Journal of Advances in Medicine
Citation: JAM: 7(1): 01-06, June 2018
DOI: 10.30954/2277-9744.1.2018.1

ORIGINAL ARTICLE

Expression of Autophagy Markers Beclin-1 and LC3 in Cervical Cancer

Namita Doharey[1], Lavina Chaubey[1*], Gopeshwar Narayan[2], T.B. Singh[3], Kirti Kaithwas[1], Arusha Modi[2] and Shilpa Chowdary Peddappolla[1]

[1]Department of Obstetrics & Gynecology, Institute of Medical Sciences, Banaras Hindu University, Varanasi (U.P.), India
[2]Department of Molecular and Human Genetics, Institute of Sciences, Banaras Hindu University, Varanasi (U.P.), India
[3]Department of Preventive and Social Medicine, Institute of Medical Sciences, Banaras Hindu University, Varanasi (U.P.), India

Keywords: Autophagy markers, Cancer progression, Genetics

Corresponding author

Lavina Chaubey
Department of Obstetrics & Gynaecology
Institute of Medical Sciences,
Banaras Hindu University,
Varanasi (U.P.), India
Email: lavinachaubey@gmail.com

ABSTRACT

Background: The accuracy of current prognostic factors in cervical cancer progression remains controversial. This progression may be understood through molecular markers of autophagy, BECLIN 1 and LC3.

Objectives: Assessment of expression of Beclin-1 & LC3 in cervical cancer to find an accurate prognostic marker of cancer progression.

Methodology: In this case control study, 43 cervical tissue biopsy samples were analysed for Beclin-1 and LC3 gene expression. A gene was considered down regulated when band intensity was lower than 2 SD of that for normal cervical tissue. The expression from normal cervix, precancerous and cancer cervix tissue sample was compared by unpaired t-test using SPSS software.

Results: Among 43 subjects of cervical pre-malignancy and malignancy, 60.5% were ≥ 55years, 65.1% had ≥ 3 live issues, 79.1% belonged to low SE status and 90.7% were post-menopausal, of which 41.7% were 6-10 years post-menopausal. All tissue samples were analysed against nine controls for Beclin-1 and LC3 expression which was up-regulated in 30.2% samples for Beclin-1& LC3 each and down-regulated in 32.5% Beclin-1 & 20.9% LC3, which was not statistically significantly. In 37.2% samples for Beclin-1 & 48.9% for LC3 the expression remained unchanged. However combined expression of Beclin-1 & LC3 in samples of pre-malignant lesions (ten) was up-regulated (70%, P=0.003). Beclin-1 & LC3 expression was also significantly up-regulated in patients less than 55 years of age (P=0.001) as against those in age group ≥ 55 years where up and down regulation was almost equal.

Conclusion: Beclin-1 & LC3 gene expression was significantly up regulated in patients < 55 years of age, and in pre-malignant tissue samples, pointing towards an operating autophagy mechanism within the cervical cells that probably protects cells from progressing to overt cervical cancer. However, this was not so in the cancer samples.

Cervical cancer is one of the leading causes of gynaecological cancer death in women worldwide and approximately 500,000 new cervical cancer cases are deduced, contributing to 280,000 deaths each year[1]. More than 80% of cervical cancer patients are diagnosed in developing countries[2]. HPV infection has been considered as the key factor responsible

for the development of cervical cancer. HPV-16 and -18 infections account for about 70% of cervical cancer cases [3]. Two primary HPV viral onco-proteins, E6 and E7, are required for the development of cervical cancer. E6 protein induces p53 degradation. E7 protein interacts with retinoblastoma (Rb) protein and preventing Rb binding to cell cycle-related transcription factor E2F leading to loss of Rb/E2F complexes and the subsequent progression of cell cycle from G1 to S phase[4-7].

