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ORIGINAL ARTICLE

Implications of the autophagy core gene variations on brain metastasis risk in non-small cell lung cancer treated with EGFR-TKI*

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Abstract

Objective The brain is the main site of failure in cancer patients with epidermal growth factor receptor (EGFR) mutations undergoing treatment. However, identifying patients who may develop brain metastases (BM) is difficult. Autophagy is critical for cancer initiation and progression. We hypothesized that genetic variants in autophagy core genes might contribute to BM risk of non-small cell lung cancer (NSCLC) following treatment with EGFR tyrosine kinase inhibitor (EGFR-TKIs).

Methods We systematically examined 16 potentially functional genetic polymorphisms in seven autophagy core genes among 105 TKI-treated NSCLC patients. Kaplan-Meier curves were plotted to assess the cumulative BM probability. Univariate and multivariate Cox proportional hazard regression analyses were utilized to calculate hazard ratios (HRs) and 95% confidence intervals (CIs). We evaluated the potential associations of these genes with subsequent BM development.

Results We found that ATG16L1: rs2241880, ATG10: rs10036653, rs3734114, and ATG3: rs7652377 are significantly associated with NSCLC treated with EGFR-TKIs (all $P < 0.05$). BM developed more often in patients with ATG3 rs7652377 CC genotype (33%), ATG10 rs10036653 AA genotype (43%), ATG10: rs3734114 CT/CC genotype (46%), and ATG16L1 rs2241880 AA genotype (37%) compared to patients with AA genotypes at rs7652377 (12%), AT/TT genotypes at rs10036653 (16%), the TT genotype at rs3734114 (13%), or AG/GG genotypes at rs2241880 (17%).

Conclusion These associations may be critical for understanding the role of autophagy in BM risk. Future prospective studies are needed to determine if prophylactic cranial irradiation (PCI) could offer a survival benefit in this group of patients.

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Key words: autophagy; non-small cell lung cancer (NSCLC); brain metastasis (BM); single nucleotide polymorphism; predictive biomarker

Brain metastases (BM) are a common problem in patients with lung cancer, and they are associated with poor prognoses. The reported incidence of BM in non-small cell lung cancer (NSCLC) ranges from 17% to 54% [1]. Mutations in the epidermal growth factor receptor (EGFR) tyrosine kinase domain occur in approximately 15% of advanced non-squamous NSCLC patients [2]. Studies have shown that EGFR tyrosine kinase inhibitor (EGFR-TKI) is an effective treatment option for lung cancer patients with EGFR mutations [3]. However, advances in the development of targeted therapy against (NSCLC) mean that patients are more likely to develop BM due to

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prolonged survival. Patients whose tumors harbored an EGFR mutation had nearly a two-fold increase in the risk of BM [4]. The incidence of central nervous system (CNS) disease in patients with EGFR mutant (EGFRm) is high and is associated with increased use of health resources. Similarly, there is financial toxicity associated with CNS disease. Prophylactic cranial irradiation (PCI) for patients with locally advanced NSCLC decreases the incidence of BM [5], but it is not routinely used because it causes toxicity without improving survival. Differences in tumor biology may impact the pattern of metastases to the brain, putting some patients at greater risk than others [6]. However, PCI might be advantageous for an identifiable subgroup of patients with a high risk of developing BM.

Prior studies have identified the clinical features (including increased size of primary tumor, higher nodal stage and histology) that are associated with increased incidence of BM in patients with NSCLC [7–9]. However, not all studies have shown a significant correlation. In one study, the expression levels of three genes, *CDH2* (N-cadherin), *KIFC1*, and *FALZ*, were found to be highly predictive of BM in early and advanced lung cancer [10]. However, none of these studies specifically addressed NSCLC treated with EGFR-TKI. Considering autophagy's essential role in cancer development, we hypothesized that genetic variants in autophagy core genes might contribute to BM risk of NSCC treated with EGFR-TKIs.

