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BNIP3L Is a New Autophagy Related Prognostic Biomarker for Melanoma Patients Treated With AGI-101H

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Abstract. *Background/Aim: Skin melanoma belongs to the most invasive malignancies with no cure for a progressing disease. Personalized therapy would allow for the selection of patients that will benefit from treatment. For this purpose, proper predictive biomarkers must be defined. Materials and Methods: Allogeneic whole-cell gene-modified therapeutic melanoma vaccine (AGI-101H) was applied in advanced melanoma patients. Humoral responses were analyzed using SEREX, and in silico gene expression analysis in TCGA melanoma patients was performed. Results: A specific antibody response was raised against an antigen identified as BNIP3L, which correlated with a good prognosis. Moreover, AGI-101H directs an immune response against autophagy, as BNIP3L is a marker of this process. Medium and high expression of BNIP3L was also linked with longer overall survival. Conclusion: BNIP3L is a candidate prognostic marker of clinical outcome of melanoma patients treated with AGI-101H, and may be considered as a prediction marker for patient survival.*

Cutaneous melanoma is responsible for 75% of skin cancer–related deaths, and its incidence has been steadily increasing worldwide (1). Metastatic melanoma is highly resistant to conventional therapies. However, novel systemic therapy approaches such as targeted therapy with BRAF and MEK inhibitors (2) or various immunotherapy approaches have been exploited. These therapies include the adoptive transfer of immune cells (3), cancer vaccines, peptides, DNA, and the use of immune checkpoint inhibitors (ICI) such as anti-CTLA-4 or programmed death factor 1 (PD-1) monoclonal antibodies (4). The latter ones significantly improved the prognosis of melanoma patients; however, as monotherapy, they still demonstrate some limitations. Accordingly, combinatorial strategies are given the most attention nowadays. They include the combination of different ICI, ICI with cancer vaccines, kinase inhibitors etc. (5). However, the predictive and prognostic biomarkers are still required (6, 7).

The specific adaptive anti-cancer immune response comprises two phases- induction and effector. The induction phase can be activated and maintained by cancer vaccines. Moreover, in combined immunotherapy approaches, cancer vaccination generates tumor specific effector T cells. Since 1997, we have been investigating the therapeutic efficacy of whole cell melanoma vaccine (AGI-101H) in patients with advanced melanoma. AGI–101H (the first in its class) is an allogeneic tumor cell vaccine consisting of two human melanoma cell lines: Mich-1 and Mich-2, which were genetically modified to secrete designer cytokine Hyper-IL6 (H6), which serves as molecular adjuvant (8). H6 is a fusion protein of interleukin 6 (IL-6) and its soluble receptor (sIL-6R, gp80). It directly targets gp130, a signal-transducing subunit, and activates the JAK1/STAT3-P/Oct4 pathway both in the paracrine and autocrine manners. Exposure of vaccine cells to H6 led to the conversion of their phenotype into melanoma stem cell (MSC) - like with high ALDH1H1

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expression (9). At the vaccination site, H6 stimulates allogeneic T cell response, inhibits T regulatory (Treg) cell formation, induces dendritic cell (DC) maturation, and presentation of cryptic antigens. It stimulates memory CD4+ and CD8+ T cell formation, activates natural killer cells (NK), stimulates granulocyte-macrophage colony-stimulating factor (GM-CSF) secretion by lymphocytes (10), and decreases the number of myeloid-derived suppressor cells (MDSCs) (9). Down-stream H6 generates specific effector T cells (CTL) targeting melanoma differentiation antigens (MAGE-A, -A1, -A9, -A12, BAGE, GAGE-1, -2, -8, GAGE-3, -4, -5, -6 -7, -7B, NY-ESO1, gp100, CTp11, PRAME, NA17A, TRP-1, TRP-2, Sox-10, SSX-1, HD-MM-05, -07,-21, -22, and -25) (11) and stem cells antigen ALDH1A1. AGI101-H reduces myeloid-derived suppressor cells (MDSC) and regulatory T cells (Treg) in circulation (9).

Here, we analyzed the humoral immune response in 38 patients with stage III and IV melanoma treated with AGI-101H. We employed a phage display technology and SEREX (Serological Analysis of Recombinant cDNA Expression Libraries). We found one peptide - BNIP3L (BCL-2/adenovirus E1B 19 kd-interacting protein-like) and enhanced production of anti-BNIP3L antibodies, which positively correlated with the clinical outcome of patients. Since BNIP3L is one of the critical factors regulating autophagy, it seems that AGI-101H is targeting the autophagy process as well. Accordingly, it may be proved to be a good prognostic factor for melanoma patients immunized with AGI-101H.

