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Publisher of open access books and journals
PJMLS
Print ISSN: 2707-4471. Online ISSN: 2707-448X

Research Article	Pak-Euro Journal of Medical and Life Sciences
DOI: 10.31580/pjmls.v6i4.2889	Copyright © All rights are reserved by Corresponding Author
Vol. 6 No. 4, 2023: pp. 387-394	
www.readersinsight.net/pjmls	**Revised:** December 09, 2023 **Accepted:** December 25, 2023
Submission: October 13, 2023	**Published Online:** December 31, 2023

INVESTIGATING ENTOSIS AND AUTOPHAGY DYNAMICS IN DIVERSE TUMOR MICROENVIRONMENTS

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Abstract

Studies on entosis, which is a homotypic invasion of an epithelial cell into the cytoplasm of another epithelial cell, have led us to the task of summarizing its importance in the context of cancer. It has been demonstrated that entosis is correlated with cancer prognosis in various types of cancers like head and neck squamous cell carcinoma, prostate cancer, lung adenocarcinoma, pancreatic ductal carcinoma, and breast carcinoma. A large number of entotic figures have been found to be associated with more malignant cancer phenotype and poorer prognosis in many types of cancers. Autophagy is a cellular process wherein the components of cells are broken down and recycled. This process has been found to be correlated with the development of cancer. We identified the role of entosis and autophagy related genes like RHOA, ROCK1, ROCK2, AMPK, BNIP3, BNIP3L that can be used as prognostic markers and prognostic signature was constructed based on these genes so that to predict the prognosis of general cancers patients. Blood samples of 60 patients of general cancer were collected. RNA was extracted and cDNA was synthesized. The quantification of entosis and autophagy genes was carried out on the DNA using RT-qPCR. The result revealed altered expression profiles of the genes under observations. We found a significant increased expression of Autophagy related gene BNIP3L and BNIP3 in breast and ovarian cancer. While in down regulation showed in AMPK of ovarian and leukemia cancer. ROCK1 and ROCK2 gene of entosis showed that upregulation in breast cancer, while ovarian cancer showed in-verse results in ROCK1 and ROCK2 expression. From this study, it is inferenced that by evaluating the expression of entosis and autophagy related biomarkers, we can devise personalized therapeutics.

Keywords: *Cancer biomarkers, Carcinomas, Cytokines, Entosis and Autophagy, Gene expression, Pathology of Malignancies, Tumor microenvironment*

INTRODUCTION

In 2020, globally there will be an expected 19.3 million new cases and a total 10 million lives lost due to cancer. Among the anticipated 2.3 million new cases, (11.7%) female breast cancer ranked highest followed closely by lung cancer at (11.4%), colorectal (10.0%), prostate (7.3%), and stomach (5.6%) cancers (1). Cell death is basically an irreversible decline in critical cellular processes which outcomes in cellular integrity loss while the "Entotic cell death" is a method of regulated cell death (RCD), which is carried out through lysosomes and results from actomyosin-dependent cell-in-cell internalization (2). A mechanism which depend on localized actin filament cause actinomycin chains to gather at the cortex of internalizing cells and a contraction that encourages engulfment is produced by the action of the Ras homolog family member A (RHOA), as well as Rho associated coiled-coil protein kinase 1(ROCK1), (ROCK2) and diaphanous related formin 1 (DIAPH1) (3). Exogenous CDH1 administration encourages entosis in CDH1-deficient breast cancer cells, while enforced increased expression of RHOA or ROCK1 plus ROCK2, which enables the internalization of entotic cells in conferring to the actinomycin-dependent cell-in-cell invasion model. Additionally, through the activation of RHOA, ROCK1, ROCK2 the entotic cell-in-cell invasion is brought on by hyperactivation of contractile myosin (4).

Autophagy is effectively activated by AMPK, when cellular energy levels decline. AMPK is triggered by the binding of AMP to the gamma subunit of the complex. This activation initiates a cascade of

events where AMPK phosphorylates various downstream targets responsible for regulating autophagy (5). The second one is Beclin 1 which is also important regulator of autophagy through AMPK mediated phosphorylation and activation. It is an essential component of the class 3 phosphatidylinositol 3 kinase complex, plays a crucial role in the assembly of autophagosome (6). BNIP3 also known as Bcl-2/adenovirus E1B 19kDa interacting protein 3, which is a member of the Bcl-2 family and functions as a pro-apoptotic BH3-only protein. It has also played a critical role in autophagy, metabolic pathway and a process related to metastasis in various types of tumors (7).

