Autophagy Related Markers (Beclin-1 and ATG4B) Are Strongly Expressed in Wilms’ Tumor and Correlate with Favorable Histology

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Autophagy Related Markers (Beclin-1 and ATG4B) Are Strongly Expressed in Wilms' Tumor and Correlate with Favorable Histology

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Abstract:

**Background:** Wilms’ tumor treatment has achieved great success in the last decade. Nevertheless, some cases still fail to respond to the current multimodality therapy. These cases fall mainly in the unfavorable histology group with very few belonging to the favorable histology group. In recent years, autophagy manipulation whether inhibition or stimulation has been shown to affect cancer cell behavior and has emerged as a novel mechanism to improve cancer cell response to currently used therapeutic regimens.

**Objective:** The current study aimed to investigate the expression of autophagy related markers (ATG4B and Beclin1) in WT, its association with the different clinic-pathological parameters and its impact on patient survival.

**Methods:** Twenty-one formalin fixed paraffin embedded (FFPE) WT specimens were immunohistochemically stained using autophagy related markers; Beclin-1 and ATG-4b. All clinical, radiological and follow up data were retrieved from the patient records.

**Results:** All specimens showed positive expression of both Beclin-1 and ATG4B. The staining score for Beclin1 varied between 50 and 300, and its expression was significantly associated with favorable histology (p=0.007). Similarly, ATG-4B expression was significantly higher in favorable histology tumors compared to unfavorable histology (p=0.046). A statistically significant positive correlation between Beclin-1 and ATG4 expression was observed. The cumulative disease-free survival in patients with favorable histology was significantly higher compared to patients with unfavorable histology (p=0.0027).

**Conclusions:** Beclin-1 and ATG4B expression were both found to be statistically significant discriminators of survival. Collectively these findings suggest that the expression of autophagy-related markers is associated with a favorable histology and could predict better survival in these patients.

**Key words:** Autophagy, Wilms tumor, Malignancy, Beclin-1.
Introduction:

Wilms’ tumor (WT) is the most common solid renal tumor in children accounting for more than 95% of all childhood renal malignancies (Davidoff, 2009, Diniz, 2016). Over the past decade, WT treatment has achieved great success with an overall 5-year survival rate reaching up to greater than 85% (Dome et al., 2013). Nevertheless, there still remain cases that fail to respond to current multimodality therapy (Szycot et al., 2014).

Despite the significant progress in development of genetic markers that predict tumor behavior and patient outcome, in the case of WT, histology remains the gold standard. Patients are stratified into high-risk (unfavorable histology) and low risk (favorable histology) groups based on the presence or absence of anaplasia. And those patients who develop recurrent disease continue to suffer a 50% mortality rate even with radical and aggressive treatment (Mizushima and Komatsu, 2011). Therefore, there is still a need to identify molecular markers that could predict tumor cell behavior.

Current models of tumor development suggest that autophagy plays an important role in carcinogenesis. Autophagy is a catabolic pathway that shuttles cytoplasmic components via double membrane vesicles into the lysosomes for recycling and energy production (Mizushima and Komatsu, 2011). It is controlled by a number of autophagy related genes and proteins (ATG) (Kirisako et al., 2000, Mizushima and Komatsu, 2011); Beclin-1 (BECNI) stimulates the formation of the isolation membrane of autophagosome, ATG4 and ATG5 genes being instrumental in the elongation phase cascade (Mizushima et al., 1998, Liang et al., 1999, Kirisako et al., 2000, Yue et al., 2003, Li et al., 2011) and in mammals, the C-terminal peptide of Light Chain 3 (LC3) which is similar to (ATG8 in yeast participates in cargo recognition and determines the size of the autophagosome. ATG8 complex is then cleaved by the ATG4 homologues and the resultant vesicle fuses with the lysosome (Hemelaar et al., 2003, Tanida et al., 2004). The role of autophagy in cancer is currently identified as an active area of research. This role is very complex and context-dependent. While some studies have demonstrated a tumor enhancing effect of autophagy, others have suggested a complete opposite effect (Mizushima and Komatsu, 2011, White, 2015, Fei et al., 2016).

