Genetic polymorphisms of ATG5 predict survival and recurrence in patients with early-stage esophageal squamous cell carcinoma

Pei-Wen Yang¹,*; Min-Shu Hsieh²,³,*; Ya-Han Chang¹; Pei-Ming Huang¹; and Jang-Ming Lee¹

¹Department of Surgery, National Taiwan University Hospital and National Taiwan University College of Medicine, Taipei, Taiwan
²Department of Pathology, National Taiwan University Hospital and National Taiwan University College of Medicine, Taipei, Taiwan
³Graduate Institute of Pathology, College of Medicine, National Taiwan University, Taipei, Taiwan

*These authors have contributed equally to this work

Correspondence to: Jang-Ming Lee, email: jmlee@ntu.edu.tw

Keywords: esophageal squamous cell carcinoma (ESCC), ATG5, SNP, recurrence

ABSTRACT

Esophageal squamous cell carcinoma (ESCC) is a deadly disease with high risk of tumor recurrence even among patients with an early pathologic stage of tumor. In the current study, we investigate the association between 20 SNPs of the ATG5 gene and prognosis of patients with early-stage ESCC. A total of 305 patients diagnosed with early-stage ESCC were enrolled in the study and randomly assigned to a training set (n=93) or replication set (n=212). The genotypes of candidate SNPs (single nucleotide polymorphisms) within ATG5 were analyzed and correlated with the prognosis of ESCC patients. We repeatedly demonstrated that 3 SNPs in ATG5, rs1322178, rs3804329, and rs671116, were significantly correlated with the prognosis of patients with early-stage ESCC (HR[95% CI]=2.01[1.19-3.40], p=0.009 for ATG5: rs1322178; HR[95% CI]=1.88 [1.08-3.26], p=0.025 for ATG5:rs3804329; HR[95% CI]=1.73[1.24-2.42], p=0.001 for ATG5:rs671116, in combined group). Both rs1322178 and rs3804329 can predict early distant metastasis of patients. Furthermore, increased expression of ATG5 was observed in ESCC tumor tissue as compared to adjacent normal tissue. Moreover, higher levels of ATG5 expression in both normal and tumor tissues exhibited a trend to correlate with poor prognosis of patients. However, the expression of ATG5 did not correlate with these 3 relevant prognostic SNPs. We concluded that hereditary genetic polymorphisms and gene expression of ATG5 can serve as prognostic predictors of patients with early-stage ESCC.

INTRODUCTION

Esophageal cancer is among the major causes of cancer death worldwide [1–2]. It presents mainly as either esophageal squamous cell carcinoma (ESCC) or esophageal adenocarcinoma (EA) in histology [1]. The standard treatment for locally advanced esophageal cancer is neoadjuvant (preoperative) concurrent chemoradiotherapy (CCRT) with or without surgery. Patients who respond well to CCRT are restaged as pathologically early-stage after treatment. A high risk of recurrence has been found even among those diagnosed with a pathologically early-stage of tumor [3–4]. More than 50% of the patients with primary esophageal cancer encounter local-regional recurrence or distant metastases within 2 to 3 years [5–7]. The median survival after recurrence of ESCC is only about 8 months [7].

Autophagy is the “self-eating” molecular machinery involved in the bulk lysosomal degradation of long-lived proteins and organelles, which serves to maintain cellular homeostasis [8–9]. The genes involved in the process of
Autophagy are termed autophagy-related genes (ATG). Autophagy has been shown to be correlated with tumor formation and progression, and with cancer therapy outcomes [9–11]. The role of autophagy in tumorigenesis is complicated and is likely to be background dependent. A functional autophagy mechanism may be a necessary homeostatic process which removes damaged organelles and thus protects against cancer [12]. However, it may also promote cancer cell survival and growth in response to growth-limiting conditions such as nutrient depletion and hypoxia [13–14]. Autophagic activity has also been demonstrated to correlate with sensitivity to radiation or chemotherapy in treating various cancers [15–17]. Numerous anti-cancer therapies are known to induce autophagy. Targeting autophagy during cancer therapy is, therefore, regarded as a potential approach to improve the clinical outcome of cancer patients [10].

Autophagy has been suggested as a potential mechanism for resistance of ESCC to therapy [18–21]. Induction of autophagy by drug-resistant esophageal cancer cells was found to promote their survival and recovery following treatment with chemotherapeutics [18]. Specific inhibition with siRNA of early autophagy induction targeted to ATG7 and Beclin 1 obviously enhanced the effect of 5-FU (5-Fluorouracil) and reduced the recovery of drug-treated esophageal cancer cells [18]. Autophagy inhibition was also observed to contribute to radiation sensitization of ESCC [21]. However, a well-known autophagy inducer, lithium, has been reported to enhance the efficacy of therapeutic agents in esophageal cancer [22].

The ATG5 gene encodes autophagy protein 5 (Atg5), which associates with Atg12 and Atg16 to form an Atg5-Atg12/Atg16 complex that is essential for the formation of autophagosomes during the process of autophagy [10]. ATG5 is thus a possible factor involved in the tumor recurrence in early-stage esophageal cancer, yet it has hardly been investigated.

We thus set out to investigate the association of ATG SNPs (single nucleotide polymorphisms) with the prognosis of early-stage ESCC [22]. The correlation of ATG5 expression in ESCC tissues and both prognosis and genotype of early-stage ESCC patients were also investigated.

