Alteration in the level of NR2F2-AS1/ miR-320b / BMI1 differentially converges with the progression of gastric adenocarcinoma toward metastasis

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Research Article

Keywords: lncRNA NR2F2-AS1, BMI1, miR-320b, pathogenesis, gastric adenocarcinoma, expression

Posted Date: March 17th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2681039/v1

Abstract

A regulatory pathway centered on lncRNA NR2F2-AS1/miR20b effects on dysregulation of BMI1 was hypothesized particularly in higher stages, which was confirmed by our bioinformatics examinations. An increase of more than 2-fold for BMI-1 and lncRNA NR2F2-AS1, respectively found in lower stages, and elevation continued with the increasing stage of the disease, and converged with significant downregulation of miR-320b and PTEN, noticing their association with tumor progression and decreased patient survival. lncRNA NR2F2-As1 acts as an oncogene to sponge miR-320b making changes in BMI1. A reduction in the amount of miR-320b against lncRNA NR2F2-AS1 and BMI1 directly correlates with a reduced overall survival rate of patients, especially if this disproportion is more than 3.0. Further study by gene ontology and KEGG pathway enrichment analysis revealed that disruption in the expression of BMI1 interplays in WNT, AKT, and RTK pathways affected the cell cycle, and provide the condition in favor of tumor progression. ROC curve analysis indicated that alteration in the level of BMI1 and lncRNA NR2F2-AS1 showed more than 94.0% sensitivity and specificity to differentiate the lower from higher stages of GC and predict the early onset of metastasis. This study can suggest another molecular aspect in the pathogenesis or prognostic marker for the progression of GC, particularly if this event is also observed in the blood of the patients.

1. Introduction

Gastric cancer (GC) is a commonly diagnosed cancer of the gastrointestinal tract, with more than one million cases and approximately 770,000 deaths worldwide by 2020. Nearly 50% of patients with gastric cancer have metastases, and the 5-year overall survival (OS) of these patients does not exceed 25% (Bray, Ferlay et al., Bernards, Creemers et al. 2013). Despite significant advances in diagnosing and treating gastric cancer using surgical methods and adjuvant chemotherapy, the prognosis for affected patients is relatively unfavorable because more than 80% of affected patients are diagnosed at an advanced stage (Huang, Zhang et al. 2017). Nevertheless, patients with metastatic GC missed the optimal treatment period due to delayed diagnosis. Epidemiological studies have shown that in addition to *Helicobacter pylori* infection, diet, lifestyle, genetics, and ethnicity are associated with the burden of gastric cancer incidence and mortality(Karimi, Islami et al. 2014). BMI1 (B Lymphoma Mo-MLV Insertion Region 1 Homolog) is a well-known oncogene located in chromosome 10p13, at first identified as a cooperator with c-myc to generate pre-b-cell lymphoma in mice (Kim, Yoon et al. 2004). BMI1 belongs to the Polycomb repressive group and has a significant role in cell cycle regulation, hematopoiesis, and senescence (Beà, Tort et al. 2001). Its increased level in various cancers, such as pancreatic adenocarcinoma (Proctor, Waghray et al. 2013), breast cancer (Guo, Feng et al. 2011), and liver cancer (Xu, Lee et al. 2009) was revealed. Wang et al. (2016) reported that BMI1 acts as a negative regulator of PTEN in pancreatic cancer and suppresses its expression (Wang, Jiao et al. 2016). PTEN functions as the primary tumor suppressor in AKT/PI3K pathway, and its suppression leads to the activation of this pathway and subsequently results in cancer invasion and progression (Song, Li et al. 2009). AKT/PI3K is

a central modulator of EMT, and BMI1 also activates this pathway by accelerating the phosphorylation of AKT and stabilizing snails as a transcription factor of N-cadherin (Du, Xia et al. 2014).

Long noncoding RNA (lncRNA) is a noncoding transcript that exceeds 200 nucleotides in length. According to the new version of LNCpedia, over 60,000 lncRNA are listed (Volders, Helsens et al. 2013). This noncoding RNA can act as a negative or positive regulator of its target genes. lncRNA performs its function in the cis or trans-acting way (Yan, Luo et al. 2017). Various studies have shown that lncRNAs dysregulated in GC and are involved in GC as tumor suppressors or oncogenes. For instance, Li et al. (2014) discovered that H19 was overexpressed in GC patients and cell lines, and its overexpression correlated with poor prognosis and tumor progression of GC (Li, Yu et al. 2014). NR2F2-AS1, a newly identified lncRNA, has a joint promoter with NR2F2 and is located on 15q26 (Baribault, Ehrlich et al. 2018). NR2F2 is implicated in angiogenesis and plays a role in cancer metastasis (Le Dily, Métivier et al. 2008, Shin, Kwon et al. 2009). In addition, its dysregulated expression has been reported in endometriosis and infertility (Hawkins, Loomans et al. 2013). NR2F2-AS1 functions as a tumor promoter lncRNA, and it has been indicated to promote cancer development and accelerates metastasis in prostate (Fu, Wang et al. 2020), nasopharyngeal (Qin and Qin 2020), non-small cell lung cancer (Zhang, Zhang et al. 2019, Liu, Li et al. 2021), clear cell renal cell carcinoma (Chen, Zhang et al. 2020), and cervical cancer (Liu, Huang et al. 2020). However, its expression and potential functions in GC are mainly unknown.