Materials and Methods

Patients and sera. This is a retrospective study using archived serum samples. The clinical studies were approved by the Local Ethical Committee (330A/96 dated 13 Sep 1996, 477A/97 dated 3 April 1997). All patients signed the informed consent form. The study was carried out in 38 patients participating in 2 clinical trials (A phase II trial: the evaluation of the efficacy and toxicity of an allogeneic melanoma vaccine, genetically modified, with interleukin 6/soluble interleukin 6 receptor complex (Hyper-IL-6), in patients with resected melanoma; A phase II trial: the evaluation of the efficacy and toxicity of an allogeneic melanoma vaccine, genetically modified, with interleukin 6/soluble interleukin 6 receptor complex (Hyper-IL-6), in patients with measurable melanoma metastases). The vaccine was administered 8 times in 2-week intervals (induction phase), and then ones per month (maintenance phase) until death. Twenty out of 38 (53%) patients developed progression of the disease (PD), 10 out of 38 (27%) responded to the treatment displaying either disease stabilization (SD), a partial response (PR), or a complete response (CR), and 8/38 patients received a vaccine in the adjuvant setting after the complete resection of melanoma. Sera used in the study were collected 6 months after the first dose of the vaccine administration and were stored in –20˚C until analyses.

Serological screening of recombinant cDNA expression libraries (SEREX). The immunological screen was performed as described previously (12). Briefly, sera were first diluted 1:10, pre-absorbed with *E. coli* and phage proteins. Pre-absorbed sera were then diluted 1:100 (final dilution) and used in screening. cDNA phage library derived from melanoma was used for serological identification of antigens by recombinant expression cloning. *E. coli* was transduced with recombinant lambda-ZAP phages and plated onto NZY-agar plates. Resultant plaque proteins were subsequently blotted into nitrocellulose membranes, which were then incubated with sera. Each serum was screened with approximately 2.5×10^4 bacterial clones expressing different genes from a melanoma gene library, in at least three separate experiments Reactive antibodies were detected with alkaline phosphatase-coupled secondary anti-human IgG antibody and visualized with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolinum (NBT). Positive monoclonal phages were subjected to *in vivo* excision of the pBluescript phagemids allowing the inserts to be analyzed by sequencing. A secondary SEREX was then performed with all of the other sera.

Sequence analysis. Sequencing was performed using the ABI Prism Genetic Analyser and Big Dye Terminator Cycle Sequencing Ready Reaction Kit with the primers recognizing the T3 and T7 promoters of the pBluescript phagemid. The homologous sequences were searched with a blast algorithm using the HUSAR package from the Biocomputing Service Group at the German Cancer Research Center.

TCGA data. The TCGA expression data of BNIP3L, expression of selected genes, and clinical data were downloaded from cBioPortal (Skin Cutaneous Melanoma, TCGA, Provisional, 479 samples) (13), from the UALCAN databases (14), and from StarBase v3.0 (15) for 472 cancers. All data is available online; access is unrestricted and does not require patient consent or other permissions. The use of the data does not violate the rights of any person or any institution.

Data analysis. The expression levels of BNIP3L were analyzed depending on the clinicopathological parameters, such as: sample type (normal, n=1 *vs*. primary, n=104 vs metastasis, n=368), gender (women *vs*. men, n=472), age (<58 *vs*. >58, n=464), ulceration (no *vs*. yes, n=315), Clark level (I *vs*. II *vs*. III-IV *vs*. V, n=323), Breslow depth (<1 *vs*. 1-2 *vs*. 2.1-4 *vs*. >4, n=361), mitotic rate (0-2 *vs*. 2-3 *vs*. >4, n=173), cancer stage (0 *vs*. I+II *vs*. III+IV, n=434), M-stage (M0 *vs*. M1, n=444), T-stage (T0 *vs*. T1+T2 *vs*. T3+T4, n=443). Next, from a group of 472 patients, high and low expression subgroups of BNIP3L were selected using the <25 and >25 percentile as cutoff: i) low (n=111) and ii) medium with high (n=351), respectively. OS was assessed in these subgroups.

Gene analysis. Using StarBase v3.0 database correlation between BNIP3L and genes connected with autophagy were analyzed in melanoma patients, and next selected genes with R- square <-0.2 and >0.2 were compared between the BNIP3L low- and medium-with high-expressing groups of patients.

Statistical analysis. All statistical analyses were performed using GraphPad Prism 5 (GraphPad, San Diego, CA, USA). The Shapiro-Wilk normality test, *t*-test, and Mann–Whitney *U*-test or one-way ANOVA performed using Dunn's multiple comparisons test were used for BNIP3L (depending on clinical parameters) and gene expressions (depending on BNIP3L subgroups). For DFS and OS analyses, the Log-Rank (Mantel-Cox) test was used. TCGA data are presented as mean with SEM, and in all analyses, $p0.05$ was used to determine statistical significance.