Autophagy is intracellular degradation, a dynamic, multi-step process[8]. It occurs when cells undergo stress like starvation, radiotherapy to promote cell survival or apoptosis[9,18]. Beclin-1 & LC3 regulate the beginning of mammalian autophagy[8]. Beclin-1 plays a central role in the signalling pathway activating autophagy and autophagosome formation[8, 16]. LC3 is responsible for the elongation of the autophagosome[13, 14]. In a study from 2012 it was shown that expression of Beclin-1 and LC3 were significantly lower in cervical squamous cancer cells and malignant epithelial ovarian cancer than normal cells [15]. This may have prognostic significance[11]. In another study a natural agent, HMDB, having growth inhibitory effect on malignant cells was seen to trigger autophagy in HeLa cervical cancer cells followed by increased expression of LC3 and Beclin-1[12].

Many onco-proteins inhibit or promote autophagy[19]. Autophagy is required for optimal anti-cancer immune-surveillance[21]. In neoplastic cells, autophagy responses cope with intracellular and environmental stress favouring tumour progression implying that in some cases, oncogenesis proceeds with temporary inhibition of autophagy or gain of molecular functions antagonizing its onco-suppressive activity[13].

This study was to find a correlation between expression of Beclin-1 and LC3, markers of autophagy, in pre-malignant and cancerous cervical cells, in the hope of finding an accurate prognostic marker of progression of cancer other than existing clinical parameters.

MATERIAL AND METHODS

The study was approved by the departmental research committee, Institute postgraduate board and Institute ethical committee of Institute of Medical Sciences, Banaras Hindu University. From August 2016 to January 2018 total number of 230 non pregnant symptomatic patients attending the OPD of Sir Sunderlal hospital, IMS, BHU, who were in a relationship (married or otherwise), were recruited into the study after informed consent. After a detailed history, PAP smears were taken and sent for cytology. For those with obvious growth on the cervix, punch biopsies were taken. The patients in whom only PAP was done were divided into 2 groups based on cytology report: (1) Normal (2) Abnormal. The second group with abnormal PAP reports were further evaluated with colposcopy and guided biopsies were taken from suspicious areas. As controls, biopsies from hysterectomy specimens with prior normal PAP report and no evidence of abnormal cervical changes were taken.

Patients with obvious growths were also evaluated regarding (1) Type and size of growth (2) Extension, parametrial or rectal mucosa involvement (3) Any palpable lymph nodes.

A total of 52 cervical tissue biopsy samples (cases and controls) were analysed for Beclin-1 and LC3 gene expression .The biopsy samples were immediately transported to the genetics laboratory in an ice chamber and thereafter stored at -80 degree centigrade. RNA was extracted from the tissue samples and checked for purity. The satisfactory samples were then converted to complementary DNA (cDNA) by PCR and integrity checked by Beta Actin gene expression. The primers of Beclin-1 and LC3 gene were applied and expression of the genes assessed by electrophoresis. A gene was considered to be down regulated when band intensity value was lower than 2 standard deviations of the value obtained for normal cervical tissue samples. Thereafter the gene expression from normal cervix, precancerous lesion and cancer cervix tissue sample was compared.

Statistical analysis: The results were tabulated and unpaired t-test applied using SPSS software.

RESULTS

Demographic analysis

Table 1: Characteristics of patients with biopsy proven pre-malignant and malignant lesions of the cervix

Age group (in years)	N (%)	Total
< 55	17 (39.5)	43 (100)
≥ 55	26 (60.5)	
Parity		
< 3	15 (34.9)	43 (100)
≥ 3	28 (65.1)	
Socioeconomic status (Kuppuswamy scale)		
Lower	34 (79.1)	43 (100)
Middle	06 (13.9)	
Upper	03 (7.0)	

Menstruation Status		
Menstruating	07 (16.3)	43 (100)
Menopausal	36 (90.7)	

When analysed, the range of age of subjects was found to be between 35-78 years. The age group ≥ 55 years had more number of patients (60.5%). Of 43 subjects, 28(65.1%) had ≥ 3 living children. Majority (79.1%) belonged to the lower socioeconomic status and 90.7% were post-menopausal.

Table 2: Total duration of menopause in post-menopausal females

Duration (in years)	N (%)
0-5	10 (27.8)
6-10	15 (41.7)
11-15	06 (16.7)
> 15	05 (13.8)
Total	36 (100)

Out of 36 postmenopausal females from a total of 43 cases with malignant and pre-malignant lesions of the cervix, a big group, 15 (41.7%) were postmenopausal since 6-10 years, followed by ten (27.8%) who were 0-5 years into menopause. The least number of subjects with abnormal cervical biopsy reports were in their late menopause that is after ten years onwards.