It has been demonstrated that defective autophagy can be associated with an increased oxidative stress, genomic instability, aneuploidy as well as evasion of the anti-tumor immune response thus promoting carcinogenesis (Dou et al., 2015) (Ladoire et al., 2016). On the other hand, other studies have shown that once malignancy is established, autophagy can support tumor cell growth and induce resistance to a variety of chemotherapeutic agents by providing
the dividing tumor cells with the necessary nutrients in the harsh hypoxic tumor microenvironment (Lazova et al., 2012).

With the current archives of literature showing this dual function of autophagy in tumorigenesis it is essential to have an in-depth probe into the mechanisms of autophagy as this could probably uncover the various factors regulating cancer growth. The characterization of autophagy manipulators inducers or inhibitors, with novel mechanisms of action has emerged as a very active area of research and to the best of our knowledge, the role of autophagy in Wilms’ tumor has not been yet fully investigated. Therefore, the current study aimed to investigate the expression of autophagy related markers Beclin1 and ATG4B in WT, its association with the different clinico-pathological parameters and its effect on patient survival.

Our hypothesis suggests that autophagy could have an important role in WT development and progression and that manipulation of autophagy in WT could help improve the outcome of those few patients who still fail to respond to current therapeutic modalities.

**Materials and Methods:**

The present study is a retrospective study conducted on twenty-one FFPE WT specimens. Specimens were retrieved from the archives of the pathology department in Alexandria College of Medicine, Egypt. All patients had undergone curative nephrectomies followed by Post-operative chemotherapy/radiotherapy according to the National Wilms Tumor Study Group (NWTSG)/ Children Oncology group (COG) guidelines.

Clinical and pathological data including patient age, recorded tumor size and weight, stage as well post-operative follow up data were retrieved from the patient records. Written informed consents to use the patient’s specimens for medical research were obtained from the parents. The median age of the patients at the time of surgery was 24 months (ranging between 3 months and 132 months). Tumor specimens were reviewed by a single pathologist in order to confirm the diagnosis, the grading as well as staging of the tumor. The study included fourteen cases of Favorable histology (FH) and seven cases with unfavorable histology (UH) which were very difficult and rare to find. Anaplasia was identified in the latter group according to the COG definition by the presence of a three-fold increase in nuclear size compared to adjacent nuclei, hyperchromasia and multipolar mitotic figures. Stage I disease was identified in 8 cases (38%), stage II disease in 6 cases (29%), stage III in 5 cases (24%) and stage IV in 2 cases (10%). The study was approved by the ethics committee of the college of Medicine, University of Alexandria.
**Immunohistochemistry procedures and interpretation:**

Twenty-one formalin fixed paraffin embedded tumor specimens were used. Unstained slides were prepared for immunohistochemical procedure. Representative samples were stained with Autophagy related markers: Beclin-1, primary mouse monoclonal antibody (dilution 1:100, clone SC-48381, Santa Cruz Biotechnology, USA) and ATG-4B rabbit polyclonal antibody (dilution 1:100, clone SC-130968, Santa Cruz Biotechnology, USA). Positive and negative controls were included in all the runs.

All sections were deparaffinised in xylene then rehydrated in descending concentrations of alcohol. After blocking endogenous peroxidase enzyme using hydrogen peroxide for 10 minutes, heat-mediated antigen retrieval in citrate buffer (pH 6) was performed using a microwave before starting the immunohistochemical staining protocol. The streptavidin–biotin–peroxidase complex method was used. This technique involves overnight incubation of the specimens with an unconjugated primary antibody (Beclin-1 and ATG-4b) in cold room (4°C), followed by incubation with a biotinylated secondary antibody that reacts with the primary antibody, then enzyme-labelled streptavidin, and DAB substrate chromogen. Sections were then counterstained using haematoxylin. The Abcam Detection kit (Mouse specific HRP/DAB (ABC) detection IHC kit, ab64259) and (Rabbit specific HRP/DAB (ABC) detection IHC kit, ab64261) were used.

The immunohistochemically stained slides were reviewed by two independent pathologists who were blinded to the clinical data.