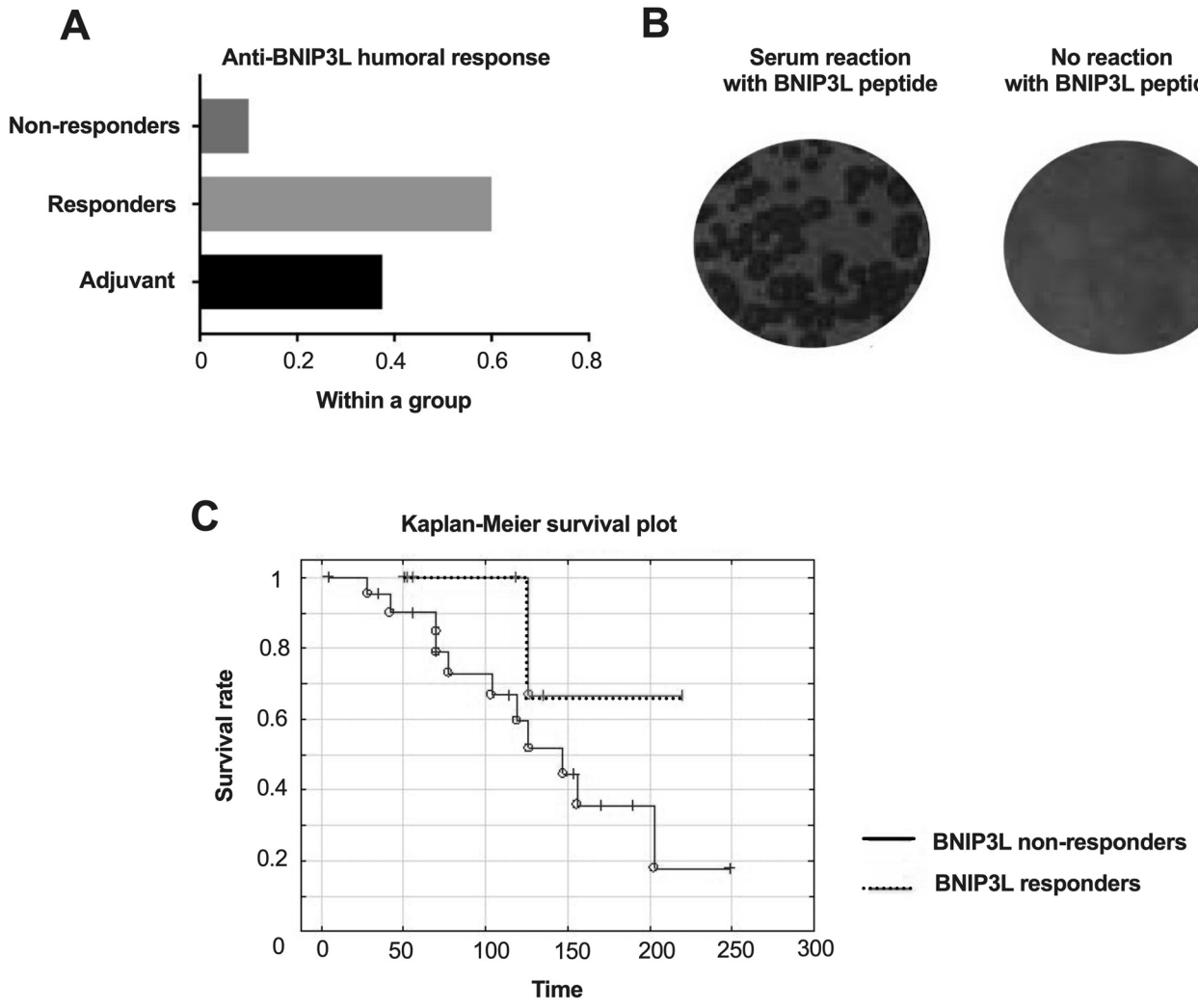

Figure 1. *Anti-BNIP3L humoral response in melanoma patients treated with AGI-101H: A) Classification of the patients depending on the response to anti-BNIP3L antibody into the non-responders, responders and adjuvant groups; B) Secondary SEREX blots eliciting seroreactivity with peptide characterized as BNIP3L; C) Progression-free survival probability curves of the two subgroups (AGI-101H responders vs. non-responders) based on the presence of BNIP3L serum response using the Kaplan–Meier survival estimates test.*

Results

AGI-101H treated patients respond to melanoma associated antigens. From the 38 patients analyzed, two patients showed the seroreactivity with the library peptides. These clones were further sequenced. The results were blasted with the mRNA sequences collected in the German Cancer Research Center's HUSAR database. One positive clone was selected and further analyzed. It showed nearly 100% mRNA homology with a sequence of *BNIP3L* (BCL-2/adenovirus E1B 19 kd-interacting protein-like).

Response to BNIP3L positively correlates with the clinical outcome. In order to evaluate the humoral response to BNIP3L in other patients, secondary SEREX was carried out. From the 38 patients analyzed, 11 showed reactivity with the clone. This group included 2 of 20 (10%) patients with disease progression (PD), 6 of 10 (60%) patients with a positive response to the treatment (CR, PR or SD) and 3/8 (38%) patients in the adjuvant setting (Figure 1A). Figure 1B presents an example of the visualized serum-clone reaction. The obtained results showed that AGI-101H-responders produced anti-BNIP3L antibodies 6 times more often than non-responders, as measured with odds ratio. The progression risk was 3 times lower in patients who produced anti-BNIP3L antibodies. Increased overall survival was observed in patients with anti-BNIP3L antibodies response. Although these differences were not statistically significant

due to the small number of individuals, there was a clear tendency towards a positive correlation of anti-BNIP3L response and clinical outcome after AGI-101H treatment as measured by Kaplan–Meier analysis (Figure 1C).