Clinical analysis

Table 3: Characteristics of growth in patients with cervical cancer

Type of growth	N (%)	Total
Ulcerative	9 (27.3)	33 (100)
Proliferative	11 (33.3)	
Infiltrative	13 (39.4)	
Parametrial involvement		
Absent	20 (60.6)	33 (100)
Present	13 (39.4)	
Histopathological type		
Squamous cell carcinoma	32 (96.9)	33 (100)
Adenocarcinoma	01 (3.1)	

It can be seen from Table 3 that among the 33 cases of biopsy proven cervical cancer subjects, there was an almost equal distribution of infiltrative (39.4%), proliferative (33.3%) and ulcerative (27.3%) type of growth and parametrial involvement was absent in 20 (60.6%) subjects. Almost all were squamous cell carcinomas (96.9%).

Gene expression analysis

Table 4: Total cervical biopsy samples

Cervical cancer*	33
Pre-cancerous**	10
Controls***	09
Total	**52**

Malignant cervical biopsy sample, **Premalignant lesion in cervical biopsy (High grade intraepithelial squamous lesion), * Biopsy from hysterectomy samples with previous normal PAP report and no evidence of abnormal cervical changes.*

Table 4 shows the distribution of cervical cancer, 33, and pre-malignant tissue samples, ten, among a total of 43 case samples in which expression of Beclin-1 and LC3 was seen as against nine controls.

Table 5: Beclin-1 & LC3 gene expression in 43 cases of pre-malignant and malignant lesions

Gene expression	Beclin-1 N (%)	LC3 N (%)
Up regulated	13 (30.2)	13 (30.2)
Down regulated	14 (32.5)	09 (20.9)
Unchanged	16 (37.2)	21(48.9)
P value	0.072	0.3672

Table 5 shows that in 43 cases of premalignant and malignant tissue sample no significant changes in expression of Beclin-1 and LC3 could be found. Each was up regulated in equal number of cases and down- regulated in 32.5% (Beclin-1) & 20.9% (LC3)

Table 6: Autophagy gene expression (Beclin-1 & LC3) in ten cases of only pre-malignant lesions

Gene expression	Beclin-1 + LC3
Up regulated	7 (70.0)
Down regulated	2 (20.0)
Unchanged	1 (10.0)
P value	0.003

When combined Beclin-1 and LC3 was analysed separately for the ten cases of pre-malignant lesions, it was seen that expression was significantly up-regulated in 70.0% samples (p=0.003) as seen in Table 6.

Table 7 shows combined Beclin-1 and LC3 gene expression was also significantly (p = 0.001) up regulated in 13 (76.5%) of the 17 cases in age group < 55 years as against the age group ≥ 55 years were there was an almost equal distribution of up and down regulation.

Table 7: Expression of autophagy genes Beclin-1 & LC3 in different age groups

Expression	Age group (in years)	
	< 55	≥ 55
	N (%)	N (%)
Up regulated	13 (76.5)	11 (42.3)
Down regulated	3 (17.6)	15 (57.7)
Unchanged	1 (5.9)	—
Total	**17 (100)**	**26 (100)**
P value	0.001	

DISCUSSION

Epidemiological studies show that the human papillomavirus (HPV) infection is closely related to more than 90% of the cervical cancer cases. However, the virus infection alone is not sufficient to cause cervical cancer. Cellular molecular mechanisms for the disease pathogenesis still needed to be established[22]. Autophagy is a cellular homeostasis mechanism and its dysfunction is associated with the development of malignant tumors[23-27] and the sensitivity to chemoradiation[28, 29]. In a study from China done in 2017 on Beclin-1 expression in normal cervical, CIN and cervical cancer tissue, it was shown that compared with the normal cervical tissue, the positive rate of Beclin1 in the CIN tissue was significantly declined ($p < 0.05$), which was further significantly down-regulated in the cervical cancer tissue ($p < 0.05$)[30].