By autophagic, necrotic or apoptotic pathways, BNIP3 can cause cell death. Three different mechanisms explain how BNIP3 controls autophagy and autophagic cell death. The first one is direct binding and inhibition of the mTOR activating GTPase, Rheb, which release the autophagy pathway from mTOR's inhibition (8). The second one is competitive binding at the BH3 domain releases the autophagy effector protein Beclin-1 from an inhibitory complex with Bcl-2. The third one is indirect activation of autophagy by mitochondrial ROS mediated by BNIP3. However, the expression levels of BNIP3 and BNIP3L genes have been studied in various types of cancer including pancreatic ductal adenocarcinoma, basal like breast cancer and ovarian cancer (9). The Objective of this study is to Evaluate the Tumor Cells Cannibalism (Entosis) and Autophagy in varied Tumor Microenvironments.

MATERIALS AND METHODS

SAMPLE COLLECTION

EDTA Blood samples (3ml) were collected from 60 patients enrolled from the oncology department of Jinnah Hospital, Lahore after an informed consent for patients fulfilling study criteria. Samples were transported to the Molecular Diagnostics Laboratory, FCCU for further processing. Additionally, 36 control samples (20 male and 16 female) were taken from FCCU.

Inclusion Criteria: Histologically confirmed stage I-IV of different cancer patients. ECOG performance of 0 to 1, Informed consent, Multimodal therapy (chemotherapy, surgery or radiotherapy)

Exclusion Criteria: Children and pregnant, breast-feeding women, Patients suffering from autoimmune disorders, Patients suffering from cardiac, renal or hepatic disorders other than cancer

RNA EXTRACTION AND cDNA SYNTHESIS

RNA extraction from blood was performed using (Invitrogen TRIzol reagent: Catalog# 15596026, USA) according to the manufacturer's protocol. The quality and quantity of RNA was analyzed by using NanoDrop 2000/2000c Spectrophotometer (Thermoscientific, USA). The samples having 260/230 and 260/280 absorbance ratio of 1.5-1.8 were selected for further cDNA processing using (M-MuLV) Reverse Transcriptase Kit (Catalogue # K1622, Thermoscientific, USA) as per manufacturer's instruction.

PRIMER DESIGNING AND OPTIMIZATION

Primers were designed using the NCBI primer designing tool for genes like RHOA, ROCK1, ROCK2, AMPK, BNIP3, BNIP3L that can be used as prognostic markers. A set of primers (forward and reversed) was designed for each of the prognostic marker. as shown in Table 1. Primers were optimized using gradient PCR thermocycler (Bio-Rad T100-Thermocycler, USA) to determine the best Tm. For confirmation of primers, In-Silico PCR was performed using UCSC Genome Browser (Table I).

REAL TIME QUANTITATIVE POLYMERASE CHAIN REACTION (RT-qPCR)

The RT-qPCR experiment was conducted using the CFX96qPCR system manufactured by Bio- Rad. The aim was to analyze the mRNA expression of AMPK, BNIP3, BNIP3L, RHOA, ROCK1, and ROCK2. Each reaction consisted of a total volume of 11ul, comprising of SYBR Green mix, cDNA Template, Forward Primer and Reverse Primer. Actin serves as a fundamental gene, which is commonly referred as a housekeeping gene, which plays a vital role as an internal positive control during analysis. Its utilization involves normalizing biomarker expression levels and assessing the quality of cDNA. The Blood samples from 18 healthy individuals were collected and cDNA was synthesized from theses samples. The resulting

cDNA served as a negative control to calibrate inflammatory markers in the patients. cDNA extracted from the blood of patients was used as positive control.

Table I. Selected primers sequencing and optimization

GENE	Primer Sequence		Tm(°C)	Size
AMPK	Forward	5′TGCGCAGACTCAGTTCCTG3′	60	196
	Reverse	5′AGGCTCCGAATCTTCTGTCG3′		
BNIP3	Forward	5′CCTCAGCATGAGGAACACGA3′	60	158
	Reverse	5′AAAAGGTGCTGGTGGAGGTT3′		
BNIP3L	Forward	5′TGCTCCCAAATCAACAGGGTT3′	60	168
	Reverse	5′GATGGTACGTGTTCCAGCCC3′		
ROCK1	Forward	5′GGAAGTGAGGTTAGGGCGAA3′	59	113
	Reverse	5′CGGGTACAACTGGTGCTACA3′		
ROCK2	Forward	5′GGCTGGAAAGCCTTTTTCCG3′	60	170
	Reverse	5′TGCCTTCATCTGTAGACCTCTG3′		

RT-qPCR DATA ANALYSIS

To analyze the data obtained from RT-qPCR, we utilized Litvak's method (2001) for assessing the relative fold change expression of inflammatory markers. The calculation of relative expression for these markers involved comparing them to the control samples.