For Beclin-1 and ATG4B staining, cytoplasmic reactions were considered positive. A semi-quantitative (Modified H-score) method was used to assess the degree of positive staining. This method assigns an IHC H-score to each patient on a continuous scale of 0–300, based on the percentage of cells at different staining intensities visualized at different magnifications (Pirker et al., 2012). Cytoplasmic staining was scored according to four categories: 0 for ‘no staining’, 1 + for ‘light staining visible only at high magnification’, 2 + for ‘intermediate staining’ and 3 + for ‘strong, dark staining, visible even at low magnification’. The percentage of cells at different staining intensities was determined by visual assessment, with the score calculated using the formula $1 \times (\% \text{ of } 1+ \text{ cells}) + 2 \times (\% \text{ of } 2+ \text{ cells}) + 3 \times (\% \text{ of } 3+ \text{ cells})$ depending on the percentage of stained cells.
Statistical analysis:

Data were collected and entered using SPSS (Statistical package for social science) program for statistical analysis (version 21). Data were entered as numerical or categorical as appropriate. Data were described using minimum, maximum, mean, standard deviation and 95% CI of the mean for the normally distributed area. Categorical variables were described using frequency and percentage of total. Comparisons were carried out between two studied independent not normally-distributed subgroups using Mann-Whitney U test. Comparisons were carried between more than two studied independent not normally distributed subgroups using Kruskal-Wallis test. Chi-square test was used to test the association between qualitative variables. Fischer’s exact test and Monte Carlo correlation was carried out when indicated. An alpha level was set to 5% with a significance level of 95% and a beta error accepted up to 20% with a power of study of 80%. Patient overall survival was defined as the number of patients who were alive during the follow up period (which ranged from 2 to 5-years post treatment). The disease-free survival was defined as the number of patients who were alive and did not develop any recurrence or metastasis during the follow up period

Results:

A total of twenty-one Wilms’ tumors were investigated in this study. All patients had undergone radical nephrectomy with post-operative chemotherapy/radiotherapy in Alexandria University hospital, Egypt, according to the NWTSG/COG guidelines. The age of the patients at surgery ranged between 3 months and 132 months with a median age of 24 months. Ten out of the twenty-one cases (47.62%) showed the typical triphasic pattern on histology whereas six had a biphasic pattern (28.57%) and only five tumors had a monophasic pattern (blastemal only) (23.81%).

Fourteen out of the 21 cases had favorable histology (FH) (66.67%) and seven cases showed unfavorable histology (uFH) (33.33%). As for the tumor stage, 14 were classified as low stage (stage I and II) and seven were classified as high stage (stages III and IV). Data concerning patients’ follow up were retrieved from the patients’ records. The follow up period ranged between 2 years and 5 years.

Beclin-1 expression in Wilms’ tumors:

Cytoplasmic Beclin-1 expression was noted in all the examined tumors. However, its degree of expression varied widely between tumors. Beclin-1 was more expressed in the
epithelial component of the tumors (primitive tubules) compared to the stromal and blastemal component which both showed less expression. Beclin-1 expression score (as measured by modified H-scoring) varied between 50 and 300 with a median value of 220. Beclin-1 expression was significantly higher in low grade tumors (favorable histology) (Median value 234) compared to a lower expression in high grade tumors (unfavorable histology) (Median value 50). A statistically significant correlation was found between Beclin-1 expression and tumor grade (p=0.007, Mann Whitney U test). Although Beclin-1 expression was higher in low stage tumors (stage I and II) compared to high stage tumors, this difference was not statistically significant (p=0.128 and 0.799, respectively) (Fig. 1 and 2). Furthermore, there was a statistically significant positive correlation between Beclin-1 and ATG-4B expression in tumor cells (Fig. 3).

**ATG-4B expression in Wilms’ tumors:**

Similar to Beclin-1, cytoplasmic ATG-4B was variably expressed in the tumor cells (Fig. 4). Modified H-score for ATG4B expression varied between 150 and 300. ATG-4B expression was also positively associated with favorable histology (FH) in the studied cases. The median H-score in FH cases was 300 compared to only 180 in uFH. (p=0.046, Mann Whitney U test). ATG4B expression did not show any statistically significant correlation with the age, gender or tumor stage.