BNIP3L expression is dependent on sample type, stage, and mitotic rate. According to the database (cBioportal and UALCAN), the expression of BNIP3L was significantly up-regulated in metastatic melanoma compared to primary samples (p=6.06e-3) (Figure 2). Significant differences between expression levels of BNIP3L were observed in patients with Clark level I *vs*. II (p=0.0291) and mitotic rate 0-2 *vs*. 2-3 (p=0.0396) and 2-3 *vs*. >4 (p=0.0376) (Figure 3).

BNIP3L expression levels positively correlate with overall survival (OS). Melanoma samples were divided into BNIP3L low, medium and high expression groups using the <25 and >25 percentile of BNIP3L expression as a cut-off, respectively. Significantly longer OS of BNIP3L medium and high expression group compared to low expression (p=0.0188) was observed with median survival of 98.32 *vs*. 58.48 months (Figure 4).

BNIP3L expression correlates with autophagy-related gene expression. Using the StarBase v3.0 database the correlation between BNIP3L and genes related to autophagy was analyzed in melanoma patients. Genes with R- square < -0.2 and >0.2 were compared between the BNIP3L low- and medium- with high-expressing groups of patients. There was down-regulation of *ATG4D* gene expression and up-regulation of *ATG16L1*, *ATG4A, ATG4C, ATG3, ATG12, ATG5, MAP1LC3B, DRAM1, GABARAPL2*, *BECN1* expression that are associated with autophagy components in the melanoma patients with medium and high compared to the low expression of BNIP3L (Figure 5). In the group of co-regulators of autophagy and apoptosis process in patients with medium and high expression of BNIP3L, down-regulation of *CLN3, AKT1, BAD, FADD* and up-regulation of *PIK3CG, TNFSF10 (TRAIL), BNIP3, NFKB1, CASP3, CASP8 (FLICE), FAS (TNFRSF6), MAPK8 (JNK1), EIF2AK3, BENC1* and *PRKAA1* (*AMPK*) were observed compared to the BNIP3L low-expressing group (Figure 6). In the patients with medium and high expression of BNIP3L, down-regulation of *CTSD, HGS* and up-regulation of *UVRAG, ULK2, ESR1 (ERα), DRAM2 (TMEM77), MAPK14 (p38ALPHA), PIK3R4, PIK3C3 (Vps34)* and *RPS6KB1*, which are associated with autophagy in response to other intracellular signals, were observed. Moreover, in the case of co-regulators of autophagy and cell cycle related genes, *CDKN1B* (*P27KIP1*) and *RB1*, as well as *HSP90AA1*, chaperone-mediated autophagy gene, up-regulation in patients with medium and high expression of BNIP3L were observed (Figure 7).

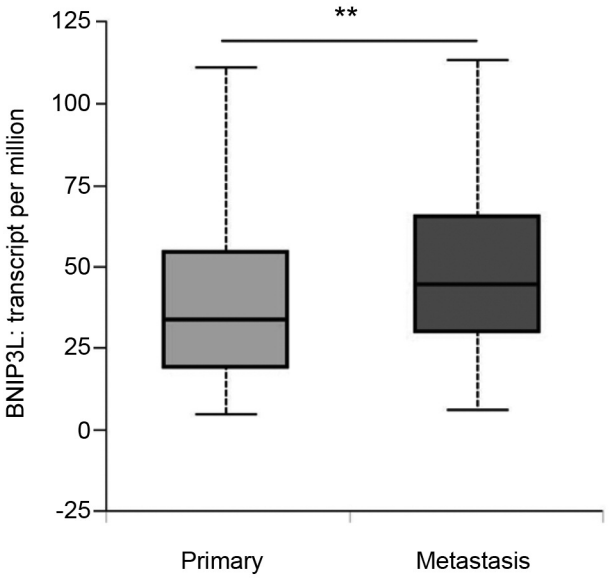

Figure 2. *BNIP3L expression in melanoma patients from The Cancer Genome Atlas (TCGA) depending on the sample type (primary: n=104 and metastasis: n=368). Box and whiskers with 5-95 percentile, **p<0.01.*

Discussion

So far, no biological marker for monitoring melanoma immunotherapy has been identified. In our study, we found a positive correlation between the production of antibodies to BNIP3L/NIX and the clinical outcome of melanoma patients treated with the whole-cell melanoma vaccine genetically modified to stem cell-like phenotype (AGI-101H). Anti-BNIP3L humoral responses were remarkably enhanced in AGI-101H responders, most likely due to the promotion of the autophagy process, which led to inhibition of melanoma proliferation and extended patient OS as well as DFS. This is the first study reporting the generation of BNIP3L-specific antibodies in patients immunized with whole-cell melanoma vaccine. Our *in silico* analysis confirmed that increased expression of BNIP3L was also positively correlated with patient OS in melanoma.