STATISTICAL ANALYSIS

Statistical analysis was conducted using Graph Pad Prism Software (version 8) to assess the significant interval relationship between two variables. The Kruskal Wallis test with a 95% confidence interval was employed for this purpose. The results were presented as mean ± standard deviation (SD). To ensure reliability, the experiments were repeated multiple times and consistent reproducibility was seen. A p-value of ≤ 0.05 was considered statistically significant.

RESULTS

DEMOGRAPHIC FEATURES OF STUDY POPULATIONS

In this study 60 general cancer patients were enrolled along with 36 healthy individuals taken as a control. Subjects were divided in groups for comparative analysis between different cancers. There were 44 disease females and 16 disease males. There were 16 healthy females and 20 healthy males. Half of the patients fell into the age group of 23-40 years while the others half were in 40-69 age group (Table II).

Table II. Demographic features of study population

Parameters	Groups	Gender		Range		Standard Deviation (± SD)	
		M	F	M	F	M	F
Age (years)	Control	56%	44%	19-30	22-25	24± 2.27	24±2.35
	Disease	27%	73%	22-65	30-51	51±10.85	40±6.50

BIOPSY PROFILE OF ENROLLED PATIENTS

The biopsy profile characterized the enrolled general cancer patients in to a various histologic type. There were total 16.7% ovarian cancer patients, 6.7% peritoneal fallopian tube cancer, 6.7% epithelial ovarian cancer and 3.3% stromal ovarian cancer, 33.3% breast cancer and 16.7% lung cancer patients.

AMPK DOWNREGULATED IN OVARIAN AND LEUKEMIA CANCER:

AMPK gene expression profiling utilizing RT-qPCR was done for cancer of female reproductive track. Liquid cancer such as leukemia and in solid tumors such as lung cancer. P-value of less than 0.05 was considered significant and relative fold change was plotted against the controls (Fig. 1).

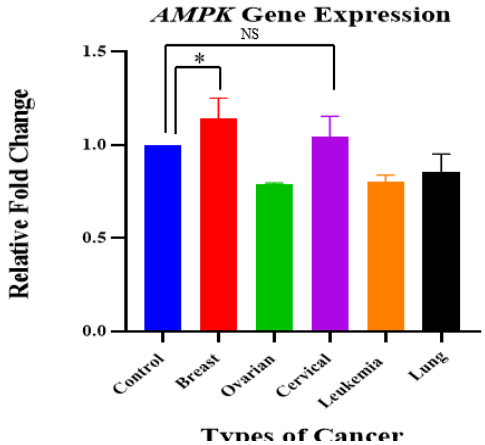

Fig. 1. Expression Profiling of AMPK expression in different cancer utilizing RT-qPCR. Statistical significance was determined using Kruskal Wallis Test. Significant differences (where *P<0.05, **P<0.005, *** P<0.0005) NS not significant (P>0.05). Kruskal Wallis values (P=0.0382) showed significant up-regulation of AMPK gene in breast cancer as compare to controls. Whereas, up-regulation of AMPK in cervical cancer is statistically in-significant. Interestingly, ovarian cancer showed significantly decreased in expression along-with Leukemia and Lung cancer. (Kruskal-Wallis statistics 11.76).

CYTOSKELETAL MODULATING ENZYMES ROCK1 AND ROCK2 PLAY CRUCIAL ROLE IN CANCER TRANSFORMATION:

ROCK1 and ROCK2 gene expression profiling utilizing RT-qPCR was done for cancer of female reproductive track. Liquid cancer such as leukemia and in solid tumors such as lung cancer. P- value of less than 0.05 was considered significant and relative fold change was plotted against the controls (Fig. 2).

