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ORIGINAL ARTICLE

Identification of Autophagy Status in Diagnosed Epithelial Ovarian Tumor/Cancer Cases

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Abstract:

Background: Physiological autophagy is a conserved process in which lysosomal degradation of damaged organelles and proteins occurs for its reutilization. During autophagy, decrease in cell size leads to a change in organ size. Deranged autophagy is observed in many neoplastic growths. However, few studies have been conducted for the examination of relationship between autophagy and Epithelial Ovarian Neoplasia (Tumor/Cancers), so this study investigated the expression of autophagy in Epithelial Ovarian Neoplasia (Tumor/Cancers) and its correlation with the age, stage and grade of the patient.

Objectives:

Methods: In this study, LC-3 marker for the detection of autophagy was analyzed by immunohistochemistry on formalin fixed paraffin embedded tissue sections. Total of 74 patient's specimens of previously diagnosed Epithelial Ovarian Tumor/Cancer were taken into consideration. The correlation between autophagy and patient's age, grade and stage were determined by using Chi square test.

Results: The findings resulting from this study showed that Adenoma is more prevalent in younger age group (14-34 years) while adenocarcinoma in older age group (54-74 years). 77.5% of adenoma and 82.5 % adenocarcinoma cases were detected with positive LC 3 autophagy marker confirming that autophagy expression is elevated.

Conclusion: From the results of this study, it is concluded that the process of Autophagy is enhanced in Epithelial Ovarian Tumor/Cancer and can be a viable target for the treatment of it in particular and other cancers in general. Future studies done to target different steps of autophagy are expected to clarify the role of this phenomenon in the biology of Epithelial Ovarian Neoplasia. Methods like FACS analysis, single cell sequencing, Microarray of different tumors and further elucidate the significance of this phenomenon in organ neoplasia .

Keywords: *Autophagy, Epithelial Ovarian Tumor, Epithelial Ovarian Cancer, immunohistochemistry.*

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Introduction

The process of cell reconstruction and cell repair occurs naturally in all living organisms. We experience cell death and cell multiplication on daily basis (1). At the time of stress, when cell survival is in danger is goes to programmed cell death (PCD). For determining the fate of the cell, PCD plays an important role in balancing the equilibrium between cell death and cell survival of healthy cells cell (2, 3). The three types of PCD are Apoptosis, Autophagy and Necrosis. Autophagy is the normal physiological process by which survival under stressful conditions of the cell is possible.

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Inducers of autophagy for the survival includes chemotherapeutic agents, radiations, oxidative stress, ER stress (4). Deregulated autophagy is observed in many different diseases like cancers, neurodegenerative disorders, inflammatory diseases and autoimmunity(5). The functionof autophagy in different diseases is under debate and research are being conducted to explore more about it.

Among the gynecological malignancy, ovarian cancer is on the second rank for causing death. Annually in United States around 22,000 new cases are diagnosed and about 14,000 deaths occurs because of ovarian tumor (6). The majority of ovarian cancer patients are diagnosed when they are in stages 3 to 4 and the disease has spread outside of the pelvic region, which explains why the survival rate is less than five years, or 27%, due to the lack of early signs and effective screening tools.

Its chemo-resistance feature may also be a contributing factor in the lower survival rate. The earliest possible diagnosis of an ovarian tumour is the most crucial factor in increasing survival rates. (Siegel et al., 2014). Among various types of ovarian tumors, epithelial ovarian tumor/cancer is the most common and accounts for about 60% mortality rate around the globe (7). According to morphology Serous (low grade and high grade), mucinous (low grade and high grade), and mucinous transitional epithelial ovarian tumor/ cancer (EOC) are the different types (Brenner type),endometrioid, mixed mesodermal, clear cell, and histologically undifferentiated subtypes (8).

Autophagy was once thought to be the mechanism of cell death, but recent studies have shown that it has two roles in tumours and malignancies. It can serve as a tumour suppressor or an activator on occasion. Autophagy is beneficial in the beginning of the tumour because it inhibits by eliminating oncogenic protein molecules, damaged organelles, and toxic unfolded proteins (9). Lately, tumor promotes autophagy by getting energy and substrates that are necessary for its survival (10).

Methodology

Patient's selection and tissue collection:

This retrospective study includes 74 cases of epithelial ovarian tumor/cancers selected in the year from Jan 2016 – Oct 2017 from the archived materials of the laboratory of histopathology, Basic Medical Sciences Institute (BMSI), Jinnah Postgraduate Medical Center (JPMC) Karachi, Pakistan. Archival Formalin-fixed, paraffin embedded [FFPE] tissues of selected candidate cases were obtained and used for the present investigation. Complete clinicopathologic information and the availability of enough paraffin-embedded tumour tissues were inclusion criteria. According to guidelines authorised by the Ethics Review Board (ERB), the patient information file was used to acquire clinicopathologic information on each case, including age, tumour size, location, histological grade, and stage.

