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# Reduction of Tumor Formation in GABARAP Knock-out Mice is Associated with Absence of H-ras Mutation

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## Abstract:

GABARAP gene has an essential role in the autophagic process through its involvement in the maturation of the autophagosome. The role of GABARAP in tumorigenesis is not yet clarified. It is ubiquitously expressed in all tested normal tissues, while its expression in tumors is divers. Autophagy could induce by Oncogenic Ras to handle the metabolic stress and support cell survival. In this study, we found that GABARAP knockout mice exhibited significantly less tumor formation than wild-type mice after 7,12-dimethylbenz(a)anthracene treatment. Different types of tumor developed in the mice (skin, mammary, lymphoma and liver tumors). Furthermore, the tumor occurrence started earlier in wild-type mice compared to GABARAP knockout animals, and the tumor sizes in wild-type mice were obviously larger in most of induced tumors compared to the tumors formed in GABARAP KO mice. No H-ras mutation detected in the tumors of GABARAP knockout mice compared to 5 mutations in 14 tumors of the wild-type mice which revealed by mutation analysis of tumors induced by DMBA. In conclusion, the absence of H-ras mutation in DMBA-induced tumors of GABARAP KO mice indicates the significance of GABARAP gene in tumor progression that need further studies to clarify the exact role.

***Key words:*** *GABARAP, Tumorigenesis, DMBA, H-ras, Mutation.*

## Introduction:

Gamma (γ)-aminobutyric acid type A (GABAA) receptor-associated protein (GABARAP) is an evolutionarily highly conserved gene family from yeast to mammals and ubiquitously expressed in a wide range of organisms and tissues. The amino acid level for mammalian forms of GABARAP showed 100% identity suggesting that the function of this gene is essential or beneficial in mammals (1). GABARAP has an essential role in the autophagic process through its involvement in the maturation of the autophagosome (2).

Autophagy is a degradation cellular pathway of proteins and organelles within the lysosome/vacuole. It recycled obsolete components of cytoplasm to generate macromolecular building blocks and energy under stress conditions (3). There are three primary types of autophagy (4): macroautophagy (hereafter referred as autophagy), microautophagy and chaperone-mediated autophagy (CMA). Autophagy begins with origination of a cup shaped membrane (termed "phagophore"). The phagophore enwraps parts of the cytoplasm to form a double-membrane vesicle, called autophagosome, which ultimately fuses with the lysosomes/vacuole. Autophagy occurs at low basal levels in most normal cells to perform homeostatic functions such as protein and organelle turnover or when the cells need to 'self-cannibalize' and to proceed to cell survival under stress in order to maintain cellular integrity (5). Observations and genetic analyses in patients, as well as studies in transgenic animal models, clearly implicate autophagy in diseases (5,6,7).

The role of autophagy in cancer is complex and likely tissue and genetic context-dependent. Autophagy could be oncogenic in some occasions, whereas in others, it clearly supports tumor suppression (8,9,10). In 2003, Qu et al. (11) and Yue et al. (12) provided the first genetic link between autophagy and tumorigenesis. They conferred evidence that mice with heterozygous loss of Beclin-1; an autophagy and apoptosis regulating gene, promoted spontaneous malignancies in aged mice. Paradoxically, Atg5, Atg7, or FIP200 knockout in various tissues did not lead to malignant tumor development in vivo (13), but the mice with systemic mosaic deletion of Atg5 and liver-specific Atg7 deletion developed benign liver adenomas originating from autophagy-deficient hepatocytes (14). Moreover, Atg4C-deficient mice showed increased sus-

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ceptibility to fibrosarcoma induction by the chemical carcinogen methylcholanthrene (MCA) (15).