RESULTS

A total of 305 patients pathologically diagnosed with early-stage (stage 0, I and II) ESCC were enrolled in the study and randomly assigned to a training set (n=93) or replication set (n=212). The distributions of the demographic and clinical characteristics in the total patient group were compared by survival and recurrence status (Table 1). As expected, stage, T-stage and N-stage were strongly associated with both mortality and tumor recurrence (Table 1). Gender was also significantly associated with both survival and disease recurrence (P=0.014 for survival and P=0.001 for recurrence, Table 1). The management of patients, including surgical resection (esophagectomy) and CCRT was also strongly correlated with disease recurrence (P=0.021 for surgical resection and P=0.026 for CCRT treatment).

The genotypes of 20 candidate ATG SNPs were analyzed from the genomic DNA of 93 ESCC patients in the training group. The genotypes of the early-stage ESCC patients were correlated with survival by multivariate Cox regression analysis using dominant (Dom), recessive (Rec) and additive models. Three ATG5 SNPs, rs1322178, rs3804329 and rs671116, were found to be significantly or borderline associated with overall survival of patients by either the dominant, the recessive or the additive model (Supplementary Table 1).

In patients with early-stage ESCC, the genetic variants of ATG5:rs1322178 (HR [95 % CI]=3.60 [1.40–9.26], P=0.008), ATG5:rs3804329 (HR [95 % CI]=3.60[1.13–8.31], P=0.029), and ATG5:rs671116 (HR [95 % CI]=1.95[1.03–3.71], P=0.041, recessive model) were significantly associated with increased risk of death in the training set (Supplementary Table 1 and Table 2). Significant association of ATG5: rs1322178 and ATG5: rs671116 with risk of death was further confirmed in the replication group (HR [95 % CI]=1.99[1.02–3.90], P=0.045 and HR [95 % CI]=1.59[1.06–2.41], P=0.027 for ATG5: rs1322178 and ATG5: rs671116, respectively, Table 2). The genotypes of ATG5:rs3804329 displayed borderline association with overall survival in the replication group (p=0.064, Table 2). All of these SNPs were significantly correlated with hazard of death in the combined group (HR [95 % CI]=2.01[1.19–3.40], p=0.009 for ATG5:rs1322178; HR [95 % CI]=1.88[1.08–3.26], p=0.025 for ATG5:rs3804329; HR [95 % CI]=1.73[1.24–2.42], p=0.001 for ATG5:rs671116, Table 2). Notably, these ATG5 SNPs can predict early recurrence (i.e., recurrence within 2 years), of early-stage ESCC. Compared to the CC variant, the CT variant of ATG5:rs1322178 had a 7.03-fold increased risk of early local recurrence (OR [95 % CI]=7.03 [0.99–49.99], P=0.051, Table 3) and a 4.50-fold increased risk of early distant metastasis (OR [95 % CI]= 4.50 [1.19–17.01], P=0.027, Table 3). Patients carrying the AG genotype of ATG5:rs3804329 also had a 4.5-fold increased hazard of early distant metastasis (OR [95 % CI]= 4.50 [1.19–17.01], P=0.027, Table 3) compared to patients with the AA genotype.

The Kaplan–Meier survival curves revealed that both OS and PFS differed significantly between patients with and without the variant allele T of ATG5:rs1322178 in early-stage patients (P=0.009 for OS and P=0.012 for PFS, Figure 1A and 1B). Patients carrying variant genotype CT exhibited decreases in both OS and PFS (mean survival time [MST] 33.54 vs. 12.69 months for OS; MST 17.80 vs. 7.74 months for PFS, Figure 1A and 1B). Both OS
### Table 1: Patient characteristics