Ethical statement:

The SZABIST (Shaheed Zulfiqar Ali Bhutto Institute of Science and Technology) Ethics Review Board Committee ERBC accepted and revised this study's design. It is acknowledged that the study is noninvasive and that there is negligible risk of harm to the study participants. The informed consent form's requirement was waived by the ERBC. In this study, there are no patient-specific data reported. All the samples underwent covert analysis.

Immunohistochemistry:

Sections were made of paraffin-embedded tissues of identified selected cases having thickness of 3-4 µm leading to deparaffinization with xylene. Antigen retrieval was done by using hydrogen peroxide at 95^{O}C. Sections were incubated with primary antibody (LC3, Mouse monoclonal antibody, Biorbyt, orb380517: dilution 1:100) and secondary antibody wit substrate chromogen solution (Biorbyt) was used for the development of antibody antigen reaction (provided with Super Sensitive IHC Detection System Kit, Biorbyt). DAB for staining sections and hematoxylin for counter stain was used. The sections were mounted with DPX mounting media (Merck) and analyzed under light microscope.

Evaluation of slides:

All the slides were examined by using proportion score method, number of positively stained cells (0=none, 1= <10%, 2= 10-50%, 3= >50%). And intensity of the stained cells was scored as (0= no staining, 1= weak, 2= moderate, 3= strong). Total score was calculated by multiplying intensity score with proportional score ranging from 0 to 9 as (0 – 4= negative, 5 – 9= positive), (Jiang et al., 2012: He et al., 2013). Two patterns of LC3 were observed: cytoplasmic and stone-likestructures.

All slides were analyzed by three histopathologists. Images were clicked by digital camera attached to bright field microscope.

Statistical analysis was performed on SPSS software 21 with the level of significance at 0.05.

Results and Discussion

For this study, histological reports of the patient's biopsy samples were analyzed for the diagnosis and the categories were made accordingly. In some reports, Adenocarcinoma (malignant growth) wasn't categorized as serous or mucinous, so for this study, adenocarcinoma is evaluated separately. In our study we observed that majority of the patients (51.3%, n=38) belong to younger age group (14-34 years).

The positive cases for autophagy (LC 3 marker) in Epithelial Ovarian Tumor/Cancer were 57 (77%) out of 74 cases while rest of the 17 (23%) cases were found to be negative for autophagy marker LC3B as shown in table 1. In Adenoma 38 samples (77.5%) were positive on the other hand 11 samples (22.5%) were negative for LC 3 marker. In borderline tumors, out of 8 cases, 5 samples (62.5%) showed positivity for autophagy marker and 3 cases (37.5%) were negative. 14 case samples (82.3%) of adenocarcinoma including serous and mucinous were positive and 3 cases were negative.

According to the FIGO classification system as described in the literature review the majority of the patients of (12) were observed at stage I of the malignant cancer while 2 cases at stage III. The Stages are further categorized into three types, A, B and C.

Further we observed that 36% cases of stage IB, 28.5% cases of stage IC and 57% of the ovarian carcinoma cases were at Grade 3 showed autophagy LC3 expression (Fig 1 and 2). The statistical analysis showed that there is no correlation (p=0.733) between stage of the cancer and status of autophagy in the cells of cancer. Also, the p value of 0.392 shows that there is no association between histological grade and autophagy positivity.

Table 1 : Types of ovarian cancer patients by frequency, age group, autophagy status.

Groups	**Frequency**	**Age group**			**Autophagy status**	
Sub-groups		***14-34 years***	***34-54 years***	***54-74 years***	***Positive***	***Negative***
Serous Adenoma	32	18	12	2	24	8
Mucinous Adenoma	17	11	5	1	14	3
Borderline Serous Tumor	5	2	1	2	3	2
Borderline Mucinous Tumor	3	2	1	0	2	1
Adenocarcinoma	9	2	2	5	7	2
Serous Adenocarcinoma	3	3	0	0	3	0
Mucinous Adenocarcinoma	5	0	4	1	4	1
Total	74	38	25	11	57	17
	Stage				**Grade**		
	IA	***IB***	***IC***	***IIIA***	***Grade 1***	***Grade 2***	***Grade 3***
Adenocarcinoma	2	2	1	2	2	3	2
Serous Adenocarcinoma		1	2		0	0	3
Mucinous Adenocarcinoma	1	2	1		1	0	3
Total	3	5	4	2	3	3	8