**Disease free survival:**

During the period of follow up which ranged between 2 and 5 years, three out of the seven cases with uFH developed recurrences or metastasis (42.85%) compared to none of the cases of FH. The cumulative disease-free survival in the low-grade group (FH) was significantly higher when compared with the high-grade group (uFH). (p=0.0027, Log-Rank test)

When comparing low stage to high stage patients, the difference in disease free survival was not statistically significant. Two out of the seven high-stage patients (7.14%) developed recurrences or metastasis during the follow up period. The median disease-free survival for the uFH tumors was 3.486 years compared to 4.667 years for the FH tumors. (p=0.135, Log-Rank test) (Fig. 5A and B).
Overall survival:

Similarly, the cumulative overall survival function was significantly higher in FH tumors compared to uFH ones (p=0.009, Log-Rank test). When comparing low-stage tumors with high-stage ones, the cumulative overall survival did not show a statistically significant difference. (p=0.176, Log-Rank test) (Fig. 5C and D).

Diagnostic test accuracy analysis:

Beclin-1 was found to be a statistically significant discriminator of survival in Wilms tumors. The area under the ROC curve (AUC) = 0.954 (95% CI 0.764-0.999) (p=0.0001). The diagnostic criterion using the Youdon index is the level of < 60 (H-score) with a sensitivity of 100%, specificity of 88.89%. Positive predictive value of 60% and negative predictive value of 100% (Fig. 6).

Discussion:

Autophagy is a cellular pathway through which cytoplasmic organelles and macromolecules are transported to the lysosomes where they are used to produce energy (Avalos et al., 2014). Interestingly, its role in cancer has shown both tumor-promoting and tumor-suppressing effects depending on the tissue type, stage of tumor progression as well as the presence of certain genetic mutations (Rabinowitz and White, 2010). Therefore, autophagy manipulators whether promotors or inhibitors have emerged as potential drugs to be added to the currently used therapeutic protocols.

In the present study, the immunohistochemical expression of autophagy-related markers Beclin-1, and ATG4B was investigated in Wilms’ tumor. The expression of Beclin-1 as well as other autophagy related markers like ATG4, ATG5, LC3, p62 have been extensively investigated in many solid tumors and were suggested as potential prognostic markers. All tumor samples in the present study (100%) showed positive expression of Beclin-1. However, the calculated H-score for Beclin-1 varied between 50 and 300. A high Beclin-1 expression was significantly associated with favorable histology (FH) compared to a lower H-score mostly seen in unfavorable histology (uFH) tumors, thus depicting a statistically significant correlation between Beclin-1 expression and favorable histology (p=0.007). This finding is quite novel as to the best of the authors knowledge these markers have not been previously studied in WTs. Furthermore, a trend towards high Beclin-1 expression in low-stage (Stage I and II) tumors
compared to high stage tumors (stage III and IV) was demonstrated, although this did not prove to be statistically significant (p=0.128).

It is well established that unfavorable histology tumors have a much worse prognosis than the favorable histology ones which according to our findings means that the high expression of Beclin-1 seen in Favorable histology WTs could point to a good prognostic significance of Beclin-1 expression in WTs. However, due to the small number of patients and the very limited number of unfavorable histology cases investigated in this study, Beclin-1 did not qualify as an independent prognostic marker and it did not show a statistically significant correlation with overall survival in these patients.