| Variables         | Total | Survival | Recurrence | p-value | p-value |
|-------------------|-------|----------|------------|---------|---------|
|                   |       |          | no recurrence |         |         |
|                   |       |          | recurrence |         |         |
|                   |       |          | p-value |         |         |
|                   | 201 (65.9) | 104 (34.1) | 78 (25.6) | 227 (74.4) | 0.057 | 0.281 |
| Age               |       |          |          |         |         |
| <40               | 70    | 38 (54.3) | 32 (45.7) | 23 (32.9) | 47 (67.1) | 0.057 | 0.281 |
| 40-60             | 144   | 98 (68.1) | 46 (31.9) | 34 (23.6) | 110 (76.4) | 0.281 | 0.281 |
| >60               | 91    | 65 (71.4) | 26 (28.6) | 21 (23.1) | 70 (76.9)  | 0.281 | 0.281 |
| Sex               |       |          |          |         |         |
| Male              | 278   | 189 (68.0) | 89 (32.0) | 64 (23.0) | 214 (77.0) | 0.014 | 0.001 |
| Female            | 27    | 12 (44.4) | 15 (55.6) | 14 (51.9) | 13 (48.1)  | 0.014 | 0.001 |
| Stage             |       |          |          |         |         |
| 0                 | 50    | 28 (56.0) | 22 (44.0) | 17 (34.0) | 33 (66.0)  | <0.001 | <0.001 |
| I                 | 112   | 62 (55.4) | 50 (44.6) | 40 (35.7) | 72 (64.3)  | <0.001 | <0.001 |
| II                | 143   | 111 (77.6) | 32 (22.4) | 21 (14.7) | 122 (85.3) | <0.001 | <0.001 |
| T-stage           |       |          |          |         |         |
| 0                 | 69    | 45 (65.2) | 24 (34.8) | 19 (27.5) | 50 (72.5)  | 0.006 | 0.004 |
| 1                 | 100   | 54 (54.0) | 46 (46.0) | 36 (36.0) | 64 (64.0)  | 0.006 | 0.004 |
| 2                 | 94    | 71 (75.5) | 23 (24.5) | 17 (18.1) | 77 (81.9)  | 0.006 | 0.004 |
| 3                 | 41    | 31 (75.6) | 10 (24.4) | 5 (12.2)  | 36 (87.8)  | 0.006 | 0.004 |
| 4                 | 1     | 0 (0)     | 1 (100)   | 1 (100)   | 0 (0)      | 0.006 | 0.004 |
| N-stage           |       |          |          |         |         |
| 0                 | 245   | 152 (62.0) | 93 (38.0) | 71 (29.0) | 174 (71.0) | 0.001 | 0.001 |
| 1                 | 59    | 49 (83.1) | 10 (16.9) | 6 (10.2)  | 53 (89.8)  | 0.001 | 0.001 |
| 2                 | 1     | 0 (0)     | 1 (100)   | 1 (100)   | 0 (0)      | 0.001 | 0.001 |
| Tumor location    |       |          |          |         |         |
| Upper             | 61    | 47 (77.0) | 14 (23.0) | 10 (16.4) | 51 (83.6)  | 0.117 | 0.107 |
| Middle            | 149   | 95 (63.8) | 54 (36.2) | 45 (30.2) | 104 (69.8) | 0.117 | 0.107 |
| Lower             | 95    | 59 (62.1) | 36 (37.9) | 23 (24.2) | 72 (75.8)  | 0.117 | 0.107 |
| Operation         |       |          |          |         |         |
| No                | 27    | 21 (77.8) | 6 (22.2)  | 2 (7.4)   | 25 (92.6)  | 0.173 | 0.021 |
| Yes               | 278   | 180 (64.7) | 98 (35.3) | 76 (27.3) | 202 (72.7) | 0.173 | 0.021 |
| CCRT              |       |          |          |         |         |
| No                | 100   | 57 (57.0) | 43 (43.0) | 34 (34.0) | 66 (66.0)  | 0.051 | 0.026 |
| Yes               | 197   | 139 (70.6) | 58 (29.4) | 41 (20.8) | 156 (79.2) | 0.051 | 0.026 |
| CT                | 1     | 1 (100.0) | 0 (0)     | 0 (0)     | 1 (100)    | 0.051 | 0.026 |
| RT                | 6     | 4 (66.7)  | 2 (33.3)  | 2 (33.3)  | 4 (66.7)   | 0.051 | 0.026 |
| CT+RT             | 1     | 0 (0)     | 1 (100.0) | 1 (100.0) | 0 (0)      | 0.051 | 0.026 |
and PFS were significantly shorter in patients with the variant allele of ATG5:rs3804329 (MST 31.71 vs. 17.47 months, P=0.029 for OS; MST 17.74 vs. 9.25 months, P= 0.037 for PFS, Figure 1C and 1D). Patients carrying the TT genotype of ATG5:rs671116 also had significantly shorter OS and PFS compared to patients with the CC or CT genotypes (MST 34.16 vs. 26.43 months, P=0.023 for OS; MST 19.87 vs. 12.26 months, P= 0.008 for PFS, Figure 1E and 1F).

To investigate whether the effect of the ATG5 SNPs on the prognosis of patients was mediated by modulating the expression of ATG5, we analyzed the expression levels of ATG5 in tumor and adjacent normal tissues from patients with early-stage ESCC by IHC. The expression levels were scored as 0+ (no detection), 1+ (low), 2+ (medium), and 3+ (high) (Figure 2A and Supplementary Table 2). Approximately 77.4 % of the ESCC tumor samples tested positive for ATG5. In adjacent normal tissues, ATG5 could be detected in about 48 % of the samples (Supplementary Table 2). The expression of ATG5 was significantly up-regulated in tumor tissues compared to its expression in adjacent normal tissue (Figure 2B, P<0.001, independent t-test). We further categorized the expression level of ATG5 into low (scoring 0+ or 1+) and high (scoring 2+ or 3+) expression groups. High expression of ATG5 in normal tissue was significantly correlated with increased risk of tumor progression compared to low expression (HR [95 % CI]=1.82 [0.99-3.35], P=0.033, Table 4). Patients whose tumor tissue had elevated expression of ATG5 exhibited a trend of higher risk of adverse clinical outcome compared to those with low expression, but without reaching statistical significance (HR [95 % CI]=1.43 [0.87-2.34], P=0.159 for OS, HR [95 % CI]=1.41 [0.88-2.27], P=0.150 for PFS, Table 4). Re-classifying the groups into low (scoring 0), middle (scoring 1 or 2), and high (scoring 3), high ATG5 expression in tumor tissue had a 2.23-fold higher hazard of death compared to low expression (HR [95 % CI]=2.23 [1.06-4.68], P=0.035, Table 4).