Available evidence suggests that lncRNA plays a central role in regulating gene expression by acting as a competing endogenous RNA (ceRNA) for miRNAs (Liu, Zhang et al. 2018). The precedent article using luciferase reporter assay approved that NR2F2-AS1 has a binding site for miR-320b (Zhang, Zhang et al. 2019). miR-320 family consists of 5 members, including miR-320a, b, c, d, and e. The previous study has shown this family downregulated in colorectal carcinoma and can be served as a reliable biomarker for its early detection (Tadano, Kakuta et al. 2016). miR-320b is positioned in 1p13.1, showing its changed expression in various human cancers, such as colorectal and nasopharyngeal(Wang, Cao et al. 2015, Li, Tang et al. 2016). Our *in-silico* study by multiple databases and applying different bioinformatics tools confirmed the interaction between NR2F2-AS1 and miR-320b (presented in the method and result of this article) about the PI3K pathway. Previous studies have shown that miR-320b plays a tumor suppressor in multiple cancers, including colorectal cancer (Wang, Cao et al. 2015) and pancreatic cancer (Jingyang, Jinhui et al. 2021). Still, there is no evidence of miR-320b expression in GC. BMI1, a well-known oncogene, is a direct target of miR-320b.

In this cross-sectional study, we hypothesized that the dysregulation in the level of the components in lncNR2F2-As1 / miR-320b / BMI1 axis might be disrupted in gastric cancer compared with normal tissues. According to databases and our bioinformatics study, we believed that this axis could have an influential role in carcinogenesis and upturning the conditions toward supremacy in favor of tumor progression, mainly through PI3K/AKT pathway. Therefore, the expression profile factors mentioned above have been evaluated in different stages of cancer. With our best knowledge and our search on the web, it seems that this study is a pioneer in this aspect. On the other hand, it was also tried to find the correlation between changes in their expression and the progression of tumors and, consequently, the

survival rate of patients with gastric cancer. We also believe that our obtained data could reveal or suggest another molecular aspect in the pathogenesis and progression of gastric cancer (GC) and even introduce new drug targets.

2. Materials And Methods

2.1- in silico study

At first, the interaction between lncRNA NR2F2-AS1 and miR-320b was bioinformatically confirmed by employing Diana tools (http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=site/index) database. The mirDB (http://www.mirdb.org) and star base (https://starbase.sysu.edu.cn/agoClipRNA.php?source=lncRNA) were also hired to appraise the interaction between miR-320b and BMI1. The expression pattern of NR2F2-AS1 in GC and adjacent normal tissues were evaluated using the lnCAR database (https://lncar.renlab.org/). lnCAR is a database of human lncRNA expression profiles obtained from the re-annotation of general microarray expression data from more than 57,000 samples of various cancers. We also used UALCAN (http://ualcan.path.uab.edu/), GEPIA (http://gepia.cancer-pku.cn/), and starbase (https://starbase.sysu.edu.cn/panGeneDiffExp.php) to predict the expression pattern of BMI1 in GC tissues in comparison with adjacent normal tissues. It is worth mentioning that GEPIA recruited TCGA and GTEx datasets of gene expression level in its turn.

Gene ontology (GO) and KEEG pathway enrichment assessment were used to explore the effect of changes in the expression pattern of our studied axis in different cellular networks. These cases include Biological Function (BF), Molecular Function (MF), and Cellular Components (CC) to justification in carcinogenesis and tumor progression were also applied. For this purpose, ENRICHR (https://maayanlab.cloud/Enrichr/) and ShinyGO (http://bioinformatics.sdstate.edu/go74/) web-based tools have been recruited. The co-expression network of BMI1, miR-320, and lncRNA NR2F2-AS1 with other genes and noncoding RNAs were displayed by using Cytoscape (version 3.7.0)

The relationship between lncNR2F2-AS1 level and overall patient survival (OS) and Relapse-Free Survival (RFS) were also predicted by employing the GEPIA2 (http://gepia2.cancer-pku.cn/#survival) database.

2–2 In vitro study

2-1-1 Specimen and patients

In the current study, 40 paired tissue specimens and matched standard adjacent tissue samples obtained from the GC patients (29 males, 11 females, in the age range of 33–86 years, mean age 59.1 ± 12.51years) underwent an operation at the Imam Khomeini Cancer Institute between June 2018 and November 2020. None of them received chemotherapy or any other kind of medical intervention. The inclusion criteria included those newly diagnosed with stomach cancer and with data of at least two years of follow-up (monthly phone calls) after hospitalization. Patients with clinical disorders other than gastric cancer, those initiating treatment for any clinical condition, and those who died from causes unrelated to GC during follow-up were excluded. The patient was diagnosed by histopathological analysis

of tumor resection. At the time of surgery, non-tumor tissue (2 cm adjacent to the tumor) and GC tissue (50–100 mg per sample) were collected from each patient. After collecting all tissue and keeping RNase-Later (cat No. R0901, Sigma; Germany), they were stored in a freezer at -80°C before use. Tissues histopathologically confirmed by the pathology department of Imam Khomeini Hospital. The stage and grade of tumors have been determined by a pathologist based on the TNM classification of collected specimens. 35% of samples belong to the lower set, 22.5% are categorized in a non-metastatic higher stage, and 42.5% of samples belong to the metastatic higher stage (**Table-I**). Based on guidelines and similar studies, 5 cm is considered a cutoff for grouping samples according to tumor size (Zu, Wang et al. 2013, Chen, Ou-Yang et al. 2017).