Autophagy plays important roles in various organismal processes like development and aging. Abnormal autophagy leads to pathologies like cancer [11–14]. During cancer development, autophagy facilitates tumorigenesis in most contexts [15–16]. The autophagosome is a spherical organelle with double layer membranes that is formed during autophagy. Establishment of the autophagosome is controlled by several autophagy core genes [17], which might be involved in cancer initiation and progression [18]. Single-nucleotide polymorphisms (SNPs) have been found to be associated with risk and/or prognosis in numerous cancer types, including breast, thyroid, prostate, colorectal, and gastric cancer [19–20]. However, to our knowledge, there are no studies examining the relationship between autophagy-associated gene (ATG) SNPs in NSCLC patients undergoing EGFR-TKI therapy. In this study, we hypothesized that genetic variants of autophagy core genes may contribute to differential BM risk of NSCLC patients treated with EGFR-TKI. To test this hypothesis, we systematically examined the clinical implications of 16 potentially functional polymorphisms in seven autophagy core genes (*ATG3*, *ATG5*, *ATG7*, *ATG10*, *ATG12*, *ATG16L1*, and *LC3*) in NSCLC patients who received EGFR-TKI therapy.

Materials and methods

Study population and data collection

A total of 105 patients with advanced lung adenocarcinoma who had been treated with EGFR-TKI were included in this study (Table 1). Patients were recruited between July 2008 and July 2012, at Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). Eligible patients had at least one measurable lesion with a minimum size of at least one diameter of ≥10 mm for liver, lung, brain, or lymph node metastases. No restrictions on age, gender, or disease stage were applied, but all patients were required to have blood samples available for analysis. The Karnofsky performance status (KPS) of all patients was at least 70, and all of them had a life expectancy of at least 6 months. CT or MRI scans had been obtained from each patient before treatment as part of the disease staging process. All patients were asked to return to the hospital for examination (which included CT scans of the chest and abdomen) every two months. Repeat brain CT or MRI scans were obtained only in the event of clinical indications like neurological symptoms per the standard of care. This study was approved by the Review Boards of Tongji Hospital, Tongji Medical College. Written informed consent was obtained from each patient for the use of his/her DNA and clinical information. All procedures were performed in accordance with the approved guidelines.

Polymorphism selection of autophagy core genes

Single nucleotide polymorphisms (SNPs) of autophagy core genes were selected as previously described [21]. Briefly, common SNPs (MAF ≥ 0.05 in Chinese Han population) in six core autophagy genes (*ATG3*, *ATG5*, *ATG7*, *ATG10*, *ATG12*, and *LC3*) were screened in the 10-kb upstream region of each gene based on the HapMap database. A total of 16 potentially functional SNPs were finally selected according to linkage disequilibrium analyses with an r^2 threshold of 0.80 and predictions from SNP info Web Server (http://snpinfo.niehs.nih.gov/). The *ATG3* rs2705507 polymorphism was excluded since it cannot be analyzed by the MassArray system (Sequenom Inc., USA). Other SNPs previously reported as being associated with survival or metastasis in general were also included, including *ATG16L1*: rs2241880 (Table 1).

Genotyping

The SNPs were genotyped as previously described [22]. Briefly, the SNPs were genotyped using MALDI-TOF mass spectrophotometry to detect allele-specific primer extension products with the Mass ARRAY platform (Sequenom, Inc., USA). Assay data were analyzed using

Table 1 Patient- and disease-related characteristics and their association with brain metastasis