The protumorigenic function of autophagy has been demonstrated in recent years through its ability to increase the glucose metabolism in tumor cells in response to diverse stresses. Lock et al. (16) showed that H-Ras transformation in autophagy-competent MEFs displayed enhancement in glucose uptake compared with autophagy-deficient MEFs. High level of glycolysis in autophagy-competent cells encourages transformation mediated by Ras, indicating that autophagy has a unique mechanism to promote the tumor growth driven by Ras. Furthermore, MCF10A cells infected with retroviral MFG-K-RasV12 undergo cellular transformation with increased levels of autophagy-related genes. In contrast, pharmacological inhibition of autophagy or targeted suppression of Atg5 and Atg7 expression by short hairpin (sh) RNA completely blocked K-RasV12-induced anchorage-independent cell growth on soft agar and inhibited tumor formation in nude mice (17).

Carcinogens are a number of agents that can cause tumors in humans and animals. The most used types of chemical carcinogens for in vivo study are polycyclic aromatic hydrocarbons (PAHs). Among PAHs, 7,12-dimethylbenz(a) anthracene (DMBA) is a reliable, potent and widely used carcinogen. In general, chemical carcinogens induce molecular changes in target organ, and the most frequently involved genes are Ras and Tp53 (18). Many previous studies reported the DNA damaging effect of environmental carcinogens, for example the induction of point mutations in genes such as H-ras by DMBA (19,20,21). The main aim of this study is to find the impact of GABARAP deletion on the tumor induced by DMBA by means of H-ras mutation analysis.

# Methods:

**Mice:**

Female wild-type (Wt) C57BL/6 mice were purchased from Charles River (Sulzfeld, Germany). GABARAP knockout (GABARAP KO) mice were obtained from the Max Planck Institute for Brain Research in Frankfurt, Germany (22). GABARAP KO mice were generated from the Omni bank of embryonic stem cell library by insertion of targeting vector 345 bp upstream of exon 1 of the GABARAP locus (23). The heterozygous animals were crossed to produce +/+ (wild-type), +/– (heterozygous) and –/– (homozygous) mice. Mice were housed in standard cages (10 animals per cage) under controlled temperature (22 ± 2ºC) and lighting conditions (monitored 12 h light/12 h dark cycles), and supplied with water and food (Altromin, Lage, Germany) ad libitum. The approval of animal experiments was authorized by the Thuringian commission for animal protection (No. 02-018/08 and 02-007/13).

**DMBA treatment:**

DMBA was dissolved in sesame oil (5 mg/ml) by vortexing for 2 h at room temperature. All steps in the preparation were carried out under minimum illumination. DMBA-treated groups of wild-type and GABARAP KO mice got orally 1 mg DMBA/mouse in 200 µl of dissolvent (6 weekly doses). Control groups got 6 weekly dosed of 200 µl sesame oil. The tumors formation was monitored weekly by palpation. The tumor-bearing mice were anesthetized and killed by cervical dislocation. The tumor specimens were fixed in 5% neutral buffered formalin.

**Mutation analysis:**

DNA was extracted by using Maxwell® 16 FFPE Plus LEV DNA Purification Kit (Promega) according to the manufacturer's instruction. FFPE blocks were sectioned to 10 µm thickness with a rotation microtome. The tumor region within the section was identified by Prof. Dr. Iver Petersen (Institute of Pathology, Gera, Germany). Thereafter, the identified tumor region, corresponding to H&E stained section, was scraped from FFPE tissue sections by scalpel. The number of sections for each tumor was determined depending on tumor size (~10 - 20 sections). Then the scraped sections were collected in microtube and briefly centrifuged at full speed to collect the sample at the bottom of the tube. The pellet was resuspended in 180 µl of incubation buffer and 20 µl proteinase K solution. The tumor sample was incubated at 70°C overnight. In the next day, 400 µl of lysis buffer was added to the sample and mixed by vortexing. Then the sample was prepared for Maxwell® 16 automated DNA purification instrument (Promega). NanoDrop was used to measure the DNA concentration and the purity (260 / 280 nm ratio) of the samples. If not used immediately for PCR the samples were stored at -20°C.