BNIP3L plays a crucial role in the clearance of damaged mitochondria in the process of mitochondrial autophagy (mitophagy) in response to the hypoxic conditions (16-18). BNIP3L is induced by tumor suppressor p53 (19-21) and exhibits tumor-suppressing activity itself. The knockdown of BNIP3L promotes tumorigenesis by avoiding p53-dependent apoptosis under hypoxic conditions in a xenograft model of breast cancer (22). BNIP3L also plays a role in immunogenic cancer cell death since BNIP3L-mediated mitophagy promotes the generation of natural killer memory cells (23). Overexpression of BNIP3L in hypoxic conditions is mediated by hypoxia-inducible factor HIF-1 (18). Considering that high

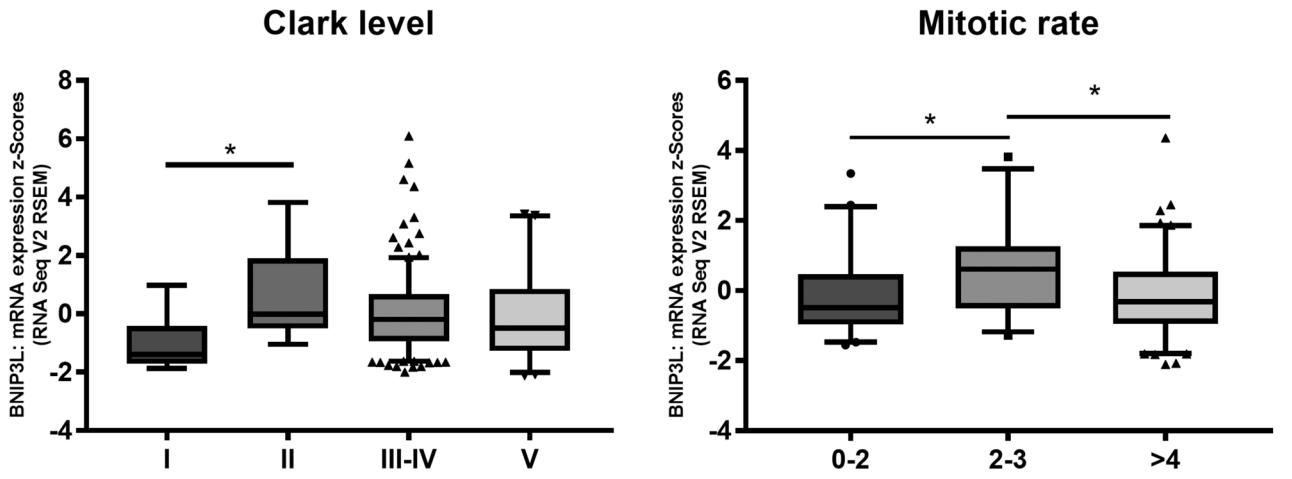

Figure 3. *The expression levels of BNIP3L in melanoma are independent of Clark stage and mitotic rate. Box and whiskers with 5-95 percentile; one-way ANOVA obtained using Dunn's multiple comparisons tests; *p<0.05.*

level of HIF-1 as an indicator of extensive tumor growth (24), overexpression of BNIP3L within the tumor mass may reflect the stage of oncogenic process. The progressive hypoxia leads then to overproduction of BNIP3L. Under the influence of the destroying power of the vaccine, a disintegrated tumor mass releases its components inducing the immune response. The bigger and more damaged a tumor tissue is, the more antigens are released, and the production of antibodies is higher. That scenario would make BNIP3L a prognostic factor for any type of immunotherapy. Also, it defines the level of hypoxia, which indicates the aggressiveness of a tumor, as well the role of autophagy in melanoma, specifically in response to AGI-101H treatment. Since AGI-101H displays CSC phenotype, of particular importance would be the fact that autophagy maintains the pluripotency of cancer stem cells (CSC) and facilitates their survival. Also, it affects epithelial-to-mesenchymal transition (EMT) (25, 26). Several markers of autophagy have been identified as potential prognostic biomarkers for melanoma. These include autophagy regulators: LC3, beclin, p62, or AMBRA1. Increased expression of light chain 3 (LC3) has been observed in malignant melanomas compared to benign nevi (27) and was associated with metastasis and poorer outcomes (28). Another study has shown that low beclin 1 expression was associated with high Breslow's depth, high Clark's level, and ulceration (29). Ellis *et al*. (30) have observed increased expression of autophagy receptor p62 in early AJCC stage melanomas, which was subsequently decreased in advanced metastatic tumors. Decreased or even complete loss of AMBRA1 expression has been observed in the epidermis overlying AJCC stage I melanomas, but not in benign nevi (31) suggesting the prognostic potential of this marker. Another study has shown that loss of Atg7 prevents melanoma development in BrafV600E mutant mice with allelic loss of *Pten* gene (32).