Characteristic	No. of patients (%)	Univariate analysis			Multivariate analysis		
		HR	95% CI	*P* Value	HR	(95% CI)	*P* value
Sex							
Female	41 (39)	1.000			1.000		
Male	64 (61)	1.426	0.615–3.305	0.408	1.016	0.346–2.981	0.977
Age (years)							
≥ 60	39 (37)	1.000			1.000		
< 60	66 (63)	1.217	0.525–2.822	0.646	1.047	0.421–2.608	0.921
Median (range)	57 (26–82)						
Disease stage at diagnosis							
I–III	48 (46)	1.000			1.000		
IV	57 (54)	3.008	1.201–7.538	0.019	2.756	1.051–7.233	0.039
Tumor histology							
Squamous cell	13 (12)	1.000			1.000		
Adenocarcinoma	85 (81)	0.974	0.290–3.266	0.966	0.787	0.217–2.863	0.717
NSCLC, NOS	7 (7)	0.589	0.061–5.661	0.646	0.461	0.046–4.645	0.511
KPS Score							
> 80	12 (11)	1.000			1.000		
80	83 (79)	2.584	0.382–21.326	0.307	1.379	0.168–11.309	0.765
< 80	10 (10)	7.955	0.928–68.205	0.059	3.960	0.392–40.023	0.244
Tobacco Smoking Status							
Current	36 (34)	1.000			1.000		
Former	11 (11)	0.831	0.237–2.916	0.772	1.191	0.309–4.587	0.800
Never	58 (55)	0.381	0.162–0.891	0.026	0.371	0.129–1.064	0.065

Multivariate analyses were adjusted for all of the factors listed in this table

the Sequenom TYPER software (version 4.0). The individual call rate threshold was at least 95%. To assess reproducibility, 5% of the DNA samples were blindly and randomly analyzed in duplicates, and the results revealed a reproducibility of 99%.

Statistical analysis

Patients were grouped according to their genotype. Statistical analyses were performed using the SPSS software (version 16.0). Univariate and multivariate Cox proportional hazard regression analyses were utilized to calculate hazard ratios (HRs) and 95% confidence intervals (CIs). Gender, age, disease stage, tumor histology, KPS, and smoking status were used as adjustment factors for the multivariate analyses. Kaplan-Meier curves were plotted to assess the cumulative BM probability. Differences between the BM risks were examined using the log-rank test. All *P* values were two-sided, and *P* values of < 0.05 were considered statistically significant.

Results

Patient characteristics and clinical outcomes

Associations according to univariate and multivariate analyses between patient and tumor-related characteristics with BM are shown in Table 1. The median age of all patients was 57 years (range, 26–82 years). Of all the cases, 54% were diagnosed at stage IV, and the others were recurrence cases from stage I, II, and III. 45% had smoked tobacco (68.7% of men and 7.3% of women). Overall, the median time from NSCLC diagnosis to detection of BM was 11 months, and median follow-up time was 25 months.

Clinical characteristics and BM risk

The association between six clinical characteristics and brain metastasis risk were studied (Table 1). Fig. 1 illustrated the cumulative BM rates for all patients according to clinical characteristics. We found that disease stage at diagnosis and KPS were associated with the risk of brain metastasis. Patients with stage IV disease were more likely to develop BM. The patients with stage IV disease also had an increased cumulative BM hazard of 33% compared to 13% in patients with state I, II, or III (log-rank $P < 0.01$, Fig. 1a). Patients with KPS > 80 were associated with an decreased BM risk (log-rank $P = 0.039$, Fig. 1b). In multivariate analysis, only stage is still associated with the risk of brain metastasis (HR = 2.756, 95% CI = 1.051–7.233, $P = 0.039$). All of these variables were adjusted in the subsequent multivariate analyses.

Individual SNPs and BM risk

A total of 16 SNPs from six genes in the autophagy pathway were analyzed (Table 2). Four SNPs from three