In our study, 43 female subjects with biopsy proven pre-malignant and malignant lesions of the cervix were recruited. Out of these, 60.5% were equal to or more than 55 years of age, 90.7% being post-menopausal. A bigger group of these females (41.7%) were 6-10 years post-menopausal whereas another sizable proportion (27.8%) was 0-5 years post-menopausal. Only small proportions were more than 10 years post-menopausal. 65.1% had previous 3 or more live issues. 79.1% belonged to the lower socioeconomic status (Kuppuswamy scale).

Clinically, there was an equal distribution of the types of cervical growths seen in 33 cases of proven malignancies. 39.4% were infiltrative, 33.3% proliferative and 27.3% ulcerative. Parametrium involvement was absent in 60.6% cases. Histologically, 96.9% were squamous cell carcinomas.

Beclin-1 and LC3 gene expression was analysed in 52 tissue samples of which 33 were biopsy proven cervical cancers and 10 pre-cancerous (high grade intra-epithelial lesions), as against 9 controls from cervical biopsies of hysterectomy specimens with previous normal PAP reports and no evidence of abnormal cervical changes.

It was found that in our study, expression of both Beclin-1 & LC3 genes were equally up-regulated (30.2%, 30.2%) and down-regulated (32.5%, 20.9%) in pre-malignant and cervical cancer tissue as against our controls. Also both remained unchanged in an appreciable number of these cases (37.2%, 48.9%), which suggests retention of autophagy capacity by cancerous cells in some cases. This appears in contrast with the belief that autophagy should always be down-regulated in cells for progression of cancer. Xu *et al.* suggested in 2012 that inhibition of autophagy may improve cisplatin chemotherapy by increasing the levels of intracellular misfolded proteins and DNA mutations which enhanced cellular apoptosis in cancer cells[20, 31]. By the same mechanism, autophagy may also promote tumor cell proliferation[23]. In another study published in 2015, Beclin-1 protein expression remained relatively unaltered in 398 ovarian high-grade serous cystadenocarcinomas[32]. So, it is possible that after an initial down-regulation of autophagy due to a variety of reasons, malignant changes are initiated within a cell by accumulation of cytotoxic material and DNA fragments after which some oncogenes or cancer causing agents may up-regulate autophagy to protect the cancer cells from apoptosis. This may be the reason that we found both up-regulation and down-regulation in our study samples.

However, when combined beclin-1 & LC3 expression was analysed in only pre-malignant cervical tissue, it was found to be significantly (P=0.003) up-regulated in 70% cases (7/9) as against our controls, probably protecting the cell by removing debris from the cellular micro-environment which may be a reason why these cases stayed in pre-malignant state and did not progress to cancer.

In a review published in 2012 from India[17], it was surmised that expression levels of both Beclin-1 and LC3 were not associated with age, FIGO stage, pathologic differentiation, and lymph node metastasis. In our study also there was no relation with stage, pathologic differentiation, and lymph node status however it was found that in patients less than 55 years of age, 76.5% had up-regulation of expression of Beclin-1 & LC3 (P=0.001).However, in patients ≥ 55years, there was almost equal distribution of up-regulation and down-regulation. So the significant association was found in the group of younger patients (13/17). This is in concurrence with the fact that most gynaecological malignancies are more common in the older age group which may in part be due to an age related down-regulation in autophagy and thus less efficient housekeeping of the cellular micro-environment.

An indirect evidence of protection from disease in younger age group was also found in a study done in mice which indicated that an age-related decline in autophagy and mitophagy responses to lung injury may contribute to the promotion and/or perpetuation of pulmonary fibrosis[33].

CONCLUSION

Although it has been seen in a number of studies that in cervical cancer, autophagy was down-regulated as evidenced by their demonstration of low expression of Beclin-1 & LC3, this was not seen in our study where we saw equal up and down-regulation of both these markers of autophagy. An up-regulation of Beclin-1 & LC3 was observed only in the pre-cancerous lesions and a significant correlation was found in patients < 55 years. Therefore, though the results could be explained, do not lead to the premise that Beclin-1 & LC3 expression in cervical cancer can replace other prognostic markers of progression in terms of accuracy.