2.1.2- Total RNA extraction and cDNA synthesis: GC and non-tumor frozen tissue were pulverized by grinding with liquid nitrogen. TRIzol® Reagent (cat No. 301-001 / 301-002, GeneAll, Korea) was mixed with tissue powder (1 ml per 50mg tissue) to extract total RNAs. All RNA samples were treated with DNase-I to eliminate genomic DNA. RNA concentration was measured by Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, CA, USA). RNA quality was evaluated by 2% agarose gel electrophoresis. The reverse transcription of miRNA was performed using the First Strand microRNA cDNA Synthesis Kit (cat No. 218160, 218161, Hilden, Germany). A cDNA synthesis kit (cat No. 22701, add Bio, Korea) was used to convert RNA to cDNA using equal amounts of RNA in all samples according to the manufacturer's instructions.

2.1.3 Primer design and real-time PCR: The sequence of primers used for real-time PCR is shown in **Table S1 (supplementary file)**. The primers used for the studied genes were obtained from the Origene (https://www.origene.com/search?q=primer) and checked by an oligo analyzer (https://eu.idtdna.com/pages/tools/oligoanalyzer). The primer used for miR-320b was designed by primer BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). SYBR Green RT-PCR Kit (cat No. A323402, Ampliqon, Denmark) was applied in Rotor-Gene™ 6000 system (Corbett Research, Australia). PCR mixture (20 µl) was prepared according to the manufacturer's instruction and briefly contained 0.5 µl of each primer and 1 µl template cDNA in addition to 12.5 µl of master mix. Each experiment in this study was performed in triplicate. The serial dilution of the genes' PCR product was utilized to standardize real-time PCR. Moreover, we applied a no-template sample as the negative control. GAPDH and U6 were also measured as housekeeping to normalize our studied genes' expression level and microRNA, respectively. Ultimately, the relative expression in transcription level was calculated using the $2^{\wedge-\Delta\Delta CT}$ method.

2.1.4 Immunohistochemistry (IHC): The protein level expression of BMI1 and PTEN were assessed by immunohistochemical staining according to the manufacturer's protocol. GC tissue samples were fixed in 10% formalin, then embedded in paraffin and cut into 4 µm segments using a microtome (Leica, Germany). After the deparaffinization of slides and the washing stage, slides were treated with 3% hydrogen peroxide and blocked with 10% goat serum for 1 hour at 37°C. Primary antibodies were added, and slides were incubated at 4°C for 24 hours. In the next step, biotinylated secondary antibodies were added, and incubation was enforced at room temperature for 1 hour. After Streptavidin-HRP addition, sections were stained with DAB substrate and Hematoxylin to stain the counter. Two pathologists

assessed slides, and 100 cells were counted randomly. Samples that more than 10% of their cells were stained moderately or strongly were considered positive, and lower than 10% were deemed harmful. The exclusion of the primary antibody was applied as a negative control.

2.1.5 Western blot analysis: Total protein was extracted from tissues. Protein concentration was assessed by bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Extracted proteins were separated using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein was transferred to polyvinylidene difluoride (PVDF) membranes. At 40C for 24h, primary antibodies against BMI1 and PTEN (Abcam) were incubated. After washing with Tris-buffered saline (TB) with Tween-20, the membrane was incubated with secondary antibodies for 1 hour at room temperature. An ECL detection reagent detected signals. GAPDH was applied as an internal control.

2.1.6 Statistical Analysis: All data are presented as mean ± SD (standard deviation). All data were analyzed using GraphPad Prism 9.3 and SPSS 21. Differences between adjacent normal tissues and GC tissues were compared using paired Student's t-test. Differences in more than three groups were compared using One-Way ANOVA. Spearman analysis revealed the correlation between studied factors (**Table S2 & S3 in supplementary files**). The ROC curve was used to indicate the specificity and sensitivity of the studied axis. Survival curves were constructed using the Kaplan-Meier method test, so we had to determine a cut-off. In this study, we used a pioneer criterion, which was not used before. The proportion of two studied genes, proven to interact directly, has been considered for this purpose. The turning point in the ratio graph in different stages seems to play as a suitable cut-off (**Fig. S1 in supplementary files)**. The calculated ratio of "changes in the expression" in location T3a was near the plot's turning point in all studies genes, which was considered a cut-off for the Kaplan-Meier method. $P < 0.05$ was considered to indicate a statistically significant difference.

3. Result

3.1 bioinformatics prediction for studied axis: Increased expression of BMI1 was observed in tumor tissues compared to normal tissues in all three databases (Fig. 1a-c). However, a significant difference was not observed in the different stages of GC compared to each other (Fig. 1d). Different expression of lncRNA NR2F2-AS1 was observed in GC tissues compared to normal tissue using lnCAR (Fig. 1e). To investigate the association of lncRNA NR2F2-AS1 expression with disease progression, the overall survival (OS) of patients was examined using the GEPIA database. The result revealed that the OS of patients in the high-expression group of lncRNA NR2F2-AS1 was worse than the patients with a low level of lncRNA NR2F2-AS1 (Fig. 1f). According to the dataset GSE93415, miR-320b is one of the miRs that has been shown to have reduced expression in gastric cancer. Searching in the Starbase revealed that lncRNA NR2F2-AS1 has a binding site for miR-320b (Fig. 1g). The interaction between miR-320b and BMI1 was proven by mirDB (Fig. 1h). The interactions of BMI lncRNA NR2F2-AS1, and miR-320b has been shown in **Fig. S2** (**supplementary file**). The interaction network of BMI1 with noncoding RNAs was displayed in Fig. 1i using Cytoscape (version 3.7).