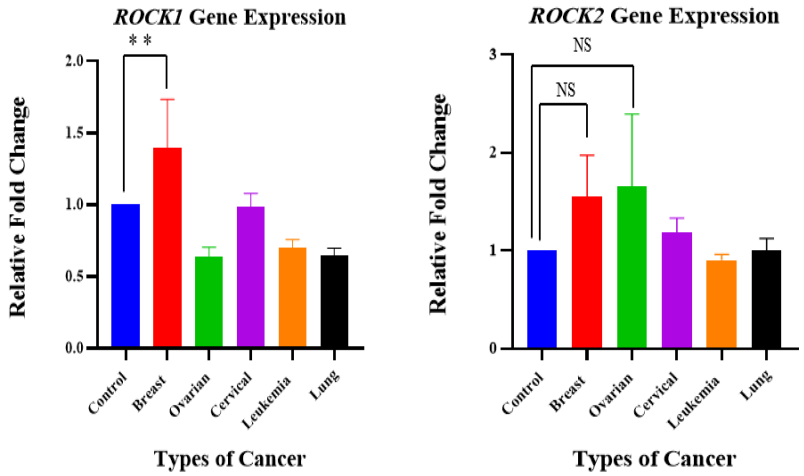

Fig. 2. Expression Profiling of ROCK1 and ROCK2 expression in different cancer utilizing RT-qPCR. Statistical significance was determined using Kruskal Wallis Test. Significant differences (where *P<0.05, **P<0.005, *** P<0.0005) NS not significant (P>0.05).

Kruskal Wallis Test showed significant (P=0.0018) increased expression of ROCK1 and ROCK2 in Breast cancer, whereas similar significant cancer was observed in ROCK2 expression in Ovarian cancer only. Whereas, Ovarian cancer showed non-significant downregulation in ROCK1 expression. Down regulation of ROCK1 in Cervical, Leukemia and Lung cancer patients showed Kruskal Wallis statistics of 19.12 which was significant in comparison of ROCK2 in similar groups.

UPREGULATION OF AUTOPHAGY RELATED GENE BNIP3L AND BNIP3 IN BREAST AND OVARIAN CANCER

BNIP3L/BNIP3 gene expression profiling utilizing RT-qPCR was done for cancer of female reproductive track. Liquid cancer such as leukemia and in solid tumors such as lung cancer. P-value of less than 0.05 was considered significant and relative fold change was plotted against the controls (Fig. 3).

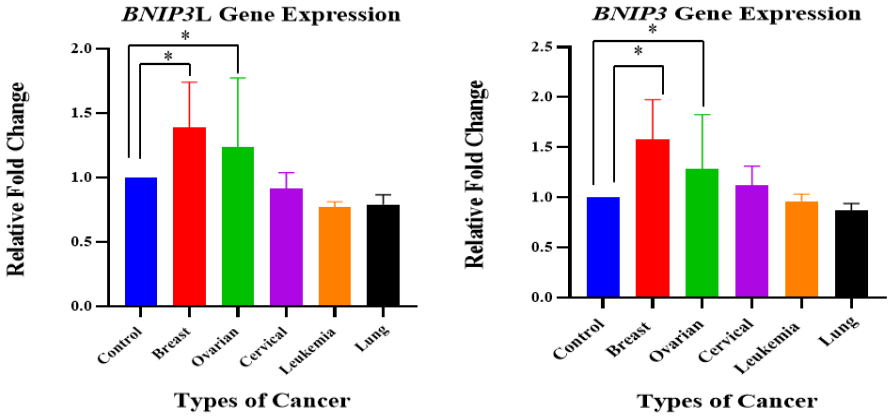

FIGURE 3: Expression Profiling of BNIP3L and BNIP3 expression in different cancer utilizing RT-qPCR. Statistical significance was determined using Kruskal Wallis Test. Significant differences (where *P<0.05, **P<0.005, *** P<0.0005) NS not significant (P>0.05).

Our result showed significant increased expression of BNIP3L gene in both breast and ovarian cancer as Compare to the controls. Whereas, cervical cancer showed no change in expression as compare to Controls. Leukemia and lung cancers similarly showed significantly decreased levels which are non-Significant in Figure 1. Interestingly, similar significant up-regulation of BNIP3 gene was noted in patients with breast and ovarian cancer as shown in Figure 2. While slightly increased expression is noted in cervical cancer. Whereas, leukemia and lung cancer showed no-significant change.