Several other genes and proteins have been previously investigated as prognostic markers in WT. Ki-67 monoclonal antibody is commonly used in evaluating cellular proliferation rates in different malignant tumors. Ki-67 is expressed in normal kidney tissues as well as in the three main components of Wilms' tumor (blastemal, epithelial and stromal cells). Ki-67 is a relevant marker for assessing the proliferative activity and tumor cell dynamics of Wilms' tumor. However, it may not be a good clinical prognostic marker in those patients who received preoperative chemotherapy, because the epithelial component is better differentiated and therefore less sensitive to preoperative chemotherapy. Therefore Beclin-1 could be superior to Ki-67 as a marker for favorable histology and good prognosis in these cases (Jurić et al., 2010). Similarly, TP53 gene mutations have been suggested as a hallmark of poor prognosis in WT as they coexist with diffuse anaplasia (Wincewicz et al., 2016). A recent study has investigated the role and significance of TP53 mutations in diffusely anaplastic Wilms tumor (DAWT), and has suggested that the use of TP53 immunoreactivity and/or gene sequencing may help refine the diagnostic and therapeutic strategy for DAWT (Ooms et al., 2016). In recent years, a new role for TP53 in regulating autophagy has been suggested and studies have shown that TP53 inhibited the autophagic response triggered by starvation via upregulation of the Rap2B-PLC-ε-IP3-Ca2+ pathway (Di et al., 2017). This may point to a possibility that the dismal prognosis associated with TP53 mutations in (uFH) WT could partly be due to its inhibitory effect on autophagy, However, more research in that area is still needed to explore this effect.

The immunohistochemical expression of Beclin-1 as well as other autophagy markers has been investigated in other human malignancies and showed variable results. In accordance with our findings, studies on human liver, brain and esophageal cancers showed that low levels of
Beclin-1 increased tumorigenesis whereas increased levels of Beclin-1 inhibited the formation of tumors (Rabinowitz and White, 2010, Park JM, 2012, Pirker et al., 2012). Similarly, previous reports showed that heterozygous deletion of the autophagy gene Beclin-1 induced spontaneous tumors [28]. Furthermore, Beclin-1 was also reported to be deleted or significantly decreased in ovarian, breast and prostate cancer (Park JM, 2012). In glial tumors of the brain, the high grade glial neoplasms (grade III and IV) had a lower Beclin-1 expression compared to low grade (Grade I and II) tumors thus signifying that loss of Beclin-1 gene function is associated with increased tumor progression and aggressiveness (Miracco et al., 2007). Similarly, in KRAS-mutated colon cancers, the low LC3B dot-like and low p62 dot-like-cytoplasmic staining, which are other autophagy related proteins, were found to be associated with a worse outcome (Niklaus et al., 2017). Together, all these studies have suggested that autophagy may exhibit a tumor suppressive effect and that lack of expression or deletion of autophagy related genes can be associated with a more aggressive phenotype.

On the other hand, several other studies have shown that autophagy may have a tumor promoting effect. A study on gastric adenocarcinoma found that mRNA and protein expression of Beclin-1 were higher in early stage gastric cancer compared to normal surrounding tissues, thus pointing to a possible role for Beclin-1 in gastric tumorigenesis (Li et al., 2013). Beclin-1 gene amplification on chromosome 5q was also found to be associated with the development of clear cell renal cell carcinoma in the kidney (Li et al., 2013) and P62 expression, another autophagy related marker, predicted a worse prognosis in a cohort of esophageal adenocarcinoma patients (Linares et al., 2011). This huge lack of consistency found in the literature regarding the significance of the expression of autophagy related genes (ATG) in the different tumors can be partly attributed to the differences in the nature of the cohorts studied as well as to the lack of standardization of the immunohistochemical scoring protocols. We tried to overcome this in our study by using the modified H-scoring method which evaluates both the intensity of staining as well as the number of positively stained cells at different magnifications. Another confounding factor to this high level of discrepancy is the use of different ATGs and their proteins to investigate the degree of autophagy activation in the different tumors. These ATGs are known to play different roles at different stages and steps of the autophagy process and their use as indicative of active autophagy should be considered with caution.

In the present study, a statistically significant positive correlation between Beclin-1 and
ATG4B expression was observed. ATG4B was also strongly expressed in our cases with the H-score varying between 150-300. Similar to Beclin-1, there was a significant positive association between ATG4B expression and favorable histology (FH) and the median H-score value in Favorable histology cases was 300 compared to 180 in unfavorable histology (p=0.046). Similar to Beclin-1, ATG4B showed no statistical significant difference in its expression in relation to patient age, gender or tumor stage. The positive correlation between Beclin-1 and ATG4B expression in the current study supports the complementary role of both genes in inducing and regulating autophagy in the tissues with Beclin-1 inducing the formation of the isolation membrane of the autophagosomes and ATG4 and ATG5 contributing to its elongation phase (Liang et al., 1999, Kirisako et al., 2000, Yue et al., 2003, Li et al., 2011).