Survival curves were also constructed for OS and PFS by the expression level of ATG5. The median survival time of OS and PFS decreased significantly as ATG5 expression elevated (MST 40.1 vs. 10.1 months, log-rank P=0.043 for OS, Figure 2C; MST 20.1 vs. 4.6 months, log-rank P=0.037 for PFS, Figure 2D). However, neither OS nor PFS differed significantly in patients with different

| SNP       | Function | genotype | Training group | Replication group | Combined group |
|-----------|----------|----------|----------------|-------------------|---------------|
|           |          |          | Training group | Replication group | Combined group |
| ATG5: rs1322178 | 3’UTR | CC | 1 | 1 | 1 |
|           |          | CT | 3.60 (1.40-9.26) | 1.99 (1.02-3.90) | 2.01 (1.19-3.40) |
| ATG5: rs3804329 | intron | AA | 1 | 1 | 1 |
|           |          | AG | 3.06 (1.13-8.31) | 1.95 (0.96-3.94) | 1.88 (1.08-3.26) |
| ATG5: rs671116 | intron | CC+CT | 1 | 1 | 1 |
|           |          | TT | 1.95 (1.03-3.71) | 1.59 (1.06-2.41) | 1.73 (1.24-2.42) |

*Adjusted for age, gender, stage, surgicalstatus and CCRT.

| SNP       | Genotype | n | Early local recurrence | Early distant metastasis |
|-----------|----------|---|------------------------|--------------------------|
| ATG5: rs1322178 | CC | 158 | 0.051 | 217 | 1 |
|           | CT | 6 | 7.03 (0.99-49.99) | 12 | 4.50 (1.19-17.01) |
| ATG5: rs3804329 | AA | 159 | 0.131 | 217 | 1 |
|           | AG | 5 | 5.23 (0.61-44.72) | 12 | 4.50 (1.19-17.01) |
| ATG5: rs671116 | CC+CT | 126 | 0.129 | 174 | 1 |
|           | TT | 38 | 2.09 (0.81-5.43) | 55 | 1.50 (0.75-2.98) |

*Adjusted for age, gender, stage, surgicalstatus and CCRT.
Figure 1: Kaplan–Meier estimates of overall survival (OS, A, C, and E) or progression-free survival (PFS, B, D, and F) by the genotypes of ATG5:rs1322178 (A and B), ATG5:rs3804329 (C and D), and ATG5:rs671116 (E and F) in early-stage ESCC patients. MST: median survival time.
Figure 2: (A) ATG5 expression in ESCC tissue was analyzed by IHC and scored as 0, 1+, 2+, and 3+. (B) Expression level of ATG5 in adjacent non-cancerous (normal) and early-stage ESCC tissues by IHC. (C-D) Kaplan–Meier estimates of OS (C) and PFS (D) by the expression levels of ATG5 (low and high) of adjacent normal tissue from early-stage ESCC patients. MST: median survival time. Low, expression score 0 or 1+; High, expression score 2+ or 3+. 
expression levels of ATG5 in tumor tissue (data not shown). The expression level of ATG5 was further evaluated with the genetic polymorphisms of ATG5. Unexpectedly, the expression level of ATG5 in both normal and tumor tissue did not exhibit significant correlation with the genotypes of ATG5:rs1322178, ATG5:rs3804329, or ATG5:rs671116 (Supplementary Table 3).

**DISCUSSION**

What role the SNPs of the ATGs might play in ESCC prognosis has not been investigated. In the current study, we demonstrated that 3 SNPs in ATG5, rs1322178, rs3804329, and rs671116, significantly correlated with the prognosis of patients with early-stage ESCC (Table 2 and Figure 1). Both ATG5:rs1322178 and ATG5: rs3804329 can predict early distant metastasis of early-stage ESCC (Table 3). Meanwhile, ATG5 expression was significantly higher in ESCC tumor tissues than in adjacent normal tissue (Figure 2A-2B). Higher expression of ATG5 in both normal and tumor esophageal tissues had a trend to correlate with adverse clinical outcome of patients (Table 4 and Figure 2C-2D). However, the ATG5 SNPs did not have an associated effect on the expression of ATG5 (Supplementary Table 3).

ATG5: rs1322178 is located within the 3’ untranslated region (3’-UTR), whereas rs3804329 and rs671116 are in the intron region of the ATG5 gene. Studies of these 3 ATG5 SNPs are rare, and their function is hardly known. A previous study found that these 3 SNPs were all located in the same haplotype block of strong LD (linkage disequilibrium) and had no significant association with childhood asthma [23]. Therefore, these SNPs may display similar associations with early ESCC prognosis due to their close proximity.

Genetic variation in 3’UTR has been shown to often correlate with mRNA stability mediated by post-translation modification or microRNA interaction [24]. We observed alteration of the nucleotide from wildtype C to variant T of ATG5: rs1322175, supporting the notion that these nucleotides might be targeted by different mature microRNAs by sequence alignment. We thus hypothesized that rs1322178 may regulate ATG5 expression by modulating RNA stability in ESCC. Unexpectedly, there was no significant correlation in esophageal tissue between ATG5 protein expression and genotype of any these 3 SNPs despite the fact ATG5 expression exhibited prognostic correlation in ESCC. Since these ATG5 SNPs did not exert an obvious effect on the regulation of ATG5 expression, we infer that these 3 ATG5 SNPs did not directly affect the prognosis of ESCC by regulating ATG5 expression. There might be some other SNP within the exon region of ATG5 and located in the same haplotype block with these 3 SNPs that influences the function of ATG5 by a structural change rather than a change in expression of ATG5.