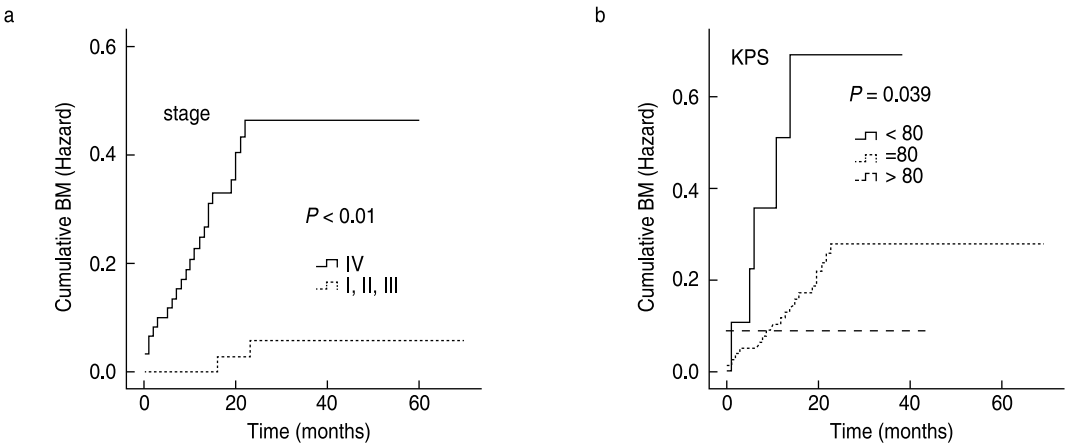

Fig. 1 Kaplan-Meier estimates of the cumulative probability of BM among patients with NSCLC according to the clinical characteristics. (a) stage at diagnosis; (b) KPS. The patients with stage IV disease had an increased cumulative BM hazard. Patients with KPS > 80 were associated with decreased BM risk.

genes showed significant associations with BM risk: two SNPs with $P < 0.05$ and two SNPs with $P < 0.01$ (Table 3). Fig. 2 illustrates the cumulative BM rates for all patients according to their genotype. The most significant association between BM risk and SNPs in this study was found with ATG10: rs3734114. The CT/CC genotype of ATG10: rs3734114 was associated with increased BM risk among all the patients in this group. The patients with the CT/CC genotype of ATG10: rs3734114 had an increased cumulative BM hazard of 46% compared to 13% in patients with the TT genotype (log-rank $P = 2.448E\text{-}4$; Fig. 2c). Multivariate Cox proportional hazard analyses showed that the CT/CC genotype of ATG10: rs3734114 was associated with a significantly higher BM risk [hazard ratio (HR) 4.593, 95% confidence interval (CI) 1.956–10.783, $P = 4.642E\text{-}4$] after adjusting for gender, patient age, disease stage, tumor histology, Karnofsky performance status (KPS), and smoking status (Table 3).

We also found three other SNPs, ATG3: rs7652377, ATG10: rs10036653 and ATG16L1: rs2241880, that were associated with BM risk. BM rates were higher for patients with the CC genotype of ATG3: rs7652377 (log-rank $P = 0.013$; Fig. 2a), the AA genotype of ATG10: rs10036653 (log-rank $P = 0.003$; Fig. 2b), and the AA genotype of ATG16L1: rs2241880 (log-rank $P = 0.018$; Fig. 2d). Multivariate Cox proportional hazard analyses showed the same results. The ATG3 rs7652377 A allele, ATG10 rs10036653 T allele, and ATG16L1 rs2241880 G allele proved to be protective alleles, which were significantly associated with lower BM risk. (HR = 0.267, 95% CI = 0.095–0.750, $P = 0.012$ for ATG3 rs7652377; HR = 0.254, 95% CI = 0.112–0.574, $P = 0.001$ for ATG10 rs10036653; HR = 0.417, 95% CI = 0.181–0.963, $P = 0.040$ for ATG16L1 rs2241880). The remaining 12 selected SNPs showed no associations between their genotype and BM risk (Table 4).

Table 2 Genes and single nucleotide polymorphisms selected for analysis

Gene (Number of SNPs)	SNP	Allelic change
ATG3 (1)	rs7652377	C > A
ATG5 (3)	rs510432	G > A
	rs688810	T > C
	rs3804338	C > T
ATG7 (3)	rs8154	T > C
	rs1375206	C > G
	rs1470612	G > A
ATG10 (5)	rs1864183	A> G
	rs1864182	T > G
	rs10514231	T > C
	rs10036653	A > T
	rs3734114	T > C
ATG12 (3)	rs26532	A > C
	rs26534	G > A
	rs26538	C > T
ATG16L1 (1)	rs 2241880	T > C