ACKNOWLEDGEMENTS

Authors acknowledge the Genetics laboratory, Institute of Sciences for technical support

Competing Interests: Authors declare that they have no competing interests

REFERENCES

1. Jemal, A.; Bray, F.; Center, M.M.; Ferlay, J.; Ward, E.; Forman, D. Global cancer statistics. CA Cancer J. Clin. 2011, **61**, 69–90.
2. Ferlay, J.; Shin, H.R.; Bray, F.; Forman, D.; Mathers, C.; Parkin, D.M. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int. J. Cancer 2010, **127**, 2893–2917
3. Roa, J.C.; Garcia, P.; Gomez, J.; Fernández,W.; Gaete, F.; Espinoza, A.; Lepetic, A.; Suarez, E. HPV genotyping from invasive cervical cancer in Chile. Int. J. Gynecol. Obstet. 2009, **105**, 150–153.
4. Lee, D.; Kwon, J.H.; Kim, E.H.; Kim, E.S.; Choi, K.Y. HMGB2 stabilizes p53 by interfering with E6/E6AP-mediated p53 degradation in human papillomavirus-positive HeLa cells. Cancer Lett. 2010, **292**, 125–132.
5. Cornelio, D.B.; Roesler, R.; Schwartsmann, G. Emerging therapeutic agents for cervical cancer. Recent Pat. Anticancer Drug Discov. 2009, **4**, 196–206.
6. Choudhari, A.S.; Suryavanshi, S.A.; Kaul-Ghanekar, R. The aqueous extract of Ficus religiosa induces cell cycle arrest in human cervical cancer cell lines SiHa (HPV-16 positive) and apoptosis in HeLa (HPV-18 positive).
7. Fan, X.; Chen, J.J. Role of Cdk1 in DNA damage-induced G1 checkpoint abrogation by the human papillomavirus E7 oncogene. Cell Cycle 2014, 13, 3249–3259. PLoS ONE 2013, 8, e70127.
8. Eskelinen EL, Saftig P. Autophagy: a lysosomal degradation pathway with a central role in health and disease. Biochim Biophys Acta 2009; **1793**: 664–73.
9. Singletary, K.; Milner, J. Diet, autophagy, and cancer: A review. Cancer Epidemiol. Biomark. Prev. 2008, **17**, 1596–1610
10. Eskelinen, E.L.; Saftig, P. Autophagy: A lysosomal degradation pathway with a central role in health and disease. Biochim. Biophys. Acta 2009, **1793**, 664–673.
11. Zhu,W.; Pan, X.; Li, F.; Zhang, Y.; Lu, X. Expression of Beclin 1 and LC3 in FIGO stage I-II cervical squamous cell carcinoma and relationship to survival. Tumour Biol. 2012, **33**, 1653–1659.
12. Jie-Heng Tsai, Li-Sung Hsu, Hsiu-Chen Huang, Chih-Li Lin, Min-Hsiung Pan, Hui-Mei Hong, Wei-Jen Chen. 1-(2-Hydroxy-5-methylphenyl)-3-phenyl-1,3-propanedione Induces G1 Cell Cycle Arrest and Autophagy in HeLa Cervical Cancer Cells. Int. J. Mol. Sci. 2016, **17**, 1274
13. Klionsky DJ, Abeliovich H, Agostinis P, Agrawal DK, Aliev G, Askew DS, et al. Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes. Autophagy 2008; 4:151-75; PMID:18188003
14. Cheng HY, Zhang YN, Wu QL, *et al.* Expression and clinical significance of LC3 in cervical cancer tissues. Chin J Cancer Prev Treat. 2011; **18**: 1247–51.
15. Shen Y, Li DD, Wang LL, *et al.* Decreased expression of autophagy-related proteins in malignant epithelial ovarian cancer. Autophagy. 2008; **16**: 1067–8.
16. Giatromanolaki A, Koukourakis MI, Koutsopoulos A, *et al.* High Beclin 1 expression defines a poor prognosis in endometrial adenocarcinomas. Gynecol Oncol. 2011; **123**: 147–51.
17. Saumya Pandey, Chandravati. Autophagy in Cervical Cancer: An Emerging Therapeutic Target APJCP, **13**, 2012 4867 doi:http://dx.doi.org/10.
18. Zois CE, Koukourakis MI. Radiation-induced autophagy in normal and cancer cells: towards novel cytoprotection and radio-sensitization policies? Autophagy. 2009; **5**: 442–50.