The findings of the present study, in relation to ATG4B expression support data from previous investigations. In a study by Marino et al., it was reported that ATG4 knockout mice had increased susceptibility to develop carcinogen-induced fibrosarcomas (Marino et al., 2007) and spontaneous hepatic tumors were observed in ATG5 mosaic-deleted mice and in mice with ATG7 liver-specific deletion respectively (Takamura et al., 2011). Bif-1, a constituent of Beclin-1/class111-P1k complex also contributed to the regulation of cell proliferation and suppression of tumors. Reports show that its expression is downregulated in prostatic and gastric tumors and the animals deficient in Bif-1 were more likely to develop tumors (Takahashi et al., 2007). Furthermore, deletions or mutations of ATG4, 5,12 and 9b were documented in various human malignancies (Liu and Ryan, 2012).

In the present study, we reported a trend towards high levels of Beclin-1 expression in those tumors from patients who did not develop any relapse or metastasis during the follow up period. All tumors (100%) that showed high expression levels (H-score above 60) showed a longer disease-free survival and overall survival. The results of the present study are in accordance with the results obtained in several other types of cancers. Increased levels of Beclin-1 expression were highly linked with a longer survival of patients in both esophageal and stage 1B colon cancers and patients with hepatocellular carcinoma having low protein levels were found to have a poorer prognosis (Ding et al., 2008, Chen et al., 2009, Shi et al., 2009). Similarly, in another study on colonic adenocarcinoma, the cytoplasmic expression of P62, another autophagy related marker, was found to be associated with increased overall survival (Schmitz et al., 2016).

On the other hand, several reports have shown that patients with Nasopharyngeal, ovarian
and breast cancers with decreased Beclin-1 expression had a significantly longer overall survival compared to the ones with higher expression (Wan et al., 2010, Zhao et al., 2014, Ueno et al., 2016). Also, high Beclin-1 expression in Colorectal, Nasopharyngeal and Breast cancers was associated with poor survival, higher cell proliferation and low clinical and pathological responses indicating a distinct role of Beclin-1 as a vital cancer prognostic marker (Koukourakis et al., 2010, Wan et al., 2010, Ueno et al., 2016).

From all the findings in our study and other similar studies, we may conclude that the role of autophagy in carcinogenesis is a complex one and that use of a single technique for identifying the exact biological significance of autophagy in tumorigenesis may lead to misleading results. However, several studies have suggested that this role will markedly depend on the time frame at which autophagy is being evaluated. In the early stages of carcinogenesis, it may have a tumor-suppressing effect, whereas, in later stages, autophagy allows survival, dormancy, growth and metastasis by providing an alternative energy source therefore exhibiting tumor-promoting properties (Levine and Kroemer, 2008, Galluzzi et al., 2015).

The tumor-suppressing effects of autophagy are said to be due to the activation of autophagy in extreme stress conditions in order to eliminate proteins and cell organelles that are damaged. Any obstruction in this process eventually results in elevated levels of reactive oxygen species leading to DNA damage, amplified gene breaks and polyploid nuclei with subsequently higher predisposition to initiation of cancer (Karantza-Wadsworth et al., 2007, Mathew et al., 2007). Another suggested mechanism is that any defect in apoptosis and autophagy will stimulate necrotic cell death and inflammation thereby triggering the cells to non-autonomous means of tumor progression by inducing prolonged wound healing response (Degenhardt et al., 2006). Altogether, all these reports and the present findings, suggest that suppression of tumors could be a feature of the autophagy machinery thereby protecting organisms against a variety of ailments.