ATG5 is a cellular factor with an ambiguous role in malignant transformation. It has been known to promote Ras-induced cell transformation since autophagy was demonstrated to be involved in the oncogenic event [25–26]. In addition to the function of autophagy, ATG5 plays

| Variables | N | Overall survival | P-value | Progression-free survival | P-value |
|-----------|---|----------------|---------|---------------------------|---------|
|           |   | HR (95 % CI)    |         | HR (95 % CI)              |         |
| Normal_ATG5 expression |   |                 |         |                           |         |
| Low       | 69 | 1              |         | 1                         |         |
| High      | 8  | 2.01 (0.82-4.93) | 0.126   | 1.82 (0.99-3.35)         | 0.033   |
| Tumor_ATG5 expression |   |                 |         |                           |         |
| Low       | 69 | 1              |         | 1                         |         |
| High      | 46 | 1.43 (0.87-2.34) | 0.159   | 1.41 (0.88-2.27)         | 0.150   |
| Tumor_ATG5 expression |   |                 |         |                           |         |
| Low       | 26 | 1              |         | 1                         |         |
| Middle    | 69 | 1.20 (0.67-2.16) | 0.540   | 0.98 (0.57-1.69)         | 0.942   |
| High      | 20 | 2.23 (1.06-4.68) | **0.035** | 1.49 (0.73-3.06) | 0.277   |

*Adjusted for age, gender, and stage.
a role as a pro-apoptotic molecule after being cleaved at residue Thr 193 by calpain (a calcium dependent protease) indicating a molecular switch between autophagy and apoptosis [27–28]. ATG5 gene knockdown by small interference RNA (siRNA) has also been reported to enhance starvation-induced cell death [27, 29].

Both up-regulation and down-regulation of ATG5 have been demonstrated in various tumor tissues. Down-regulation of ATG5 has been found in colorectal cancer and early-stage cutaneous melanoma tissue compared to their normal counterpart parts [30–31]. Partial loss of ATG5 has also been observed in gastric and hepatocellular carcinomas [32]. Notably, elevated ATG5 was correlated with lymph vascular invasion even though ATG5 was decreased in colorectal cancer [30]. Increased expression of ATG5 has been observed in oral squamous cell carcinoma (OSCC) and prostate cancers [33–34]. ATG5 expression was found associated with tumor grade, tumor size, clinical stage and lymph node metastasis and clinical outcome in OSCC [33]. Meanwhile, increased expression of ATG5 was also significantly correlated with adverse prognosis and chemoresistance in gastric cancer [35].

The expression of ATG5 in ESCC has never been previously reported. Our current study demonstrates that ATG5 expression was markedly increased in early-stage ESCC tissue compared to adjacent non-tumorous tissue even though we did not observe correlation of ATG5 expression with pathologic characteristics (data not shown). We thus reasonably suggest that ATG5 might be involved in malignant transformation of esophageal squamous cells. Even though K-Ras mutation in esophageal cancer is rare [36], upstream factors such as EGFR (epidermal growth factor receptor) are frequently over-expressed in esophageal cancer [37]. Therefore, ATG5 might participate in autophagy and promote esophageal cell transformation mediated by EGFR-Ras signaling. Moreover, ATG5 might also be induced by hypoxia, a cellular stress known to induce autophagy [38], since HIF-1α (hypoxia-inducible factor-1 alpha) was found to express in ESCC cells [39–40].

A high level of ATG5 expression in normal tissue is, even more so than in cancer tissue, significantly associated with an adverse clinical outcome in early-stage ESCC. Over-expression of ATG5 in ESCC cells is possibly correlated with autophagic events which induce drug resistance and tumor growth to lead to poor prognosis. Adjacent normal cells expressing ATG5 might induce cell apoptosis to prevent tumorigenesis. However, the apoptosis of surrounding normal cells might also promote tumor growth based on the concept of cell competition [41], and lead to cancer progression. Blocking apoptosis of the adjacent normal cells has been hypothesized to be a novel pathway to prevent tumor growth [42].

In conclusion, our study demonstrates for the first time the prognostic relevance of the genetic polymorphisms and expression of ATG5 in patients with ESCC. These results reveal a novel functional mechanism involved in tumor progression of ESCC and provide a novel biomarker for predicting the clinical outcome of patients with ESCC. A limitation of our study is that no elucidation of the underlying mechanism regarding the prognostic function of ATG5 was provided, a goal well worth pursuing in further research.

MATERIALS AND METHODS

Study population

This retrospective study, investigating a total of 305 patients with early-stage (pathological stage (stage 0, I or II) ESCC collected in the surgical department of National Taiwan University Hospital (NTUH) from 2000 to 2013, was approved by the research ethics committee (201205090R1C). Patients histologically confirmed with early-stage (stage 0, I or II) primary ESCC, or those with locally advanced ESCC who were restaged as early-stage after CCRT, were included. Pregnant women, pediatric patients, and those unable to give informed consent were excluded. Cisplatin-based neoadjuvant concurrent chemoradiation therapy (CCRT) was administered to patients with locally advanced ESCC. Esophagectomy was performed on those patients with resectable disease status and acceptable surgical risk after CCRT. Information regarding demographics, tumor location, treatment protocols, recurrence status, and TNM stage according to the AJCC 7th edition [43], was obtained through medical chart review. Overall survival (OS) duration was defined as the interval between initial diagnosis of the disease (in patients who did not undergo surgery) or surgery for the disease and mortality of the patient. Progression-free survival (PFS) was defined as the interval between diagnosis of or surgery for the disease and detection of local recurrence, disease progression of the tumor, or death. Recurrence within 2 years after surgery (or initial diagnosis in patients without surgery) were considered as early recurrence, whether recurrence was local only or included distant metastasis.