Agarose gel electrophoresis was used to qualify the DNA extraction from the samples. The DNA Clean & Concentrator™-5 Kit (Zymo Research) was used for the purification of PCR products for sequencing according to manufacturer's instructions. Purified PCR products (100 ng) were applied for direct sequencing by capillary electrophoresis (LGC Genomics GmbH, Berlin, Germany), and the sequencing-profiling was analyzed by the Finch TV 1.4.0 software program (Geospiza, PerkinElmer, MA, USA).

**Statistical analysis:**

Differences between groups were calculated using the two-tailed Student's t-test for unpaired values. Normal distribution of the values was checked using the Kolmogorov-Smirnov test (K–S test). Statistical significance was calculated by the SPSS software package (v.16.0, Chicago, USA), p-values below 0.05 were considered as being significant (* $p < 0.05$, ** $p < 0.01$, *** $p = 0$).

# Results:

**GABARAP knockout mice exhibited low tumor formation**

The GABARAP role in tumor formation was investigated by treatment of our transgenic animals with a reliable potent carcinogen DMBA. Female GABARAP knockout (KO) and C57BL/6 wild-type (Wt) mice were given 1 mg doses

of DMBA for 6 consecutive weeks beginning at 6 - 8 weeks of age. Mice were checked weekly for the presence of palpable tumor masses. Tumors arose in 14 of 29 (48.3%) of wild-type mice within 35 weeks after the last DMBA dose (Table 1). Surprisingly, the numbers of GABARAP KO mice exhibiting tumors were significantly less than their wild-type counterparts (4 of 33; 12.1%; $p < 0.002$) (Table 1). Different types of tumors formed in each group of mice, for instance mammary, skin, lymphoma and liver tumors. Figure 1 showed gross morphologic and histologic features of tumors induced by DMBA. If only mammary tumors are considered, this represents a reduction from 3 of 14 (21.4%) in wild-type mice to 0 of 4 (0%) in GABARAP KO mice (Table 1).

***Table 1.*** *Tumors formation in C57BL/6 wild-type (Wt) and GABARAP KO (KO) mice treated with DMBA*

| **Tumor type**<br>*DMBA treatment* | **Wt**<br>*n* = 29 | **KO**<br>*n* =33 |
|---|---|---|
| Mammary | 3 | - |
| Skin | 7 | 2 |
| Lymphoma | 2 | - |
| Liver | - | 1 |
| Undifferentiated tumors | 2 | 1 |
| **Total** | **(48.3%) 14** | ****(12.1%) 4** |

The low percentage of tumor incidence in C57BL/6 (Wt) mice is approximately consistent with the data reported by other authors following DMBA treatment and/or spontaneous tumors development, and this is due to the fact that the C57BL/6 mouse strain is less sensitive to DMBA-induced tumorigenesis than other rodent strains (24,25,26,27).

The median onset time and latency period of tumors induced by DMBA were not available for all tumor types due to a number of mice that had interior tumors; for example liver tumors were difficult to detect by palpation. Figure 2 (A and B) showed the latency periods and sizes of tumors induced by DMBA in mice groups. The tumor occurrence started earlier in wild-type mice compared to GABARAP knockout animals, at the 9th week after the last DMBA dose (Fig. 2 A). The tumor sizes in wild-type mice were obviously larger in most of induced tumors compared to the tumors formed in GABARAP KO mice which were smallest in general (Fig. 2 B). The age in Figure 2 (B) represented the age of mice when they are sacrificed.

**GABARAP-deficient tumors did not exhibit Ras mutation**

Many studies indicated that carcinogens, in general, induce molecular changes in target organs (18). DMBA has been reported to induce point mutations in the H-ras gene resulting in an A > T transversion at the middle adenosine nucleotide of codon 61 (19,20,21). Recently, the protumorigenic function of autophagy has been demonstrated through its ability to increase the glucose metabolism and thereby facilitating Ras-mediated cell transformation and promoting Ras-driven tumor growth. For this reason, we performed H-ras mutation analysis in DMBA-induced tumors from GABARAP KO and wild-type mice. Our analysis of the hotspot codons 12 and 13 in exon 1 and codon 61 in exon 2 revealed no mutations in the 4 tumors of the GABARAP KO mice compared to 5 mutations in 14 tumors of the wild-type mice (Table 2). The mutations included transversion of CAA > CTA or CAA > CAT (Fig. 3). This result could explain the importance of GABARAP gene, through its involvement in autophagy, in Ras-mediated cellular transformation to induce tumors.