3.2 Over-expression of BMI1 in GC tissues: A significant increase in BMI1 transcription was found in GC tissues compared to its normal adjacent tissues (Fig. 2a). The expression of BMI1 at the protein level was also examined by employing western blot and IHC (Fig. 2b & 2c), showed higher expression in higher stages as compared with lower stages. The upward trend in the BMI1 face continues with the increasing stage of the disease (Fig. 2d). The BMI1 level was significantly elevated by more than 2 -fold in all stages compared with the normal adjacent tissues. BMI1 expression was also compared between metastatic and non-metastatic groups. Results demonstrate that the level of BMI1 was increased in gastric tumors of individuals with metastatic stages in comparison with the non-metastatic (Fig. 2e). Tumors larger than 5 cm showed a significantly higher level of BMI1 near 1.5-fold ($p < 0.0001$). At the base of age and gender, there wasn't a significant difference ($p = 0.029$ and $p = 0.82$, respectively). The demographic data was shown in **Table S3.**

3.3 lncRNA NR2F2-AS1 is overexpressed in GC tissue: The result has been presented in Fig. 3a. Our results demonstrate that lncRNA NR2F2-AS1 was markedly upregulated in cancerous tissues compared to adjacent normal tissues ($P < 0.0001$) in most of the cases. Further investigation has been carried out to find the relationship between lncRNA NR2F2-AS1 level and clinicopathology characteristics in patients with GC, such as tumor stage, grade, size, and metastasis status. It is no significant difference in the amount of lncRNA NR2F2-AS1 between men and women with gastric cancer ($p = 0.828$) and age groups (cut-off 60 years old) ($p = 0.291$) (data has not been shown). The level of lncRNA-NR2F2-AS1 was average in cancerous tissue by 3.2-fold in comparison to normal tissues ($P < 0.0001$) (Fig. 3b). The expression of LncRNA NR2F2-As1 was also compared in different stages. The expression was observed to elevate for 1.7-fold in stage of T2. The expression was observed to elevate 1.7-fold in the stage of T2, whereas this became more than 3.5-fold for the stage of ≥ T3a (Fig. 3c). Examination of the relationship between tumor size and lncRNA NR2F2-AS1 expression revealed that tumors with more significant than 5 cm had significantly higher expression compared to tumors smaller than 5 cm ($P = 0.02$). There was a noteworthy difference between the expression level of lncRNA NR2F2-AS1 within the non-metastatic group with patients who had metastasized ($P < 0.0001$). The level of lncRNA NR2F2-As1 was significantly raised 1.8-fold in metastatic patients compared to tumor tissues of non-metastatic patients (Fig. 3d).

3.4 Down-regulation of miR-320b in GC tissues: The level of miR-320b expression was noticeably reduced in cancerous tissues compared to adjacent normal tissues ($p < 0.0001$) in each case (Fig. 4a & 4b). That revealed an inverse correlation between miR-320b expression and the stage of the disease. The higher stage was associated with a decrease in miR-320b expression (Fig. 4c). The same trend was observed for tumor size in that tumor smaller than 5 cm showed higher expression of miR-320b ($P = 0.01$). Moreover, a significant difference between the level of miR-320b within the non-metastatic group of patients as compared with metastasized patients was observed ($p = 0.0003$) (Fig. 4d). Interestingly a significant relationship was observed between miR-320b expression and age. Patients under 60 years had a low level of miR-320b expression in comparison to patients over 60 years ($p = 0.039$). No difference was observed between the male and female groups at the base of the miR-320b level ($p = 0.8$) (data was not shown).

3.5 Changes in expression of BMI/ miR-320b/ lncRNA NR2F2-AS1 converged with downregulation of PTEN expression: PTEN also showed a reduced level in cancerous tissues compared to healthy tissues (Fig. 5a). Higher stages of the tumor demonstrated lower PTEN levels compared to the lower settings (Fig. 5b). They were performed in western blot analysis. The result showed that PTEN was reduced in protein level with an increase in the stage of cancer (Fig. 5c). But, PTEN expression in tumors larger than 5 cm was not seen a remarkable difference as compared to tumors < 5 cm ($p = 0.5$). There was not also a vast distinction in PTEN expression in gastric tumor tissues between metastatic and non-metastatic ($p = 0.01$) (Fig. 5d). There wasn't any significant difference between PTEN expression and age ($p = 0.66$) and gender ($p = 0.6$) indexes.

3.6 Correlation analysis between studied genes was apprised in Fig. 6. BMI and lncNR2F2-AS1 showed a positive correlation. It means that upregulation was observed in both. Both BMI and lncRNA NR2F2-AS1 showed a negative correlation with miR-320b.

3.7 ROC curve analysis has been done to confirm the potential diagnostic value of the studied genes (Fig. 7). Upregulation of BMI significantly shows more than 94% specificity and sensitivity to differentiate the higher from lower and metastasis from non-metastatic stages (Fig. 7a & 7b). The same trend was observed for lncRNA NR2F2-AS1, but a specific sensitivity was about 87% obtained to detect metastasis from the non-metastatic stage (Fig. 7c & 7d). Even though the level of miR-320b was significantly reduced in higher stages and metastasis but AUC of around 78% was obtained to differentiate higher from lower settings, which could not be so noteworthy. ROC curve analysis has also been done for the grade. However, only BMI showed significant specificity and sensitivity to differentiate higher from lower stages (AUC: 0.7353; $p = 0.0118$), which is not also remarkable. Roc curve analysis of lncRNA NR2F2-AS1 and miR-320b for grades was also insignificant.