Discussion

EGFR-TKIs have proved to be promising in NSCLC treatment, especially in lung adenocarcinoma patients harboring *EGFR* mutations. However, these patients seem more inclined to develop BM. Here, we determined whether genetic variations in the autophagy core genes are associated with brain metastasis risk. Multiple genetic variations in autophagy core genes, including *ATG3*: *rs7652377*, *ATG10*: rs10036653, *ATG10*: rs3734114 and *ATG16L1*: rs2241880, were found to be significantly associated with brain metastasis. To the best of our

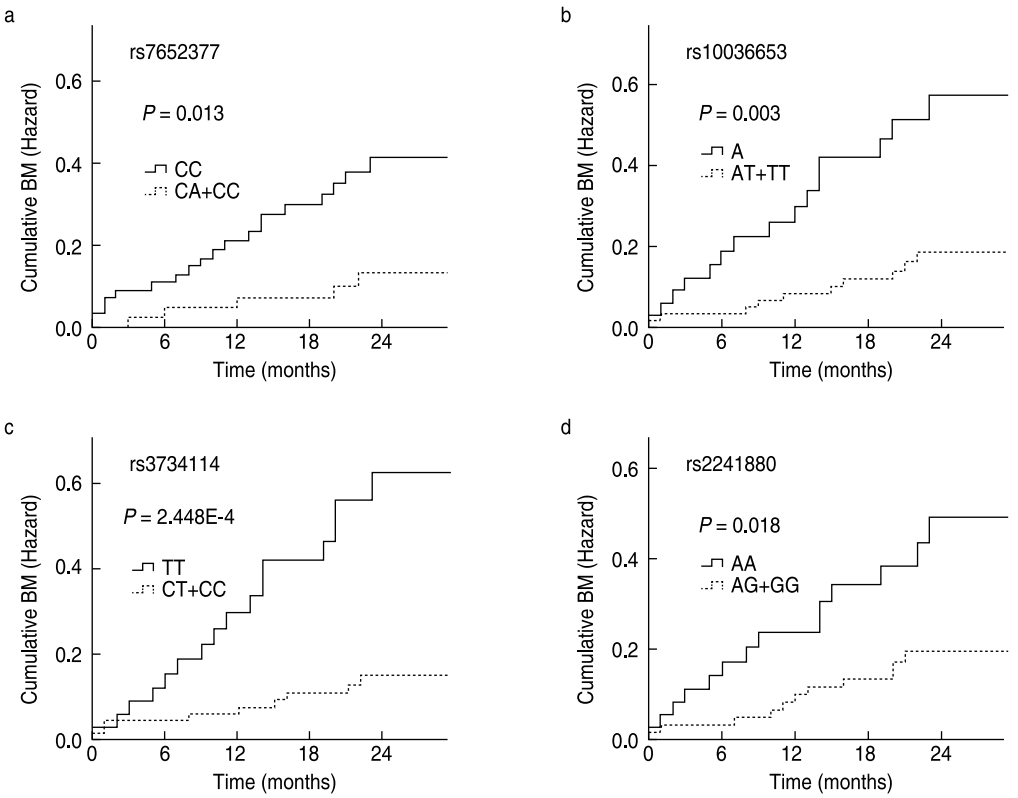

Fig. 2 Kaplan-Meier estimates of the cumulative probability of BM among patients with NSCLC according to the following genotypes: (a) ATG3: rs7652377; (b) ATG10: rs10036653; (c) ATG10: rs3734114; (d) ATG16L1: rs2241880. The CC genotype at rs7652377, the AA genotype at rs10036653, the CT/CC genotype at rs3734114, and the AA genotype at rs2241880 were associated with higher cumulative probability of brain metastasis than the other genotypes