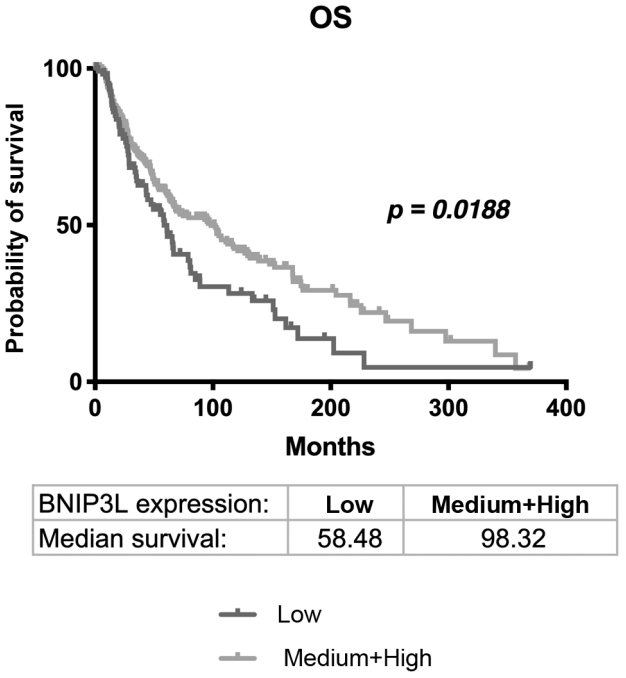

Figure 4. *OS in TCGA melanoma patients with low (n=111) and medium with high (n=351) expression levels of BNIP3L. Log-rank (Mantel-Cox) test was used for the analysis. p<0.05 was considered as significant.*

Although in many reports autophagy has been described as cancer progression related factor, there are also a few which show that autophagy displays suppressive functions with a positive impact on clinical outcome (33-35). There exists a general conclusion that autophagy has tumor-suppressive functions in the early stages of cancer and is tumor-promoting in established tumors (36). Interestingly,

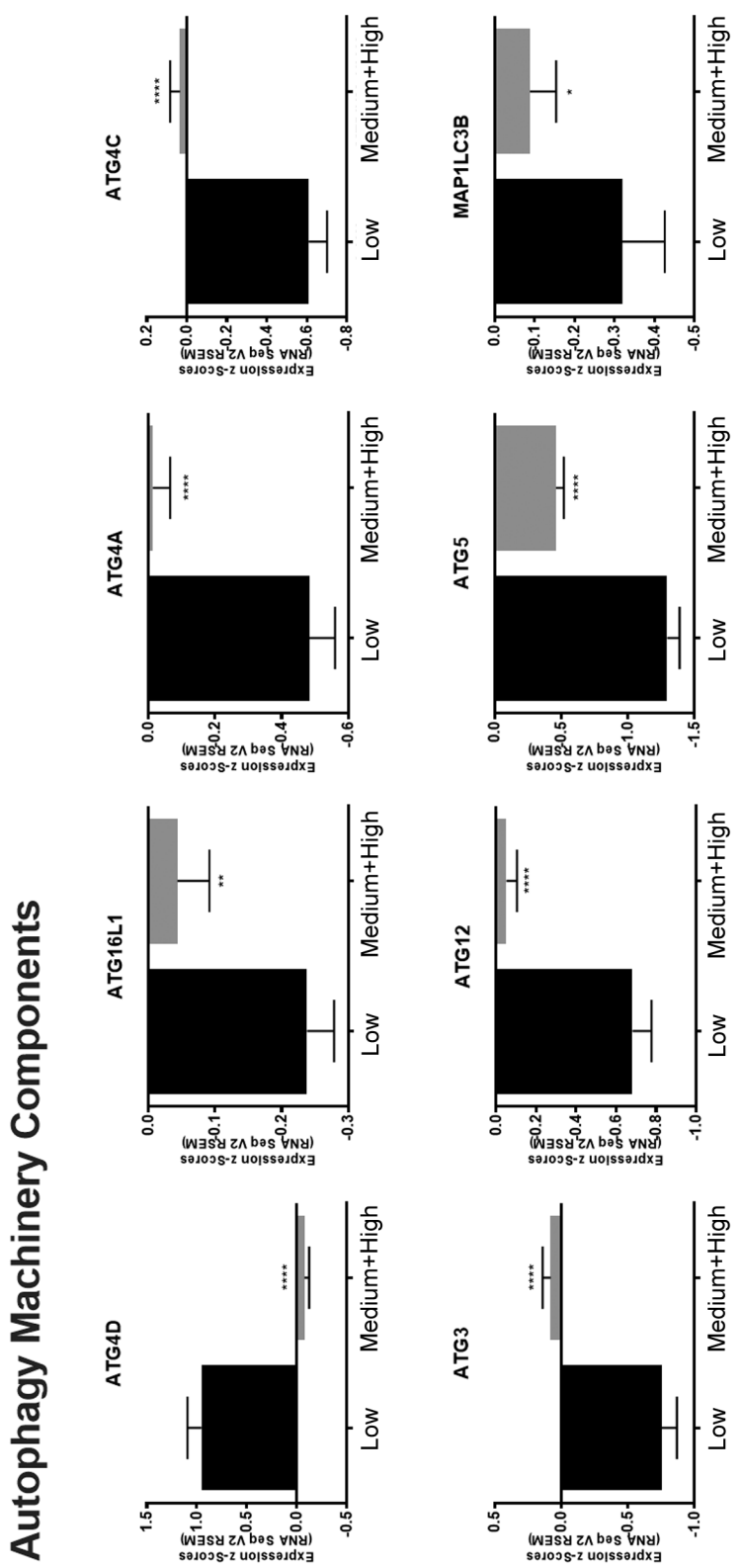

Figure 5. *The expression level of the genes associated with autophagy in groups of patients with low (n=115), medium and high (n=357) expression level of BNIP3L. Expression levels are presented as mean with SEM; un-paired t-test; *p<0.05, **p<0.01, ****p<0.0001.*