3.8 Survival analysis of studied axis: The survival rate of patients according to the ratio of changes in expression of NR2F2-AS1/ miR-320b has been shown in Fig. 8a. The 50 percent of patients with a ratio $\geq$ 6.7 showed a survival rate of near to 18 months, but it reaches to 42 months for ratio < 6.7. At 50% of survival probability, patients with a high BMI1 against miR-320b and PTEN showed reduced survival time (12 months) compared to patients with a low BMI1 to miR-320b and PTEN (Fig. 8b & 8c).

3.9 GO and KEGG pathway enrichment analysis were applied. Investigate the possible mechanism by which BMI1 participates in carcinogenesis and progression of gastric cancer on the base of the co-expression of similar genes with the same expression pattern. The results suggest that transcription regulation and Histones modification were regulated by BMI1 (**Fig. S3a**). Ubiquitin ligase complex and heterochromatin status were also controlled by BMI1 (**Fig. S3b & S3c**). **Figure S3d** shows the top 10 pathways that relate to BMI1. Cell cycle and ubiquitin regulation pathways are two critical pathways regulated by BMI1.

4. Discussion

The metastasis and recurrence of gastric cancer (GC) have resulted in considerable mortality; therefore, it is logical to find practical diagnostic tools for early prediction of metastasis in therapeutic strategy. BMI1 promotes tumor progression and interacts with different genes involved in invasion and metastasis **(Guo, Feng et al. 2011)**. Numerous endeavors have been made in recent years to find gastric cancer (GC) molecular mechanisms and the role of ncRNAs underlying disease development. It has been assumed that the essential role in regulating BMI1 is epigenetically disrupting some noncoding RNAs. It can interact with Wnt, Akt, Notch, Hedgehog, and receptor tyrosine kinase (RTK) pathways **(Douglas, Hsu et al. 2008)**. Bioinformatically, data revealed that the lncRNA NR2F2-AS1 is overexpressed in cancerous tissues compared to adjacent normal tissues. Several studies show the role of ceRNA for lncRNA NR2F2-AS1 to modulate various microRNAs, such as miR-4429 and miR-545-5p. Experimental techniques such as luciferase assay and RNA immunoprecipitation (RIP) chip have been assumed (Liu, Huang et al. 2020, Liu, Li et al. 2021). The microRNAs, down-regulated in GEO data sets such as GSE93415, were searched and considered due to the ceRNA mechanism of lncRNA NR2F2-AS1. Several microRNAs were selected, which were consequently examined and confirmed in different noncoding databases, which resulted in Cytoscape interaction analysis. Finally, miR-320b was chosen, and its downregulation was reported in various cancer, including gastric. An interaction prediction tool demonstrates that lncRNA NR2F2-AS1 has a binding site for miR-320b. Zhang et al. (2018) approved this interaction using a dual luciferase assay **(Zhang**, Zhang et al. 2019**)**. On the other hand, BMI1 is one of the direct targets of miR-320b, and its level seems to be controlled by miR-320b **(Zhang**, Zhang et al. 2019**)**. On the other hand, the bioinformatic study showed no interaction between miR-320b and PTEN (Cytoscape interaction analysis).

Overexpression of BMI1 was reported in various databases. Its upregulation continues with an increase in the stage of the disease. Growing evidence demonstrates that BMI1 dysregulation plays a significant role in several human cancer developments **(Hoenerhoff, Chu et al. 2009, Paranjape, Balaji et al. 2014)**. BMI1 belongs to a polycomb repressive family, acts as a transcriptional repressor, and silences gene expression by changing the chromatin structure **(Yang, Hsu et al. 2010)**. In the current study, our results confirmed that the level of BMI1 in gastric cancer is higher than in normal adjacent tissue. IHC staining also confirmed that BMI1 in protein level was significantly elevated in cancer cells. After applied of western blot, the data was consistent with the qPCR and IHC findings. Our research has demonstrated that over-expression of BMI1 is associated with advanced tumor stage, larger tumor size, metastasis status, and reduced overall survival rate. Other studies on various cancers, including medulloblastoma **(Leung, Lingbeek et al. 2004)**, glioma **[40]**, and hepatocellular **(Sasaki, Ikeda et al. 2008)** showed similar results. Evaluation of studies on BMI1 level in gastric cancer showed that its positive regulation leads to invasion and promotion of metastasis **(Li**, Li et al. 2010, **Xu**, Li et al. 2014, **You, Wang et al. 2020)**. Chen et al. (2013) reported that BMI1 overexpression in gastric cancer influences the cell cycle and inhibits the apoptosis of cancer cells **(Chen, Lian et al. 2013)**. The previous study of the high level of BMI1 in the gastric cancer cell line (BGC823) was shown that knocking down BMI1 enhances the cell senescence rate and promotes metastasis **(Gao, Li et al. 2013)**.

Gene ontology (GO) and KEGG enrichment analyses were conducted to justify the different modes of action of BMI1 in tumor development. Data have shown that BMI1 was mainly involved in processes

such as Histon modification, chromatin remodeling, and transcription regulation. KEGG enrichment analysis also revealed that BMI1 regulates cell cycle progression and controls multiple gene expressions. Therefore, it plays a role as a transcription regulator in cancers. Seeking previous literature has shown that BMI1 cooperates with several factors involved in the Epithelial-Mesenchymal transition (EMT), and regulating their expression, contributes to tumor development **(Joensuu, Hagström et al. 2011, Du**, Xia et al. 2014**)**. BMI1 stabilizes Snail and mediates its expression level as a transcription factor involved in the invasion of cancer cells and down-regulates E-cadherin, leading to head and neck metastasis **(Yu, Lo et al. 2011)**. A study conducted by Wang and his colleagues (2022) demonstrated that BMI1 acts as a transcription repressor by binding to the SIK1 promoter. BMI1 recruits RING1B to induce histone H2A monoubiquitination and suppress H3K4 trimethylation. These features lead to cell proliferation and enhance osteosarcoma metastasis **(Wang, Wu et al. 2022)**. These findings are converged with the results of GO and KEGG enrichment analysis.