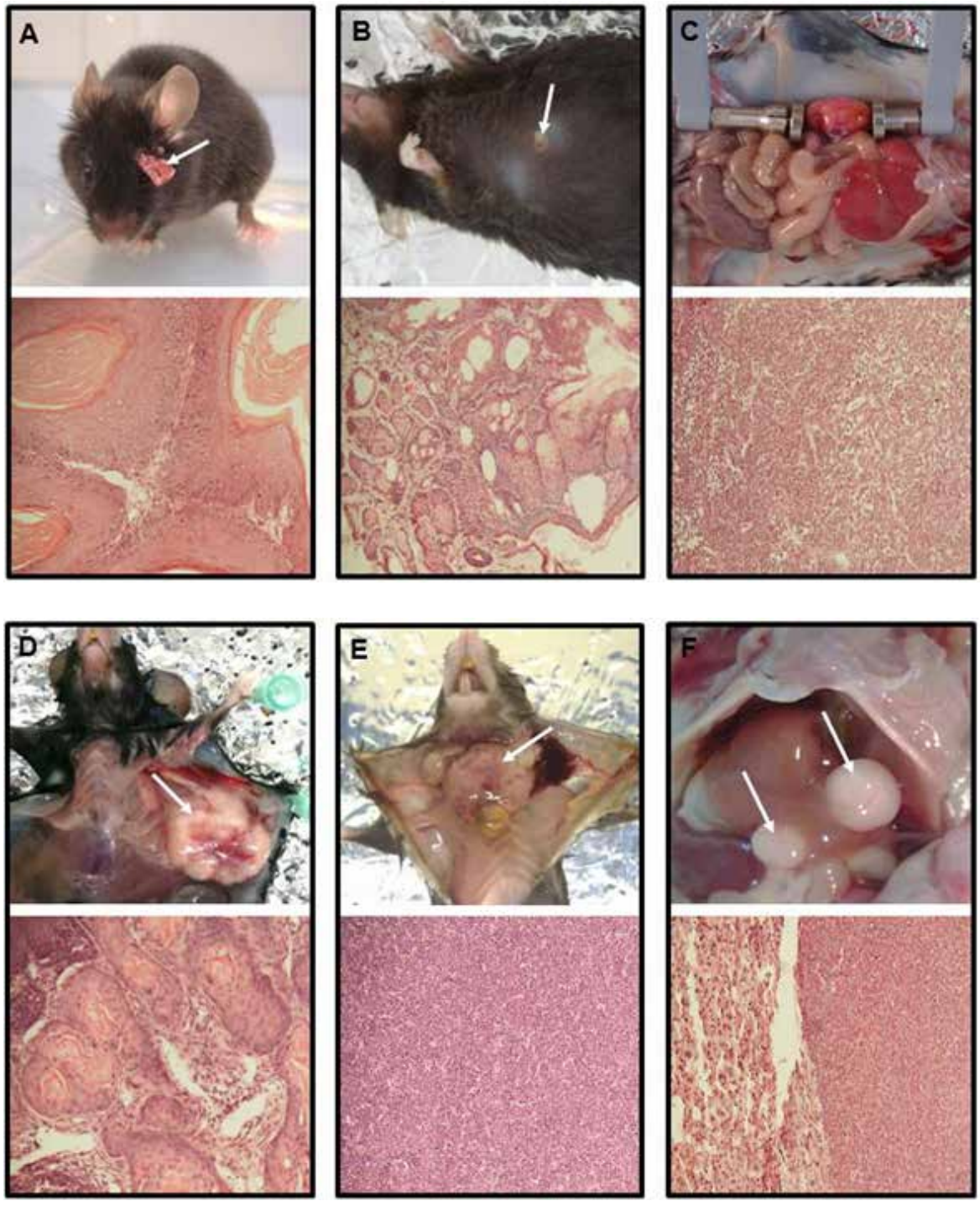



***Figure 1.*** *Gross morphologic and histologic features of mice tumors induced by DMBA. A) Upper panel: skin tumor in wild-type mouse, bottom panel: H&E staining of skin squamous cell carcinoma. B) Upper panel: skin tumor in GABARAP KO mouse, lower panel: H&E staining of skin sebaceous epithelioma. C) Upper panel: undifferentiated tumor mass in abdomen of wild-type mouse, bottom panel: H&E staining of histologic section of undifferentiated