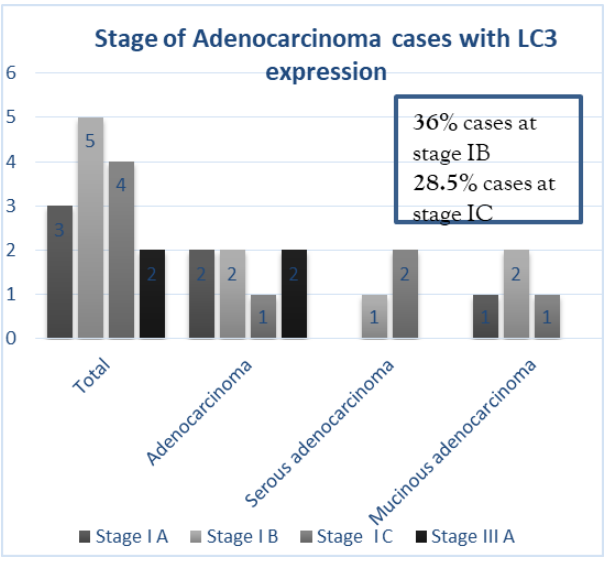

Figure 2: The value of 0.733 showed that there is no relation between stage of the cancer and status of autophagy in the cells of cancer.

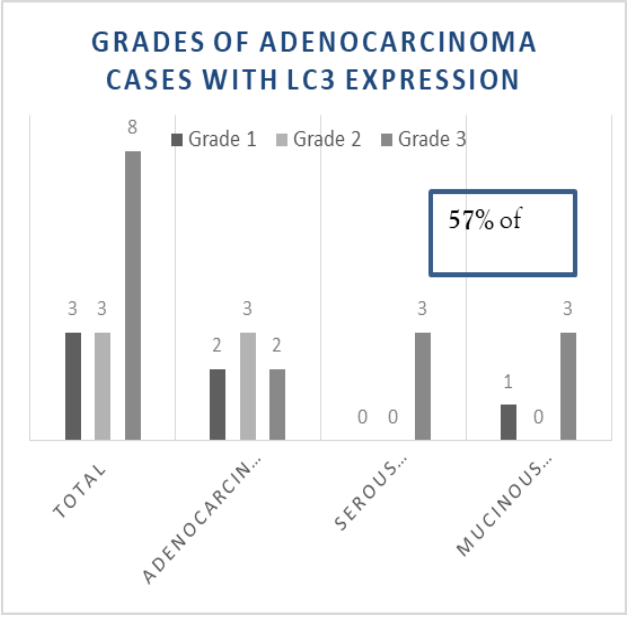

Figure 3: The value of 0.392 shows that there is no correlation between histological grade and autophagy positivity.

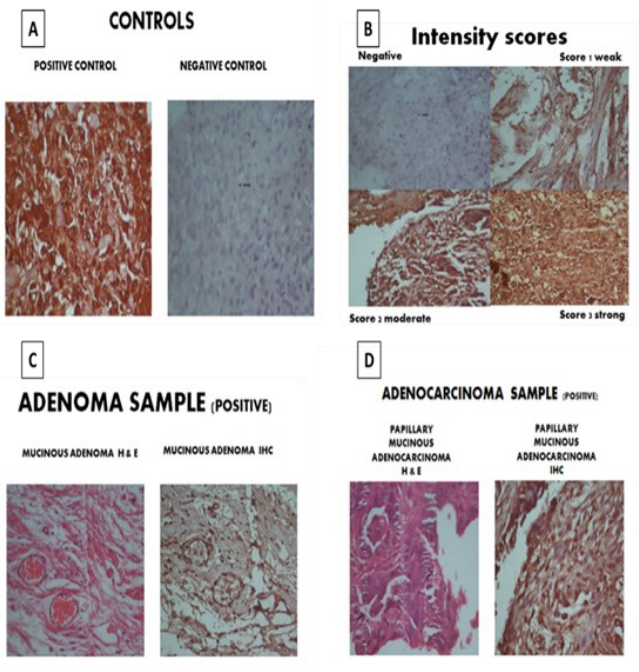

Figure 3: Representative IHC stains of LC3B in ovarian cancer patients (A) shows positive control and negative control. (B) shows intensity scores (none, low, moderate, and high). (C and D) immunohistochemical and H &E staining of adenoma and adenocarcinoma samples.