The investigation shows dysregulation of lncRNAs plays vital roles in tumors, progression, and metastasis of gastric cancer through induction of the disorder in cell proliferation and apoptosis**(Xia, Liao et al. 2014)**. LncRNA NR2F2-AS1 shows as an oncogenic lncRNA in several human cancers **(Ghorbanzadeh, Poor-Ghassem et al. 2022)** including colorectal **(Li, Jiang et al. 2020, Liu, Qian et al. 2020)**, nasopharynx **(**Qin and Qin 2020**)**, and cervical **(Liu, Huang et al. 2020)**. lncRNA NR2F2-AS1 is the antisense transcript of the NR2F2 gene. The oncogenic role of NR2F2 has been reported in several cancers **(Le Dily, Métivier et al. 2008, Shin, Kwon et al. 2009)**. Due to having a joint promoter, it seems that the expression of the two is dependent on each other, and the elevated expression of NR2F2 can lead to the upregulation of lncRNA NR2F2-AS1 **(Baribault, Ehrlich et al. 2018)**. At the base of our best search on the web in most databases, there is no evidence to measure the expression level of lncRNA NR2F2-AS1 in gastric tissues and compare in different stages. It seems this is a pioneer study in its turn. The current research has found that lncRNA NR2F2-AS1 is over-expressed in GC tissues. High expression of lncRNA NR2F2-AS1 remarkably correlates with advanced TNM stage, histological grade, reduced overall survival, and poor prognosis in GC patients.

Decreased expression of miR-320b has been reported in many human cancers, including colorectal **(Wang, Cao et al. 2015)**, glioblastoma **(Roth, Wischhusen et al. 2011)**, bladder **(Song, Xia et al. 2010)**, pancreatic **(Jingyang, Jinhui et al. 2021)**, and osteosarcoma **(Lv, Miao et al. 2018)**. They play a significant role in tumorigenesis. This research showed that the expression of miR-320b in cancerous tissues was significantly reduced compared to normal tissues, in contrast to the face of NR2F2-AS1. As the stage and grade of the disease increased, a significant decrease in the expression of miR-320b was observed, which indicates the importance of this microRNA in the progression of the disease. To date, no evidence reports miR-320b expression level in gastric cancer, and the current study appraised miR-320b expression and its association with clinicopathological features for the first time. Examination of miR-320b level in pancreatic cancer revealed that its downregulation connects with cancer cell proliferation **(Jingyang, Jinhui et al. 2021)**.

A study performed by Song et al. (2009) has shown that BMI1 directly binds to the PTEN locus and suppresses its expression at the mRNA level, consequently leading to activation of the AKT/ PI3K pathway. On the other hand, BMI1 accelerates AKT phosphorylation and activates this oncogene, and results in AKT/ PI3K pathway activation **(Song, Li et al. 2009)**. We measured the present study's PTEN amount in transcription and translation levels. The results indicate that PTEN expression is low in the cancerous tissue compared to adjacent normal tissue. Guo et al. (2020) have shown that PTEN level is significantly lower in gastric cancerous tissues compared to matched normal tissues and revealed its possible role in tumor development **(Fei, Ebert et al. 2002)**. Our evaluation of the correlation between PTEN expression and clinicopathological factors indicated an inverse link. But no significant difference was found in the expression of PTEN levels among stages of T2 and T3, though the decrease of PTEN was significantly more in the T4 location compared to T3.

Interestingly, a lower amount of PTEN converged with larger tumors. This obtained trend shows that alteration in PTEN occurred at the early onset of gastric cancer. It seems to strengthen its slope at higher stages. Perhaps the significant upward trend of BMI1 can be one of its reasons. An imbalance in the level of miR-320b and lncRNA NR2F2-As1 can epigenetically lead to increasing BMI1 in gastric cancerous tissue.

To find out the role of the studied axis in patients' survival and evaluation of their biomarker potency, we carried out an overall survival analysis and ROC curve assessment, respectively. The Kaplan-Meier test was used to appraise the overall survival rate. But, in this research, we applied a pioneer criterion, which has not been used before. Based on our best search in databases, this is the first time that the overall survival rate is calculated according to the proportion of changes in the expression of two genes that directly interact. In the 50% of survival probability, according to Fig. 8b, the patients with a high level of BMI1 against a low amount of miR-320b have shown reduced overall survival (18 months) compared to those have a low ratio of BMI1 to miR-320b (42 months). It means that their survival rates decline more than 2-fold (around 2.3) if the percentage of changes in BMI to miR-320b is higher than 3.7. 50% of patients with a higher level of lncRNA NR2F2-AS1 who almost belong to the higher stages have a reduced survival rate (12 months) compared to those who express a low amount of lncRNA NR2F2-AS1 (42 months). It indicates that patients' survival was reduced by about 3.5-fold in a high expression of lncRNA NR2F2-AS1 compared to a low amount of lncRNA NR2F2-AS1. A reduction in the amount of miR-320b against lncRNA NR2F2-AS1 and BMI1 directly correlates with a reduced overall survival rate of patients. Our study revealed that BMI1 could potentially be considered a marker for the differentiate lower stage from the higher stage of GC and predicting the early onset of metastasis with 94% specificity and sensitivity. ROC curve analysis results demonstrate that lncRNA NR2F2-AS1 showed remarkable specificity and sensitivity to differentiate higher metastasis stages from lower stages. But it seems that miR-320b could not be a reliable marker due to its calculated specificity and sensitivity to distinguish a lower stage of disease from higher stages and metastasis.