tumor. D) Upper panel: mammary tumor in wild-type mouse, bottom panel: H&E staining of mammary squamous cell carcinoma. E) Upper panel: lymphoma in the neck of wild-type mouse, bottom panel: H&E staining of histologic section of lymphoma. F) Upper panel: two masses of liver tumor in GABARAP KO mouse, bottom panel: H&E staining of liver cell carcinoma. The magnifications of all histologic sections were 20X.*

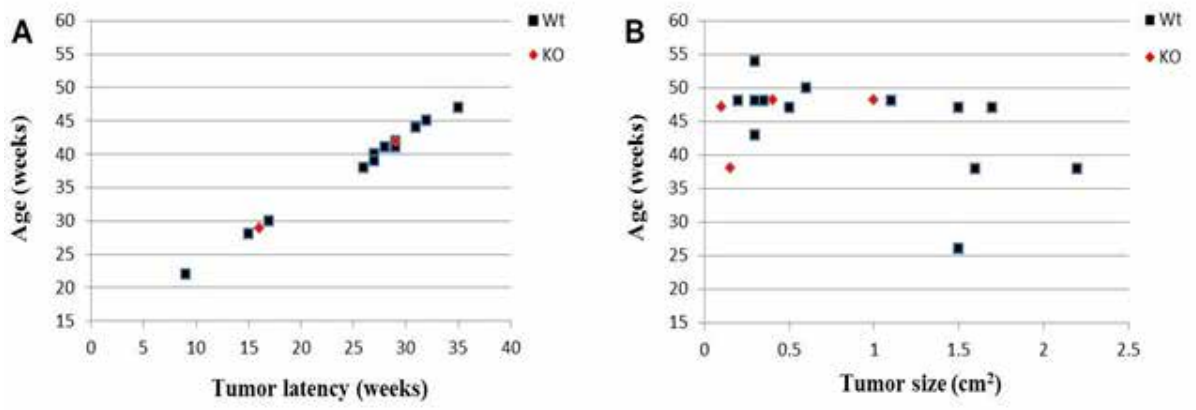
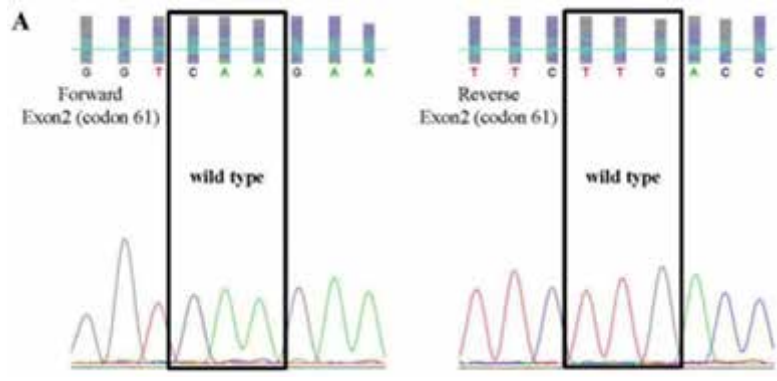