Table 3 Associations between genotypes and BM

Characteristic	No. of patients	No. of events (%)	Univariate analysis			Multivariate analysis		
			HR	95% CI	*P* value	HR	95% CI	*P* value
ATG3: rs7652377								
CC	58	19 (33)	1.000			1.000		
AC + AA	43	5 (12)	0.309	0.115-0.828	0.019	0.267	0.095–0.750	0.012
ATG10: rs10036653								
AA	35	15 (43)	1.000			1.000		
AT + TT	63	10 (16)	0.312	0.140-0.694	0.004	0.254	0.112–0.574	0.001
ATG10: rs3734114								
TT	68	9 (13)	1.000			1.000		
CT + CC	35	16 (46)	4.094	1.807-9.277	0.001	4.593	1.956–10.783	4.642E-4
ATG16L1: rs2241880								
AA	38	14 (37)	1.000			1.000		
AG + GG	64	11 (17)	0.399	0.181-0.880	0.023	0.417	0.181–0.963	0.040

Multivariate analyses in this table were adjusted for sex, patient age, tumor histology, disease stage, Karnofsky Performance Status, and smoking status. HR, hazard ratio; CI, confidence interval; BM, brain metastases

knowledge, this is the first study to show this association in NSCLC patients who received EGFR-TKI.

Here, we also found one SNP (rs2241880) in ATG16L and two SNPs (rs10036653 and rs3734114) in ATG10 that were associated with BM risk in NSCLC patients treated with EGFR-TKI. Variations in genes in the autophagy pathway are detected in several human cancers. Autophagy in cancer is context-dependent, acting as both a tumor suppressor and tumor promotor, depending on the tumor development stage [23]. The SNPs

Table 4 Associations between genotypes and BM (the other 12 selected SNPs)

Characteristic	No. of patients	No. of events (%)	Univariate analysis				Multivariate analysis		
			HR	95% CI	*P* value	[a]*P* value	HR	95% CI	*P* value
ATG5: rs510432									
GG	30	3 (10)	1.000				1.000		
AG + AA	73	22 (30)	3.323	0.994–11.104	0.051	0.061	3.581	0.949–13.515	0.060
ATG5: rs688810									
TT	37	10 (27)	1.000				1.000		
CT + CC	65	15 (23)	0.844	0.379–1.880	0.678		1.506	0.621–3.656	0.365
ATG165: rs3804338									
CC	77	19 (25)	1.000				1.000		
CT + TT	27	6 (22)	0.882	0.352–2.210	0.790		0.655	0.255–1.683	0.380
ATG7: rs8154									
TT	79	17 (22)	1.000				1.000		
CT + CC	21	8 (38)	2.050	0.884–4.754	0.094		2.185	0.905–5.273	0.082
ATG7: rs1375206									
CC	45	11 (24)	1.000				1.000		
CG + GG	55	12 (22)	0.896	0.395–2.030	0.792		0.911	0.381–2.180	0.835
ATG7: rs1470612									
GG	56	13 (23)	1.000				1.000		
AG + AA	48	12 (25)	1.105	0.504–2.422	0.803		1.171	0.504–2.724	0.713
ATG10: rs1864183									
AA	81	21(26)	1.000				1.000		
AG + GG	22	4 (18)	0.657	0.225–1.914	0.441	0.580	0.711	0.223–2.266	0.564
ATG10: rs1864182									
TT	86	21 (24)	1.000				1.000		
GT + GG	16	4 (25)	1.014	0.348–2.954	0.980	1.000	1.078	0.347–3.342	0.897
ATG10: rs10514231									
TT	84	19 (23)	1.000				1.000		
CT + CC	19	6 (32)	1.525	0.609–3.818	0.368		1.455	0.535–3.957	0.463
ATG12: rs26532									
AA	36	5 (14)	1.000				1.000		
CA + CC	66	20 (30)	2.216	0.831–5.904	0.112		1.798	0.656–4.931	0.254
ATG12: rs26534									
GG	53	14 (26)	1.000				1.000		
AG + AA	49	11 (22)	0.827	0.375–1.821	0.637		0.843	0.362–1.963	0.692
ATG12: rs26538									
CC	56	13 (23)	1.000				1.000		
CT + TT	47	12 (26)	1.133	0.517–2.483	0.756		1.025	0.430–2.442	0.956