19. Eisenberg-Lerner A, Kimchi A. The paradox of autophagy and its implication in cancer etiology and therapy. Apoptosis. 2009; **14**: 376–91.

20. Sun Y, Liu JH, Jin L, et al. Over-expression of the Beclin1 gene upregulates chemosensitivity to anti-cancer drugs by enhancing therapy-induced apoptosis in cervix squamous carcinoma CaSki cells. Cancer Lett. 2010; **294**: 204–10.

21. Galluzzi L, Pietrocola F, Bravo-San Pedro JM, Amaravadi RK, Baehrecke EH, Cecconi F, Codogno P, Debnath J, Gewirtz DA, Karantza V, Kimmelman A, Kumar S, Levine B, Maiuri MC, Martin SJ, Penninger J, Piacentini M, Rubinsztein DC, Simon HU, Simonsen A, Thorburn AM, Velasco G, Ryan KM, Kroemer G. Autophagy in malignant transformation and cancer progression. EMBO J. 2015 Apr 1; **34**(7): 856-80.doi: 10.15252/embj.201490784. Epub 2015 Feb 23.

22. Kumar V, Abbas AK, Fausto N. Robbins basic pathology. 8th ed. Saunders Elsevier 2007; pp. 718-721.

23. White E. Deconvoluting the context; dependent role for autophagy in cancer. Nat Rev Cancer 2012; **12**: 401-410

24. Mathew R, Kongara S, Beaudoin B, Karp CM, Bray K, Degenhardt K, Chen G, Jin S, White E. Autophagy suppresses tumor progression by limiting chromosomal instability. Genes Dev 2007; **21**: 1367-1381.

25. Duffy A, Le J, Sausville E, Emadi A. Autophagy modulation: a target for cancer treatment development. Cancer Chemother Pharmacol 2015; **75**: 439-447.

26. Ávalos Y, Canales J, Bravo-Sagua R, Criollo A, Lavandero S, Quest AF. Tumor suppression and promotion by autophagy. Biomed Res Int 2014; 2014:603980.

27. Zarzynska JM. The importance of autophagy regulation in breast cancer development and treatment. Biomed Res Int 2014; 710345.

28. Wang W, Liu J, Wu Q. MiR-205 suppresses autophagy and enhances radiosensitivity of prostate cancer cells by targeting TP53INP1. Eur Rev Med Pharmacol Sci 2016; **20**: 92-100.

29. Zhang N, Yang GQ, Shao XM, Wei L. GAS5 modulated autophagy is a mechanism modulating cisplatin sensitivity in NSCLC cells. Eur Rev Med Pharmacol Sci 2016; **20**: 2271-2277.

30. M.-X. Shen, J.B. Ding. Expression levels and roles of EMC-6, Beclin1 and Rab5a in the cervical cancer. Eur Rev Med Pharmacol Sci: 2017; **21**: 3038-3046

31. Xu Y, Yu H, Qin H, Kang J, Yu C, Zhong J, Su J, Li H, Sun L. Inhibition of autophagy enhances cisplatin cytotoxicity through endoplasmic reticulum stress in human cervical cancer cells. Cancer Lett. 2012 Jan 28; **314**(2):232-43. doi: 10.1016/j.canlet.2011.09.034. Epub 2011 Oct 2.

32. Rohann J. M. Correa, Yudith Ramos Valdes, Trevor G. Shepherd, Gabriel E. DiMattia. Beclin-1 expression is retained in high-grade serous ovarian cancer yet is not essential for autophagy induction *in vitro*. J Ovarian Res. 2015; **8**: 52. doi: 10.1186/s13048-015-0182-y PMCID: PMC4524172 PMID: 26239434.

33. Sosulski ML, Gongora R, Danchuk S, Dong C, Luo F, Sanchez CG. Deregulation of selective autophagy during aging and pulmonary fibrosis: the role of TGFβ1. Aging Cell. 2015 Oct; **14**(5):774-83. doi: 10.1111/acel.12357. Epub 2015 Jun 9.