***Figure 2.*** *A) Latency period of palpable tumors (skin, lymphoma and mammary tumors) in wild-type (Wt) and GABARAP KO (KO) mice after 6 weekly oral doses of 1 mg DMBA. The latency period represented the date after the last dose of DMBA, which was indicated as (0) in figure. B) Size of all the tumors induced by DMBA in wild-type (Wt) and GABARAP KO (KO) mice. In figure B, the age of mice represented the date when the mice are sacrificed.*

***Table 2.*** *Tumor types and H-ras mutation in C57BL/6 wild-type (Wt) and GABARAP KO mice*

| Tumor type | C57BL/6 | | | | GABARAP KO | | | |
|---|---|---|---|---|---|---|---|---|
| *DMBA treatment* | *n* = 29 | *H-ras mutation* | | | *n* =33 | *H-ras mutation* | | |
| | *Tumors No.* | 12 | 13 | 61 | *Tumors No.* | 12 | 13 | 61 |
| Mammary | 3 | - | - | **2** | - | - | - | - |
| Skin | 7 | - | - | **3** | 2 | - | - | - |
| Lymphoma | 2 | - | - | - | - | - | - | - |
| Liver | - | - | - | - | 1 | - | - | - |
| Undifferentiated tumors | 2 | - | - | - | 1 | - | - | - |
| Total | **14** | **-** | **-** | **5** | **4** | **-** | **-** | **-** |



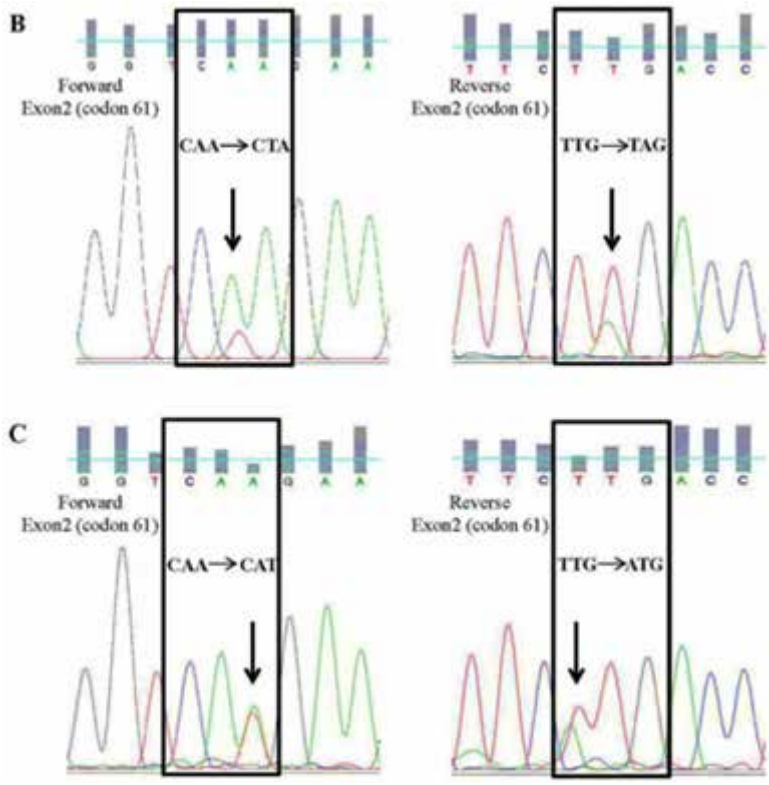



***Figure 3.*** *H-ras mutation analysis of GABARAP KO and wild-type tumors. A) Exon 2 (codon 61) of H-ras in GABARAP tumor showed wild type sequences of forward and reverse primers. B) Wild-type tumor showed mutation at forward (CAA > CTA) and reverse (TTG > TAG) sequences of exon 2 (codon 61). C) Wild-type tumor showed mutation at forward (CAA > CAT) and reverse (TTG > ATG) sequences of exon 2 (codon 61).*