Overall, the obtained data of this investigation demonstrated a significant correlation between changes in BMI1 / lncRNA NR2F2-AS1 / miR-320b axis and the progression of gastric tumors toward invasion and

metastasis (Fig. 9). It seems that upregulation of lncRNA NR2F2-AS1 can lead to increasing BMI1 levels by sponging miR-320b. These findings suggest lncRNA NR2F2-AS1 can play a role as a potent oncogene, especially in higher stages. Overexpression of lncRNA NR2F2-AS1 converged with downregulation of miR-320b, and correlation analysis showed their inverse relation. It was probable that the decrease in miR-320b level (as important tumor suppressor microRNA) is because of increasing long noncoding RNAs such as NR2F2-AS1, which have microRNA regulatory elements (MREs) site for miR-320b. Overexpression of BMI1 converged with the higher stage of the disease and reduced the overall survival rate. These results introduced another molecular mechanism involved in the onset of the invasion of cancer cells. It seems that altering the expression profile of the BMI1/ lncRNA NR2F2-AS1/ miR-320b axis was effective in upturning the conditions for the supremacy of tumor progression because of interplaying the BMI1 in several pathways involved in tumorigenesis. Remarkably, the onset of progression toward higher stages converged with a disproportion of more than 3.0 between BMI and miR-320b, and more than 6.0 was between lncRNA NR2F2-AS1 and miR-320b in this axis. If these changes were also significantly taken place in blood, therefore the member of this axis, particularly BMI1 and lncRNA NR2F2-AS1, could be a candidate as a diagnostic marker to detect early-onset or pre-onset metastatic stage. They can also be suggested as a prognostic marker to determine the progress rate of gastric cancer, mainly to evaluate treatment efficiency. Hence, further study in blood and a larger group can reach reliable results that are practical in medicine.

Declarations

Acknowledgement

Thanks to all patients and their families for participating in this study and the Hormozgan Molecular Medicine Research Center.

Conflict of Interest

The authors declare that they have no conflict of interest.

Data availability statement

The datasets generated during the current study are available from the corresponding author on reasonable request.

Funding Information

This work was supported by a grant from the office of the Vice-Chancellor of Research at the Hormozgan University of Medical Sciences, Bandar Abbas, Iran.

Ethics Approval

This study was approved by the ethics committee of Hormozgan University of Medical Sciences (Ethical code: IR.HUMS.REC.1399.459).