DNA extraction

The buffy coat was isolated from a 5 ml whole blood sample collected from each patient before treatment and was stored in a -80 °C freezer. Genomic DNA was extracted from the buffy coat with the QIAamp DNA Mini Kit (Qiagen, Hamburg Germany) following the manufacturer’s instructions.

Genotyping

Based on the results of previous studies, 20 candidate SNPs were selected, which consisted of 1 SNP at autophagy related 3 (ATG3), 6 SNPs at autophagy related 5 (ATG5), 9 SNPs at autophagy related 7 (ATG7), 3 SNPs at autophagy related 7 (ATG7), 3 SNPs at autophagy related 3 (ATG3), 6 SNPs at autophagy related 5 (ATG5), 9 SNPs at autophagy related 7 (ATG7), 3 SNPs
at autophagy related 16-like 1 (ATG16L1), and 1 SNP at beclin 1 (BECN1) (Supplementary Table 1) [23, 44–47]. The SNP genotyping was performed with the Sequenom MassARRAY platform and iPLEX gold chemistry following manufacturer's instructions (Sequenom, San Diego, CA, USA). Briefly, specific PCR primer and extension primer sequences for multiplex PCR reaction were designed using the Assay Designer software package (v.4.0). After multiplex PCR, the residual deoxynucleotides were deactivated by incubation with 0.3 U of shrimp alkaline phosphatase followed by single base extension reaction. Seven μl of purified reaction mixture was loaded onto a matrix pad of a SpectroCHIP (Sequenom) and analyzed by MassARRAY Analyzer 4. Genotypes were called by cluster analysis using MassARRAY TYPER 4.0 software and call rates higher than 80 % were accepted. Artifact data were removed manually. Data integrity and accuracy were confirmed by repeated measures.

Immunohistochemistry (IHC)

Formalin-fixed paraffin-embedded (FFPE) blocks of ESCC patient tissue collected during surgical intervention were obtained from the department of pathology in National Taiwan University Hospital. Cancer and normal esophagus FFPE sections were dewaxed and rehydrated. Details of the IHC protocol were described in a previous study [48]. The primary antibody used was a rabbit polyclonal antibody against ATG5 (1:200, NB110-53818, Novus Biologicals).

Statistical analysis

Patient characteristics and ATG5 expression among the subgroups with different genotypes of ATG SNPs were compared using a Pearson’s χ^2 test or Fisher’s exact test. The hazard ratios (HRs) of death and disease progression were obtained from multivariate Cox regression analysis adjusted for potential significant covariates. The odds ratios (ORs) obtained by logistic regression were used to describe correlations between genotypes and early recurrence or protein expression of ATG5.

The correlations between genotypes or ATG5 expression and OS or PFS were obtained using the Kaplan–Meier method and compared using the log-rank test. The ATG5 protein expression levels of esophageal tissues among normal and cancer tissue were analyzed by box-plot and independent t-test. All statistical analyses were conducted with SPSS 17.0 for Windows (SPSS Institute, Chicago, IL, USA). A p-value ≤ 0.05 was considered statistically significant.

Author contributions

Conception and design: Pei-Wen Yang, Min-Shu Hsieh, and Jang-Ming Lee; Provision of study patients: Jang-Ming Lee and Pei-Ming Huang; Performed the experiments: Pei-Wen Yang, Min-Shu Hsieh, and Ya-Han Chang; Data analysis: Pei-Wen Yang and Min-Shu Hsieh; Manuscript writing: Pei-Wen Yang, Min-Shu Hsieh, and Jang-Ming Lee.

ACKNOWLEDGMENTS

We thank the staff of the Second Core Lab, Department of Medical Research, NTUH for technical support.

CONFLICTS OF INTEREST

There is no conflicts of interest for any author regarding the publication of this manuscript.

FUNDING

This study was supported by the National Taiwan University Hospital (NTUH.102-S2140 and NTUH.105-S3005), Ministry of Science and Technology (MOST 104-2314-B-002-182-MY3, MOST 105-2320-B-002-049, and MOST-106-2320-B002-029-MY3), and Taiwan Health Foundation of the Republic of China.

REFERENCES

1. Enzinger PC, Mayer RJ. Esophageal cancer. N Engl J Med. 2003; 349:2241–52.
2. Jemal A, Siegel R, Xu J, Ward E. Cancer statistics, 2010. CA Cancer J Clin. 2010; 60:277–300.
3. Lagergren J, Lagergren P. Oesophageal cancer. BMJ. 2010; 341:c6280.
4. Allum WH, Stenning SP, Bancewicz J, Clark PI, Langley RE. Long-term results of a randomized trial of surgery with or without preoperative chemotherapy in esophageal cancer. J Clin Oncol. 2009; 27:5062–67.
5. Sugimachi K, Inokuchi K, Kuwano H, Kai H, Okamura T, Okudaira Y. Patterns of recurrence after curative resection for carcinoma of the thoracic part of the esophagus. Surg Gynecol Obstet. 1983; 157:537–40.
6. Nakagawa S, Kanda T, Kosugi I, Ohashi M, Suzuki T, Hatakeyama K. Recurrence pattern of squamous cell carcinoma of the thoracic esophagus after extended radical esophagectomy with three-field lymphadenectomy. J Am Coll Surg. 2004; 198:205–11.
7. Su XD, Zhang DK, Zhang X, Lin P, Long H, Rong TH. Prognostic factors in patients with recurrence after complete resection of esophageal squamous cell carcinoma. J Thorac Dis. 2014; 6:949–57.
8. Yorimitsu T, Klionsky DJ. Autophagy: molecular machinery for self-eating. Cell Death Differ. 2005; 12:1542–52.
9. Lozy F, Karantza V. Autophagy and cancer cell metabolism. Semin Cell Dev Biol. 2012; 23:395–401.
10. Levy JM, Thorburn A. Targeting autophagy during cancer therapy to improve clinical outcomes. Pharmacol Ther. 2011; 131:130–41.