Discussion

Autophagy is a catabolic process in which cellular components like cytoplasmic organelles and related proteins are degraded by the action of lysosomes (11, 12). It is also termed as conserved, type 2 programmed cell death which is critical for the hemostatic balance as it helps in the redistribution of nutrients to the nutrient deprived parts of body in order to continue critical metabolic activities (13). But the role of autophagy in terms of tumorigenesis is quite different as it is known to have dual role; that is why this subject is debatable because of the engagement of multiple unknown pathways in this phenomena (14, 15). Studied data suggested that autophagy at one side supports tumor growth by acting as tumor promoter while on the other hand, it suppresses tumor proliferation either at the cellular level or at the nuclear level by altering the transcription of target molecule (15). It is also known to induce chronic tissue damage and hinders release

of certain inflammatory mediators and thus down regulate tumor progression (16). LC3 and Beclin-1are the extensively used markers of autophagy detection in different types and stages of cancer (17). The investigations have been performed on the expression of LC3 and Beclin-1, both of these markers are extensively used for the diagnosis of different types of tumor for autophagy (18). The expression of Beclin-1 is not same in all cases of cancer as it may vary on the basis of type and nature of origin of cancer. Beclin-1 is highly expressed in nasopharyngeal and pancreatic cancer with poor clinical outcomes (19, 20).In another study, the expression of Beclin-1 is moderate in malignant cancers i.e. esophageal, cervical, breast, gastric, and colon cancer with poor prognosis (21). Pancreatic and pulmonary cancer presented with adverse results with the elevated expression of Beclin-1 (22, 23). In patients with early stage of breast cancer and squamous cell carcinoma, LC3 expression was observed with poor prognosis and tumorigenesis (24).

Accumulating different study outcome, it can be observed that autophagy detecting markers are inconsistent depends on various factors like type and stage of cancer, origin of cancer is the other main factor which reflects different sets of markers to confirm the diagnosis (25). Infact, our study findings also revealed that autophagy has diverse role in Epithelial Ovarian Tumor/Cancer progression and prognosis is strictly based on the origin, tumor grade and type of cancer cells.

It has been observed that the expression of autophagy may vary in diverse groups of cancer (26). Claudia et al. reported that autophagy is expressed in all types of cancers but with different expression level i.e., 82.35% in cases of malignant adenocarcinoma, 77.5% in benign adenomas and 62.5% in borderline tumors. An estimated 85.7% of patient were diagnosed at early stage of Adenocarcinoma and has reportedly single ovary malfunctioning. Adding on, LC 3 expression observed same either in case of malignant Epithelial Ovarian tumors (adenocarcinoma) or benign Epithelial tumors (adenoma) but no significant correlation was observed between grades of adenocarcinoma. Patients presented with Ovarian Tumor were treated with different chemotherapeutic agents and interestingly, over expression of LC3 and Beclin 1 was observed in response to the chemotherapeutic medications (27). It is commonly known that age factor of women is also the risk factor for different forms of cancer i.e. mucinous adenocarcinoma is more common in women with age < 45 years whereas the frequency of adenoma is higher in younger females or females with age between 14-34 years (28). In some cases, elevated expression of LC3 could not reflect the exact activity of autophagy as it sometimes reflects the fault/blockade at the level of lysosome despite of increased in the number of autophagosomes. However, the quantification of functional autophagy can be assessed directly by counting the LC3 containing vesicles with subsequent autophagy flux. Immunohistochemistry (IHC) has some limitations as staining with IHC does not clearly discriminate LC3-I and LC3-II containing vesicles (29), so we other relevant diagnostic tool could be used for this purpose. According to histological grade, the expression of LC3 was detected but the diagnosis was confirmed at different stages of disease progression. Around 21% cases with adenocarcinoma were diagnosed at stage 1 and stage 2 whereas 57.14% were detected on advance stage or stage 3.

According to this study, epithelial ovarian tumours and cancers have very rarely been the subject of investigations in Pakistan that have found the presence of autophagy markers. Yet, a noteworthy component of this study is that the samples were drawn from government institutions and included individuals from all socioeconomic and racial backgrounds. Although small sample size is the limitation of this study, but this study can be made effective by enrolling more volunteers with the application of other expression markers i.e. Beclin-1 and p62 using different diagnostic tools.

Conclusion

Study finding reveals that the function of autophagy is altered in Benign, Borderline Tumors and in Malignant Epithelial Ovarian Cancers, when compared with healthy human samples. LC3 expression or autophagy marker is almost same in benign and malignant ovarian cancers but with discrepancy in age of females.

For future understandings, this study might be helpful to insight the mechanism of tumor progression and help in designing therapeutic interventions to mitigate the consequences of this condition.

Ethical approval and consent

The study was approved by the institutional board of studies and informed consent was obtained from each participants included in the study.

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We thank the study subjects for participating in this study.

Disclosure

The authors report no conflicts of interest.

Author's contributions

FNwas involved in the execution and supervision of the project. AQS designed the study and wrote the manuscript. MFB helped in organization of data. TGM did critical analysis.NR helped in the editing. All named authors have read and approved the final version of the manuscript.

Data availability

Available from the corresponding author on reasonable request.

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