptosis in breast cancer cells.
- Protopine, cyclonovobiocic acid, hypoxylone and OSI-7904 exhibited minimal structural fluctuation during MD simulations, suggesting stable binding with HER-2.

TABLE OF CONTENT

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS & SYMBOLS

%	Percent
~	Approximately
°C	Degree Celsius
µg	Microgram
2D	Two-dimensional
3D	Three-dimensional
Å	Angstrom
AChE	Acetylcholinesterase
ADT	AutoDock Tools
AFM	Atomic Force Microscopy
ANOVA	Analysis of Variance
ANXA1	Annexin A1
AO	Acridine Orange
Bax	Bcl-2-associated protein x
Bcl-2	B-cell lymphoma 2
BRCA	Breast cancer gene
CAP1	Cyclase-associated actin cytoskeleton regulatory protein 1
CD	Cluster of differentiation
cDNA	Complementary Deoxyribonucleic Acid
CEACAM	Carcinoembryonic antigen-related adhesion molecules
CGenFF	CHARMM General Force Field
CHARMM	Chemistry at Harvard Molecular Mechanics
CST4	Cystatin 4
CSTB	Cystatin B
DAPI	4′,6-diamidino-2-phenylindole
DEPs	Differentially Expressed Proteins
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide

DNA	Deoxyribonucleic acid
DNTB	5,5′-Dithiobis 2-nitrobenzoic acid
DTT	1, 4-Dithiothreitol
EB	Ethidium Bromide
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal Bovine Serum
FITC	Fluorescein isothiocyanate
FLNA	Filamin A
FOXM1	Forkhead Box Protein M1
g	gram
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GO	Gene Ontology
HCl	Hydrochloric Acid
HER2	Human Epidermal Growth Factor Receptor 2
HSPB1	Heat shock protein family B (small) member 1
IC50	Half-maximal inhibitory concentration
ILVs	Intraluminal vesicles
JAB1	c-Jun activation domain-binding protein-1
kb	Kilobase pair
kcal	kilocalorie
kDa	Kilo Dalton
KEGG	Kyoto Encyclopedia of Genes and Genomes
kJ	kilojoule
LC-MS	Liquid Chromatography-Mass Spectrometry
MD	Molecular Dynamics
mg	Milli gram
miRNA	microRNA
mM	Milli molar
mRNA	Messenger Ribonucleic acid
MVBs	Multivesicular bodies
Na2HPO4	Sodium phosphate dibasic
NaCl	Sodium Chloride

NaOH	Sodium Hydroxide
nm	Nanometer
ns	nanosecond
nSMase2	Neutral sphingomyelinase 2
NTA	Nanoparticle Tracking Analysis
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate buffer saline
PCR	Polymerase Chain Reaction
PDB	Protein Data Bank
PEG	Polyethylene glycol
PI	Propidium Iodide
PMSF	Phenyl methyl sulfonyl fluoride
PSMA8	Proteasome 20S Subunit Alpha 8
RMSD	Root Mean Square Deviations
RMSF	Root mean square fluctuation
RNA	Ribonucleic Acid
rpm	Revolutions per minute
RT	Room temperature
RT-PCR	Real-Time Polymerase Chain Reaction
SDS	Sodium dodecyl sulphate
SEM	Standard Error of Mean
SERPINB3	Serpin Family B Member 3
SRB	Sulforhodamine B
STRING	Search Tool for the Retrieval of Interacting Genes/Proteins
TAE	Tris-acetate-EDTA
TEM	Transmission Electron Microscopy
TEMED	N, N, N’, N’-Tetramethylethylenediamine
TIP3P	Transferable intermolecular potential 3P
TNF	Tumor Necrosis Factor
VEGF	Vascular endothelial growth factor
µl	Microliter
µM	Micromolar