11. Martinez-Outschoorn UE, Balliet RM, Rivadeneira DB, Chiavarina B, Pavlides S, Wang C, Whitaker-Menezes D, Daumer KM, Lin Z, Witkiewicz AK, Flomenberg N, Howell A, Pestell RG, et al. Oxidative stress in cancer associated fibroblasts drives tumor-stroma co-evolution: A new paradigm for understanding tumor metabolism, the field effect and genomic instability in cancer cells. Cell Cycle. 2010; 9:3256–76.

12. Mizushima N, Levine B, Cuervo AM, Klionsky DJ. Autophagy fights disease through cellular self-digestion. Nature. 2008; 451:1069–75.

13. Martinez-Outschoorn UE, Whitaker-Menezes D, Pavlides S, Chiavarina B, Bonucelli G, Casey T, Tsirigos A, Migneco G, Witkiewicz A, Balliet R, Mercier I, Wang C, Flomenberg N, et al. The autophagic tumor stroma model of cancer or “battery-operated tumor growth”: A simple solution to the autophagy paradox. Cell Cycle. 2010; 9:4297–306.

14. Degenhardt K, Mathew R, Beaudoin B, Bray K, Anderson D, Chen G, Mukherjee C, Shi Y, Gélinas C, Fan Y, Nelson DA, Jin S, White E. Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis. Cancer Cell. 2006; 10:51–64.

15. Hönscheid P, Datta K, Muders MH. Autophagy: detection, regulation and its role in cancer and therapy response. Int J Radiat Biol. 2014; 90:628–35.

16. Chittaranjan S, Bortnik S, Dragowska WH, Xu J, Abeyesundara N, Leung A, Go NE, DeVorkin L, Weppler SA, Gelmon K, Yapp DT, Bally MB, Gorski SM. Autophagy inhibition augments the anticancer effects of epirubicin in anthracycline-sensitive and -resistant triple-negative breast cancer. Clin Cancer Res. 2014; 20:3159–73.

17. Zou Z, Yuan Z, Zhang Q, Long Z, Chen J, Tang Z, Zhu Y, Chen S, Xu J, Yan M, Wang J, Liu Q. Aurora kinase A inhibition-induced autophagy triggers drug resistance in breast cancer cells. Autophagy. 2012; 8:1798–810.

18. O’Donovan TR, O’Sullivan GC, McKenna SL. Induction of autophagy by drug-resistant esophageal cancer cells promotes their survival and recovery following treatment with chemotherapeutics. Autophagy. 2011; 7:509–24.

19. Liu DL, Zhang JG, Wang JJ. Autophagy: a potential mechanism for resistance of esophageal squamous cell carcinoma to therapy. J Formos Med Assoc. 2010; 109:775–76.

20. Liu D, Yang Y, Liu Q, Wang J. Inhibition of autophagy by 3-MA potentiates cisplatin-induced apoptosis in esophageal squamous cell carcinoma cells. Med Oncol. 2011; 28:105–11.

21. Chen YS, Song HX, Lu Y, Li X, Chen T, Zhang Y, Xue JX, Liu H, Kan B, Yang G, Fu T. Autophagy inhibition contributes to radiation sensitization of esophageal squamous carcinoma cells. Dis Esophagus. 2011; 24:437–43.

22. O’Donovan TR, Rajendran S, O’Reilly S, O’Sullivan GC, McKenna SL. Lithium Modulates Autophagy in Esophageal and Colorectal Cancer Cells and Enhances the Efficacy of Therapeutic Agents In Vitro and In Vivo. PLoS One. 2015; 10:e0134676.

23. Martin LJ, Gupta J, Jyothula SS, Butsch Kovacic M, Biagini Myers JM, Patterson TL, Ericksen MB, He H, Gibson AM, Baye TM, Amirissetty S, Tsonas AM, Sha Y, et al. Functional variant in the autophagy-related 5 gene promoter is associated with childhood asthma. PLoS One. 2012; 7:e33454.

24. Lai EC. Micro RNAs are complementary to 3′ UTR sequence motifs that mediate negative post-transcriptional regulation. Nat Genet. 2002; 30:563–64.

25. Kim MJ, Woo SJ, Yoon CH, Lee JS, An S, Choi YH, Hwang SG, Yoon G, Lee SJ. Involvement of autophagy in oncogenic K-Ras-induced malignant cell transformation. J Biol Chem. 2011; 286:12924–32.

26. Lock R, Roy S, Kenific CM, Su JS, Salas E, Ronen SM, Debnath J. Autophagy facilitates glycolysis during Ras-mediated oncogenic transformation. Mol Biol Cell. 2011; 22:165–78.

27. Codogno P, Meijer AJ. Atg5: more than an autophagy factor. Nat Cell Biol. 2006; 8:1045–47.

28. Yousefi S, Perozzo R, Schmid I, Ziemiacki A, Schaffner T, Scapozza L, Brunner T, Simon HU. Calpain-mediated cleavage of Atg5 switches autophagy to apoptosis. Nat Cell Biol. 2006; 8:1124–32.