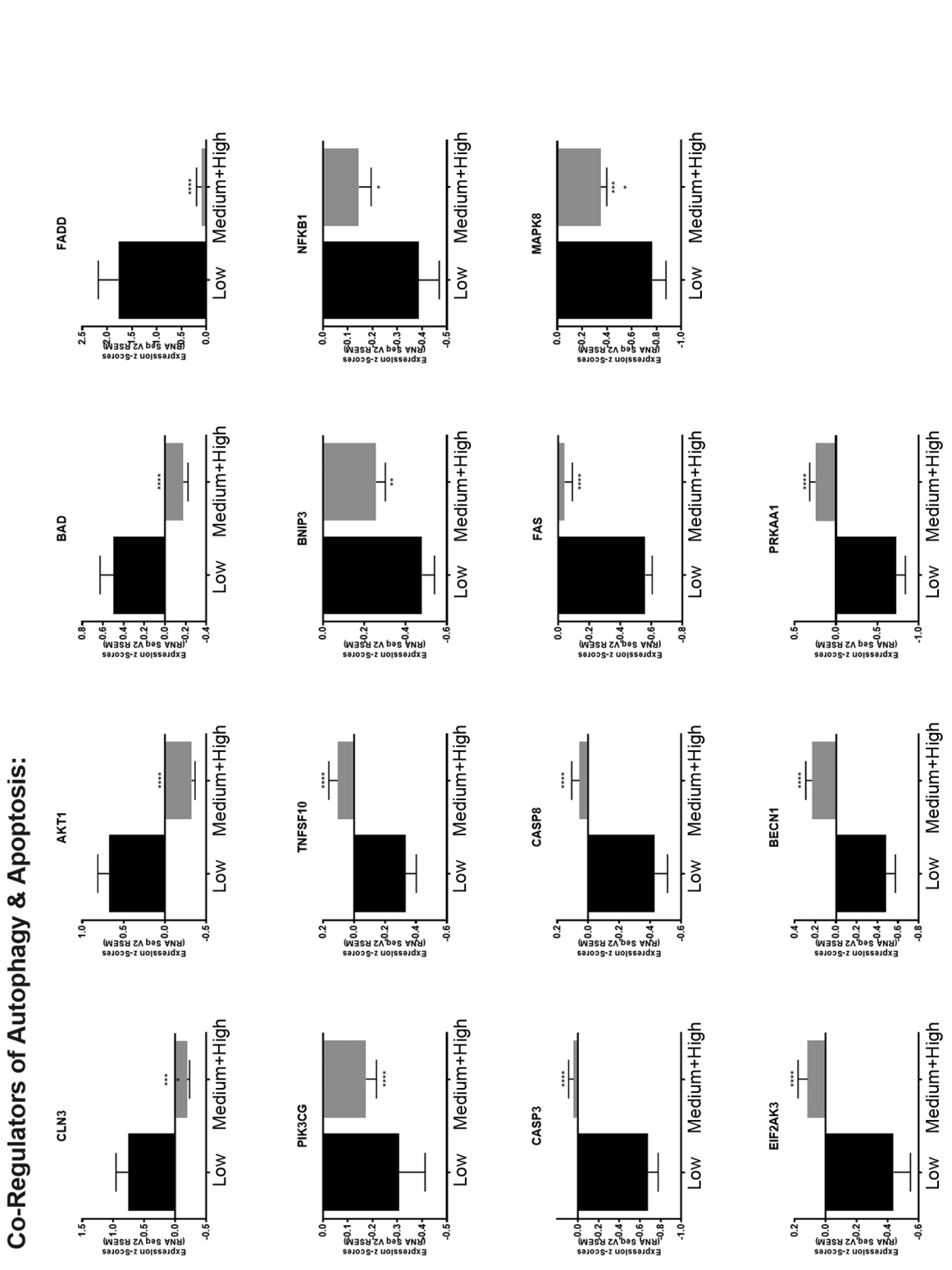

Figure 6. *The expression level of co-regulators of autophagy and apoptosis in groups of patients with low (n=115) and medium and high (n=357) expression level of BNIP3L. Expression levels are presented as mean with SEM; un-paired t-test; *p<0.05, ****p<0.0001.*