DISCUSSION

The impact of tumor microenvironment (TME) on autophagy signaling pathways is noteworthy since it provides support the growth and progression of cancer. Autophagy in the TME can regulate tumor associated fibroblasts, facilitate immune evasion. Our results showed significant upregulation of BNIP3 in breast, ovarian and cervical cancer as compared to controls. Similar study with the increase of BNIP3 is mediated through the ERK/HIF-1 pathway, whereas the subsequent consequences are mediated by the mTORC1 signaling pathway. These findings showed the therapeutic potential of BNIP3 targeting and anchorage deprivation induced autophagy as a method of preventing HCC metastasis (10). Breast cancer tissues and cell lines had increased BNIP3 expression and the length of hypoxic exposure was positively linked with BNIP3 expression. These results indicated that BNIP3 promotes autophagy in breast cancer. It brings attention to BNIP3 as a possible treatment target for breast cancer. Overall, the study finding showed that as compared to non-tumor tissues, the expression of BNIP3 is downregulated in ccRCC tumor tissues (11).

In an extension, our results similarly showed significant upregulation of BNIP3 in breast and ovarian as compared to leukemias and lung Cancer. Some results were reported on two apoptotic proteins that are found in mitochondria, BNIP3L and BAX which are directly transactivated by EGR2 (12). Further to extend our results which showed ROCK1 was significant increased expressed in breast cancer but in ovarian cancer showed inverse relationship i.e. significantly downregulated. Overall, the result of this study indicated that TMEM16A activation, through the interaction with the ROCK1 and moesin promotes breast cancer metastasis by enhancing cell migration and invasion (13, 14).

Gleason grade. Strong ROCK1 staining was detected in 3% of tumors that were androgen receptor (AR) negative but in 27% of tumors were significantly AR positive. The result of this study, ROCK1 expression is related to the prognosis of prostate cancer (15).A study conducted by M. Zhang and his coworkers in patients with pancreatic adenocarcinoma, there was a positive connection between the plasma levels of LINK-A lncRNA and ROCK1. The inhibitory effect of LINK-A silencing on cancer cell proliferation, migration and invasion was lessened by increased expression of ROCK1. By encouraging ROCK1 expression, LINK-A lncRNA may play role inpancreatic cancer (16).

Zhou and his co-workers studied that RhoA and ROCK1 were involved in actin-induced apoptosis as a suppression of RhoA and ROCK1 expression. Actin administration induced ROCK1 expression in human

leukemia cells as shown by the suppression of ROCK1 expression when RhoA was suppressed. Actein treatment in U937 cells resulted in ROCK1 suppression, which was related to the inhibition of U937 tumor growth in vivo (17). A significantly decreased the expression of ROCK1 was observed in vivo model showed similar results, highlighting the crucial role of miR-335-5p as a tumor suppressor to modulate cell proliferation and cell cycle progression by downregulating ROCK1 expression (18).Increased expression of ROCK2 was recorded in breast, ovarian and cervical cancer. Similar study supported our results stating that the mRNA and protein expression levels of ROCK2 were significantly increased in hepatocellular carcinoma (HCC) group compared to the normal group nut not in hepatitis B patients (CHB) and liver cirrhosis patients (LC) groups (19).

CONCLUSION

In conclusion our results showed that autophagy (AMPK, BNIP3L and BNIP3) and entosis (ROCK1 and ROCK2) related genes play a major role in modulating cell death phenomenon such as autophagy and entosis with regard to tumor microenvironment. The role of entosis in particular need to be emphasized in future studies. So that, we will be able to devise personalized therapeutics for alleviating the lives of cancer patients.

However, our study isn't without limitations. We were not able to enroll significant subjects for the study due to low turnover of the patients related to certain cancers and faced problem in getting the required information from patients records for our study due to unorganized patient's data and management in public sector hospitals. We would like to address this issue in future studies by opening multiple sites for increased enrollment. Secondly, gene panel for autophagy and entosis should be extended and protein analysis will give us the right picture of tumor behavior. Invitro analysis for proposed therapeutics for autophagy and entosis should be done, in order to devise effective therapies.

Ethical Statement:

The study received approval by Ethical Review committee (ERC-113-2022) and institution Review board (IRB-415/11-2022) of Forman Christian College and university (FCCU). The collection of blood samples adhered to all relevant guidance, administrative rules and regulations as documented in the declaration of Helsinki. Samples were collected from Jinnah hospital Lahore after approval of Ethical Review Board of Jinnah hospital under Ref No: (ERB133/7/01-12- 2022/S1ERB).

Conflict of Interest:

Authors have no conflict of interest.

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