Multivariate analyses in this table were adjusted for sex, patient age, tumor histology,disease stage, Karnofsky Performance Status, and smoking status. HR, hazard ratio; CI, confidence interval; BM, BM. [a] *P* values were calculated by the Fisher exact test

investigated in our study are located in the genes that are critical in the early stage of the autophagy pathway, and they are necessary for autophagosome formation [24]. ATG10 is essential for the conjugation of ATG12 to ATG5 and ultimately to ATG16L. Previously, variants in ATG genes have been associated with risk and/or prognosis in other cancers [20, 25]. Huang *et al.* observed an association between *ATG16L1* rs78835907 and recurrence of localized disease, which was replicated in more advanced disease [26]. In head and neck squamous cell carcinoma, Fernández-Mateos *et al.* observed an association between ATG10 rs1864183 and a higher susceptibility to develop laryngeal cancer and an association between *ATG16L1* rs2241880 and oral carcinoma [27]. A nonsynonymous polymorphism in *ATG16L*, rs2241880 (T300A), has been extensively studied in Crohn's disease [28]. This ATG16L SNP (GG) creates a caspase 3 and caspase 7 cleavage site, reducing protein stability and resulting in decreased autophagy. The presence of this variant is clinically associated with increased risk of ileal Crohn's disease in adults and decreased survival [28]. *ATG10* is an E2-like enzyme involved in E2 ubiquitin-like modifications, and it is essential for autophagosome formation. Jo *et al.* found that ATG10 was increased in colorectal cancer and

associated with lymphovascular invasion and lymph node metastasis [29]. Qin *et al*. demonstrated that potentially functional polymorphisms in *ATG10* were associated with breast cancer risk in the Chinese population [20]. These results indicate that *ATG10* and its genetic polymorphisms might be an important component during carcinogenesis.

Furthermore, we found *ATG3*: rs7652377 polymorphisms to be associated with brain metastasis risk. ATG3, an E2-like enzyme, catalyzes ATG8 phosphatidylethanolamine conjugation, which is essential for autophagy [30]. Wang *et al*. observed that ATG3 knockdown together with oncogenic RAS activation achieved a synergistic effect in inducing epithelial mesenchymal transformation (EMT) [31]. Another study revealed that ATG3 was significantly upregulated in patients with NSCLC [32]. Cells expressed high basal autophagy-related 3 protein (ATG3) in erlotinib-resistant lung adenocarcinoma [33]. ATG3-mediated autophagy also plays an important role in apoptotic cell death of NSCLC cells [33].

The brain is the main site of treatment failure in EGFR mutant patients. Although we have shown that PCI can decrease the incidence of BM and prolong disease-free survival in patients with high risk of BM in a randomized phase III trial [34], it remains to be determined whether conducting early intervention on patients with EGFR mutations who received a first-line EGFR-TKI with PCI could prolong disease-free survival or even overall survival of these patients.

Here, the incidence of BM was 24% (25 of 105 patients), which is slightly lower than in some other studies. We obtained post-treatment computed tomography (CT) or magnetic resonance imaging (MRI) scans only if clinical evaluation revealed suggestive findings like neurological symptoms. As is true in other studies analyzing BM risk factors, this could limit the accuracy of a putative molecular marker of BM risk. These differences may explain the relatively low incidence of BM in our population. As with all retrospective analyses, interpretation of these results is limited by bias. Another limitation is the small size of some subgroups, which could have affected some analyses. Further studies will be needed to determine if PCI in this group of patients will offer a survival benefit.

In conclusion, to our knowledge, this study is the first to evaluate the associations between genetic variations in the autophagy pathway and BM risk. We found that four SNPs (ATG16L1: rs2241880, ATG10: rs10036653, rs3734114, and ATG3: rs7652377), are significantly associated with NSCLC treated with EGFR-TKI. Further studies will be needed to determine if PCI in this group of patients will offer a survival benefit.

Conflicts of interest

The authors declared that they have no conflicts of interest.

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