29. Boya P, González-Polo RA, Casares N, Perfettini JL, Dessen P, Larochette N, Métivier D, Meley D, Souquere S, Yoshimori T, Pierron G, Codogno P, Kroemer G. Inhibition of macroautophagy triggers apoptosis. Mol Cell Biol. 2005; 25:1025–40.

30. Cho DH, Jo YK, Kim SC, Park IJ, Kim JC. Down-regulated expression of ATG5 in colorectal cancer. Anticancer Res. 2012; 32:4091–96.

31. Liu H, He Z, von Rütte T, Yousefi S, Hunger RE, Simon HU. Down-regulation of autophagy-related protein 5 (ATG5) contributes to the pathogenesis of early-stage cutaneous melanoma. Sci Transl Med. 2013; 5:202ra123.

32. An CH, Kim MS, Yoo NJ, Park SW, Lee SH. Mutational and expression analyses of ATG5, an autophagy-related gene, in gastrointestinal cancers. Pathol Res Pract. 2011; 207:433–37.

33. Tang JY, Fang YY, Hsi E, Huang YC, Hsu NC, Yang WC, Chang HW, Chai CY, Chu PY. Immunopositivity of Beclin-1 and ATG5 as indicators of survival and disease recurrence in oral squamous cell carcinoma. Anticancer Res. 2013; 33:5611–16.

34. Kim MS, Song SY, Lee JY, Yoo NJ, Lee SH. Expressional and mutational analyses of ATG5 gene in prostate cancers. APMIS. 2011; 119:802–07.

35. Ge J, Chen Z, Huang J, Chen J, Yuan W, Deng Z, Chen Z. Upregulation of autophagy-related gene-5 (ATG-5) is
associated with chemoresistance in human gastric cancer. PLoS One. 2014; 9:e110293.
36. Shigaki H, Baba Y, Watanabe M, Miyake K, Murata A, Iwagami S, Ishimoto T, Iwatsuki M, Yoshida N, Baba H. KRAS and BRAF mutations in 203 esophageal squamous cell carcinomas: pyrosequencing technology and literature review. Ann Surg Oncol. 2013; 20:S485–91.
37. Ekman S, Bergqvist M, Heldin CH, Lennartsson J. Activation of growth factor receptors in esophageal cancer—implications for therapy. Oncologist. 2007; 12:1165–77.
38. Mazure NM, Pouysségur J. Hypoxia-induced autophagy: cell death or cell survival? Curr Opin Cell Biol. 2010; 22:177–80.
39. Matsuyama T, Nakanishi K, Hayashi T, Yoshizumi Y, Aiko S, Sugiura Y, Tanimoto T, Uenoyma M, Ozeki Y, Maehara T. Expression of hypoxia-inducible factor-1alpha in esophageal squamous cell carcinoma. Cancer Sci. 2005; 96:176–82.
40. Yang PW, Hsu IJ, Chang CW, Wang YC, Hsieh CY, Shih KH, Wong LF, Shih NY, Hsieh MS, Hou MT, Lee JM. Visible-absorption spectroscopy as a biomarker to predict treatment response and prognosis of surgically resected esophageal cancer. Sci Rep. 2016; 6:33414.
41. Hogan C. Impact of interactions between normal and transformed epithelial cells and the relevance to cancer. Cell Mol Life Sci. 2012; 69:203–13.
42. Moreno E. Is cell competition relevant to cancer? Nat Rev Cancer. 2008; 8:141–47.
43. Edge SB, Byrd DR, Compton CC, Fritz AG, Greene FL, Trotti A, editors. AJCC Cancer Staging Manual. 7th ed. New York, USA: Springer; 2009. pp. 143–59.
44. Huijbers A, Plantinga TS, Joosten LA, Aben KK, Gudmundsson J, den Heijer M, Kiemeney LA, Netea MG, Hermus AR, Netea-Maier RT. The effect of the ATG16L1 Thr300Ala polymorphism on susceptibility and outcome of patients with epithelial cell-derived thyroid carcinoma. Endocr Relat Cancer. 2012; 19:15–18.
45. Lauriola M, Ugolini G, Rivetti S, Nani S, Rosati G, Zanotti S, Montroni I, Manaresi A, Zattoni D, Belluzi A, Castellani L, D’Uva G, Mattei G, et al. IL23R, NOD2/CARD15, ATG16L1 and PHOX2B polymorphisms in a group of patients with Crohn’s disease and correlation with subphenotypes. Int J Mol Med. 2011; 27:469–77.
46. Liu LC, Su CH, Wang HC, Tsai CW, Chang WS, Ho CY, Wu CI, Li FJ, Lin CH, Lane HY, Bau DT. Significant association of caveolin-1 (CAV1) genotypes with breast cancer in Taiwan. Anticancer Res. 2011; 31:3511–15.
47. Metzger S, Saukko M, Van Che H, Tong L, Puder Y, Riess O, Nguyen HP. Age at onset in Huntington’s disease is modified by the autophagy pathway: implication of the V471A polymorphism in Atg7. Hum Genet. 2010; 128:453–59.
48. Hsieh MS, Yang PW, Wong LF, Lee JM. The AXL receptor tyrosine kinase is associated with adverse prognosis and distant metastasis in esophageal squamous cell carcinoma. Oncotarget. 2016; 7:36956–70. https://doi.org/10.18632/oncotarget.9231.