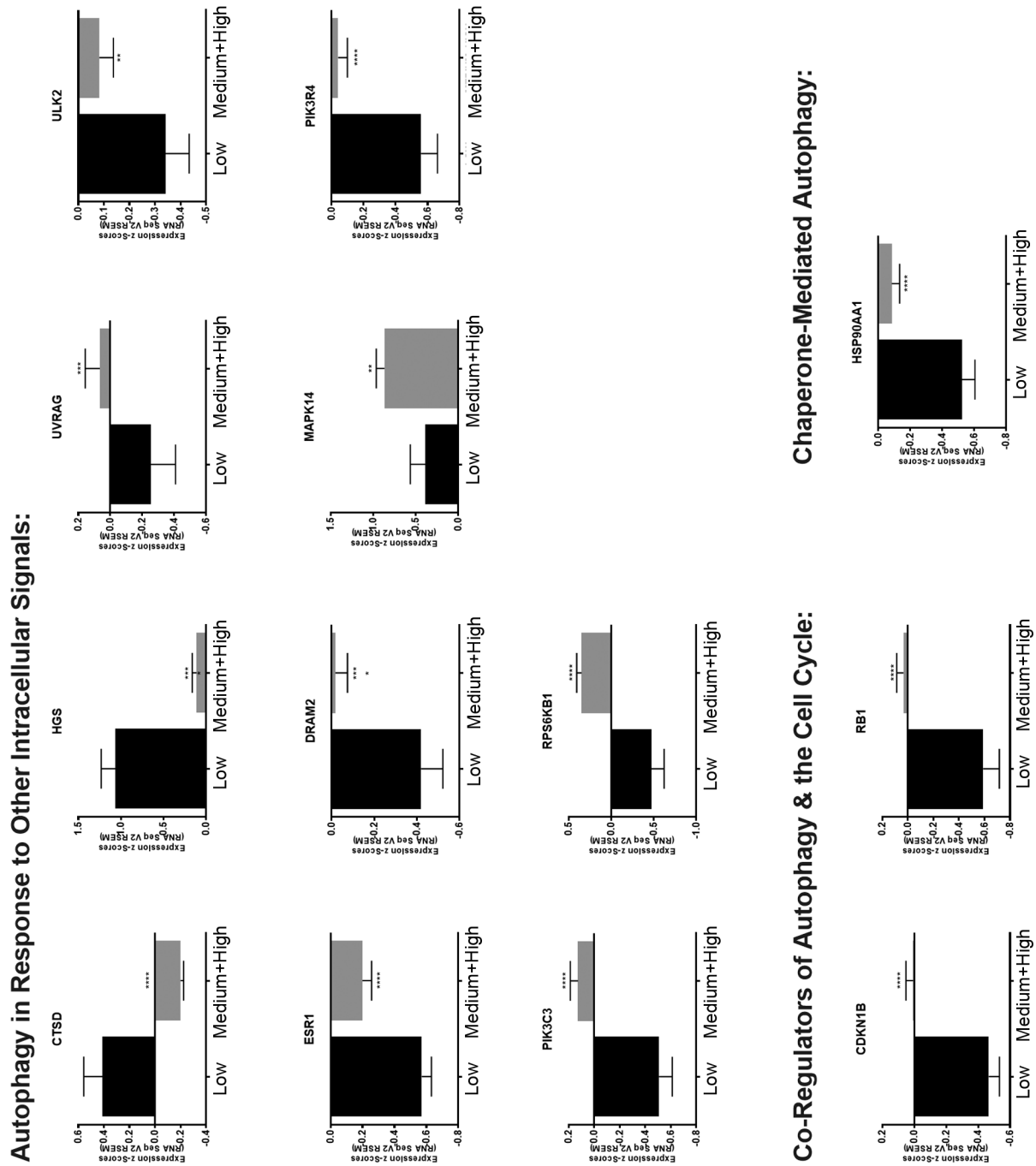

Figure 7. *The expression level of the genes associated with autophagy in response to other intracellular signals, co-regulators of autophagy and cell cycle, and chaperone-mediated autophagy in groups of patients with low (n=115), medium and high (n=357) expression level of BNIP3L. Expression levels are presented as mean with SEM; un-paired t-test; **p<0.01, ***p<0.001, ****p<0.0001.*

also in metastasis, autophagy reveals its dual role (37) with anti-metastatic capabilities at early stages and pro-metastatic at later stages. Our SEREX analysis showed that AGI-101H stimulates an anti-BNIP3L immune reaction in a group of responders. Thus, we hypothesize that in this group the expression of BNIP3L is enhanced, so does the process of autophagy. However, the level of BNIP3L in tumor samples has not been investigated due to the lack of material. Non-responders did not produce antibodies to BNIP3L, most likely due to a low level of autophagy. Accordingly, we drew three conclusions: 1) AGI-101H stimulates autophagy which acts as tumor-suppressor, 2) lack of autophagy in advanced melanoma maintains tumor growth, and 3) AGI-101H response is indirectly related with enhanced autophagy, and there are other mechanisms ensuring good clinical outcome.

There is an urgent need to identify predictive/prognostic markers for more efficient, personalized treatment in order to improve clinical outcomes. Here, we demonstrated that BNIP3L is a strong candidate to be considered as a new biomarker for the prognosis of a disease course and melanoma diagnostics. The antibody response in patients receiving specific treatment could determine the effectiveness of therapy.

Since BNIP3L is a critical factor involved in autophagy, which is indicated by enhanced production of BNIP3L antibodies, AGI-101H directs immune response towards autophagy. In view of existing knowledge targeting autophagy may prove to be successful in fighting melanoma.

Conflicts of Interest

The Authors declare that there are no conflicts of interest regarding this study.

Authors' Contributions

UK carried out the experiments, analyzed the data and prepared the manuscript. TK performed *in silico* analyses and prepared the figures. JM prepared the clinical data of analyzed patients. DK and AM revised the manuscript.

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