In summary, based on the results of our study, we propose that a high immunohistochemical expression of of autophagy gene Beclin-1 and ATG4B might characterize a positive prognostic factor in WT as it is more likely to be associated with favorable histology, a lower stage of the tumor as well as a longer disease free and overall survival in our patients. However, the regulation of Beclin-1 pathway is still being fervently investigated due to the incongruous results of Beclin-1 performing different functions in different tumors. This further emphasizes
the need for more investigations of the exact role of Beclin-1 and other ATG genes as they present a novel potential target for cancer therapy.

**Limitations:**

There are several limitations to our study, not the least of which is the comparatively small number of samples analyzed and the concomitant unusual distribution of sample. While this may limit the extent to which definite conclusions may be drawn from the work herein presented, it is certainly a motivation for future studies including a larger number of cases and ideally also samples of recurrent tumors. However, since there is no published report on the correlation between BECN1 and ATG-4 in autophagy in WT, it is important to conduct further analysis in the same field to further elucidate the role of autophagy in WT and provide an appropriate prognostic marker for WT targeted research.

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Figure Legends:

**Fig.1:** Association between Beclin-1 and ATG4B immunohistochemical expression and Wilms’ tumor grade and stage. (A) Significant positive association between Beclin-1 immunohistochemical expression and favorable histology (low grade tumours) (p= 0.007, Mann Whitney U test). (B) Significant positive association between ATG4B immunohistochemical expression and favorable histology (low grade tumours) (p=0.046, Mann Whitney U test). (C) Positive association between Beclin-1 immunohistochemical expression and low stage tumor (p=0128, Mann Whitney U test). (D) Positive association between ATG4B immunohistochemical expression and low stage tumor (p=0.799, Mann Whitney U test).

**Figure 2: Beclin-1 immunohistochemical staining in Wilms’ tumor tissue.** (A)Epithelial compartment of favorable histology (FH) Wilms’ tumor showing strong cytoplasmic staining for Beclin-1 in primitive tubules. (B) Blastemal component of FH Wilms’ tumor showing strong cytoplasmic staining for Beclin-1. (C) Unfavorable histology Wilms’ tumor showing minimal cytoplasmic staining for Beclin-1. Note the large size of the nuclei and the tripolar mitotic figure (arrow) (magnification 40X).

**Figure 3:** A statistically significant association between Beclin-1 and ATG4B immunohistochemical expression in Wilms tumor (p=0.002)

**Figure 4:** ATG4B immunohistochemical staining in Wilms’ tumor tissue: (A) Favorable histology Wilms’ tumor showing strong cytoplasmic staining for ATG4B more evident in the epithelial component (magnification 20X). (B) Favorable histology Wilm’s tumor with evident cytoplasmic staining for ATG4B in the blastemal component. (Magnification 40X).

**Figure 5:** (A) Kaplan Meier curve showing overall survival for favorable and Unfavorable histology WTs. (B) High and low stage WTs. (C) Kaplan Meier curves showing disease free survival for favorable and Unfavorable histology WTs. (D) High and low stage WT

**Figure 6:** ROC curve showing that Beclin-1 was found to be a statistically significant discriminator of survival in Wilms tumors. The area under the ROC curve (AUC) = 0.954 (p=0.0001)
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Histologic grade

Favourable histology (No anaplasia)
Unfavourable histology

Beclin

ATG4

Low Stage
High Stage

Favourable histology (No anaplasia)
Unfavourable histology

Histologic grade

Low
High
Survival Functions

A

Histologic grade
- Favourable histology (No anaplasia)
- Unfavourable Histology
- Favourable histology (No anaplasia)-censored
- Unfavourable Histology-censored

Cum Survival

Overall survival (OS) (years)

B

Stage
- Low
- High
- Low-censored
- High-censored

Cum Survival

Overall survival (OS) (years)

C

Survival Functions

Histologic grade
- Favourable histology (No anaplasia)
- Unfavourable Histology
- Favourable histology (No anaplasia)-censored
- Unfavourable Histology-censored

Cum Survival

Disease free survival (DFS) (years)

D

Stage
- Low
- High
- Low-censored
- High-censored

Cum Survival

Disease free survival (DFS) (years)
Sensitivity: 100.0
Specificity: 88.9
Criterion: ≤60