## Discussion:

The role of autophagy in tumorigenesis is complex and likely tissue and genetic context-dependent. Contradictory roles of autophagy in tumor initiation and progression have been mentioned in several reports. In some instances autophagy may serve as protumorigenic mechanism, whereas in others, it contributes to tumour suppression (8,9,10,28). Beclin-1 represented the first genetic link between autophagy and tumorigenesis (11,12). Heterozygous loss of Beclin-1 promoted spontaneous malignancies in mice. Later on, studies showed that the increased rate of tumor formations in mice with heterozygous loss of Beclin-1 is not due to autophagy, since that Beclin-1 has been shown to interact and associated with apoptotic protein (Bcl-2) and tumor suppressor p53 (29,30,31). Moreover, Atg4C-deficient mice showed increased susceptibility to fibrosarcoma induced by the intradermal injection of chemical carcinogen MCA in a tissue-specific mode (15). In contrast, knockout of FIP200, an important regulator of autophagy, suppressed mammary tumor initiation and progression in a mouse model of breast cancer driven by the PyMT oncogene (13). In our investigation, we used the chemical carcinogen (DMBA) in order to enhance the tumor induction and to explore the potential role of GABARAP in tumorigenesis. DMBA is a widely used carcinogen and can induce various types of cancer in animal models (32). Most studies have used DMBA to induce mammary tumors following administration by oral gavage in mouse models (33,34). We found that GABARAP KO mice significantly reduced the tumor formation after DMBA treatment compared with wild-type mice. For this reason we concluded that the GABARAP gene may play an influential role in the process of tumor development. Based on our knowledge, this is the first report providing evidence for the role of GABARAP in tumorigenesis in a mouse model.

Many chemical carcinogens induce molecular alterations in target organ, and the most frequently involved genes are Ras and Tp53 (18). It is well known that DNA damaging effect of DMBA mostly induced point mutations in genes such as c-

H-ras (19,20,21). Ras oncogenes play a fundamental role in regulation of cell growth and survival and they are frequently activated in cancer (35). Previous studies showed that active oncogenic Ras induced autophagy to promote and facilitate oncogenic transformation by maintaining and improving cell metabolism (16,17,36). Indeed, Ras-transformed cells have been shown to enhance glucose uptake, whereas autophagy-deficient counterparts have decreased rates of glycolysis (16). Bhatt et al. (37) suggested that a combination of autophagy inhibition and lipid metabolism interruption could be an effective therapy for LKB1 deficient RAS-driven lung cancer. On the other hand, the combinatorial BRAFV600E and autophagy inhibition may enhance the outcomes of therapy in patients whose tumors have BRAFV600E/K mutations (38). Moreover, Wei et al. (13) observed that FIP200-null mammary tumor cells and transformed MEFs had reduced glycolysis and suppressed mammary tumor initiation and progression in a mouse model of breast cancer driven by the PyMT oncogene. These findings elucidated the essential role of autophagy-related genes in tumorigenesis induced by oncogenic signaling. Since DMBA has been reported to induce point mutations in H-ras gene leading to A > T transversion in codon 12, 13 and 61 (19,20,21), we wanted to investigate the H-ras status in the tumors obtained from GABARAP KO and wild-type mice after treatment with 6 weekly oral doses of DMBA. Interestingly, we did not find any mutations within the hot spots of exon 1 (codon 12, 13) and exon 2 (codon 61) of H-ras in the tumors of GABARAP KO mice. In contrast, tumors of wild-type mice were shown to harbour H-ras mutation within codon 61 of exon 2 in about 36% of total tumors number.

We suggested an essential role of GABARAP gene in tumorigenesis, and this role may be through its involvement in the autophagy process. Furthermore, the non-occurrence of H-ras mutations in the tumors of GABARAP KO mice may indicate the involvement of autophagy/GABARAP in Ras-mediated cellular transformation.

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