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Table

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Figures

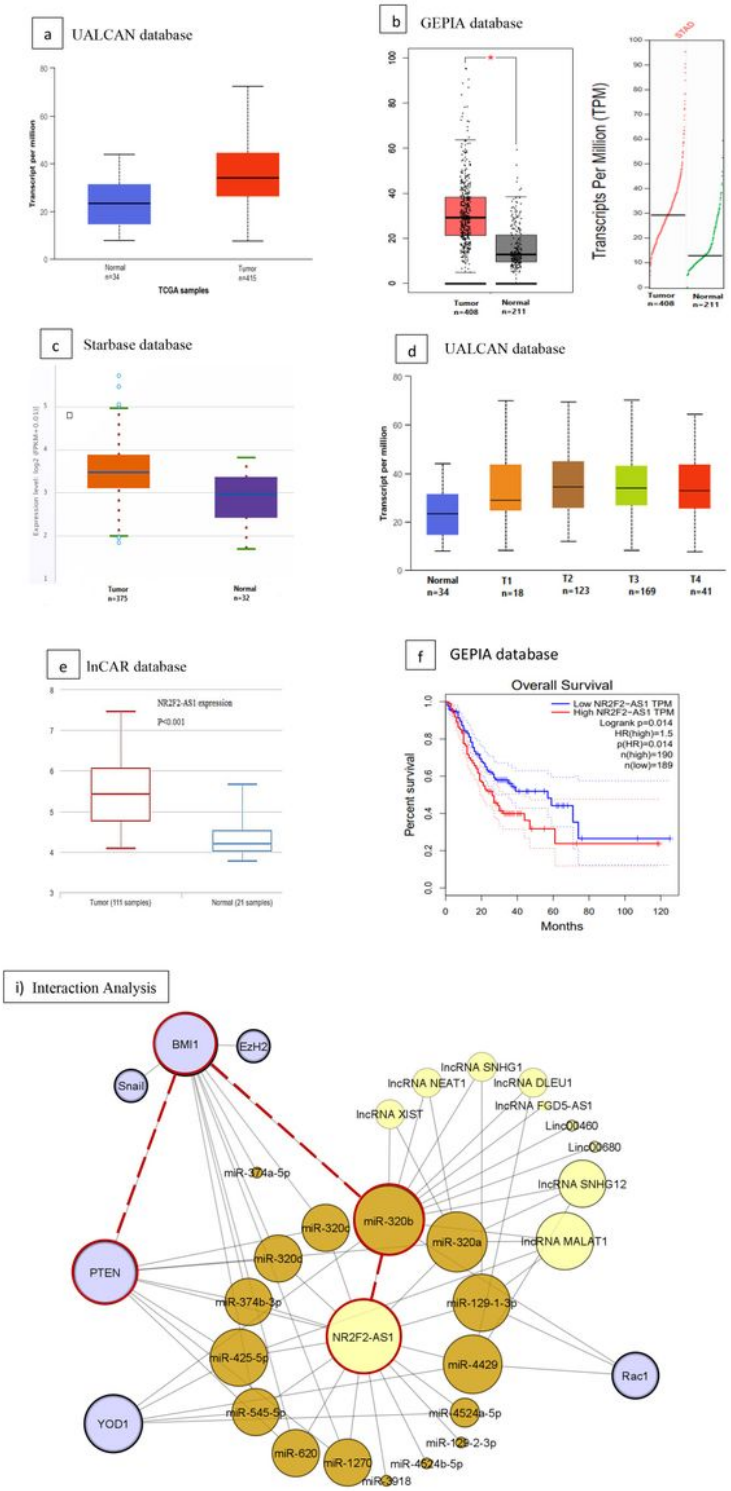

Figure 1

Overexpression of BMI1 in gastric tissues and stages according to UALCAN **(a, d)**, GEPIA **(b)**, StarBase **(c)**. Overexpression of lncRNA NR2F2-AS1 in gastric tissues **(e)**, and its correlation with overall survival **(f)**. Interaction analysis **(i)**.

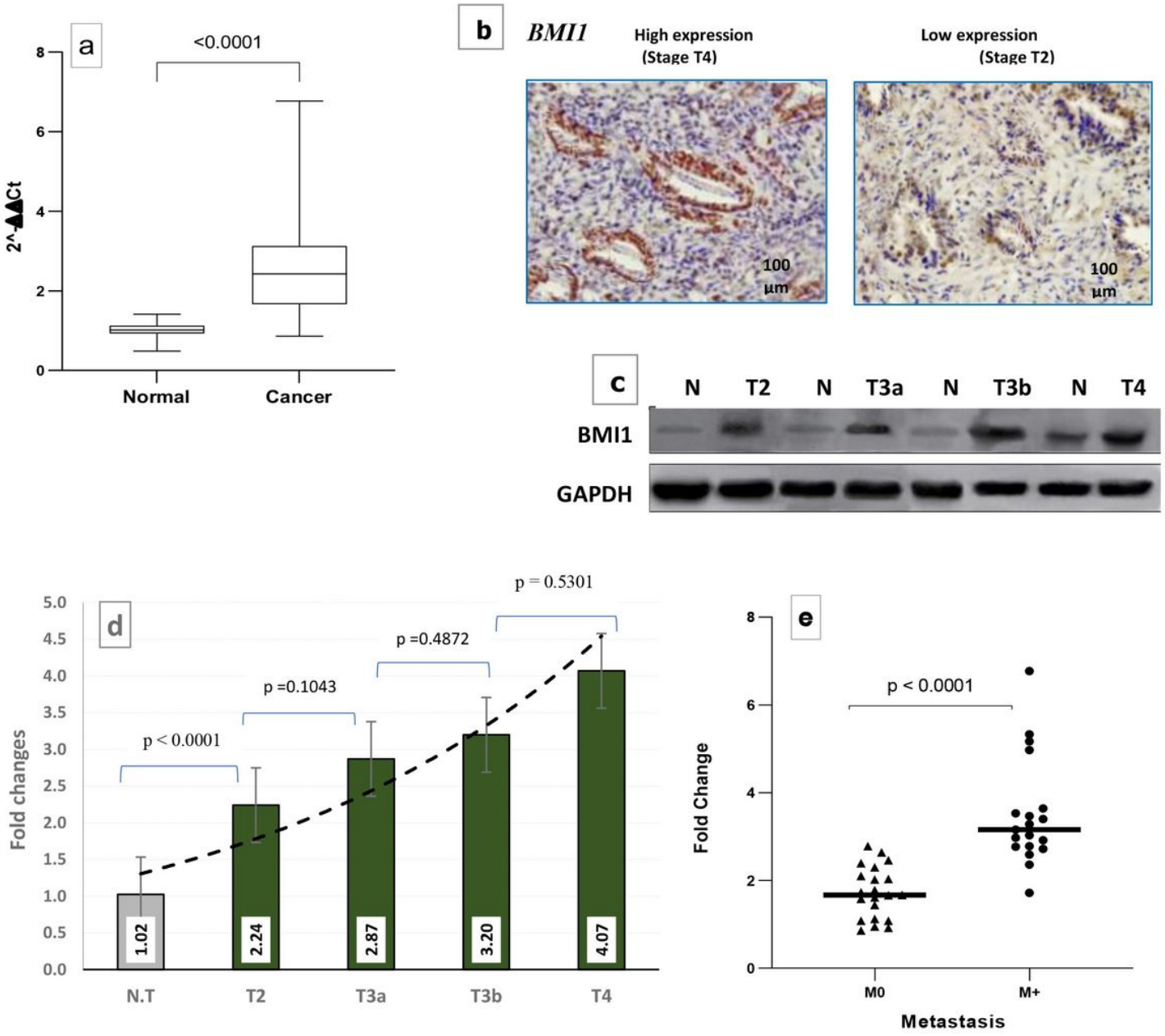

Figure 2

Relative BMI1 level in gastric tumor in all malignant tissues as compared to normal **(a)** immunohistochemistry of Bmi1 (Scale bars, 100um) **(b)**, western blot analysis **(c)**, in different stages **(d),** comparison between metastasis and non-metastasis cases **(e)**.

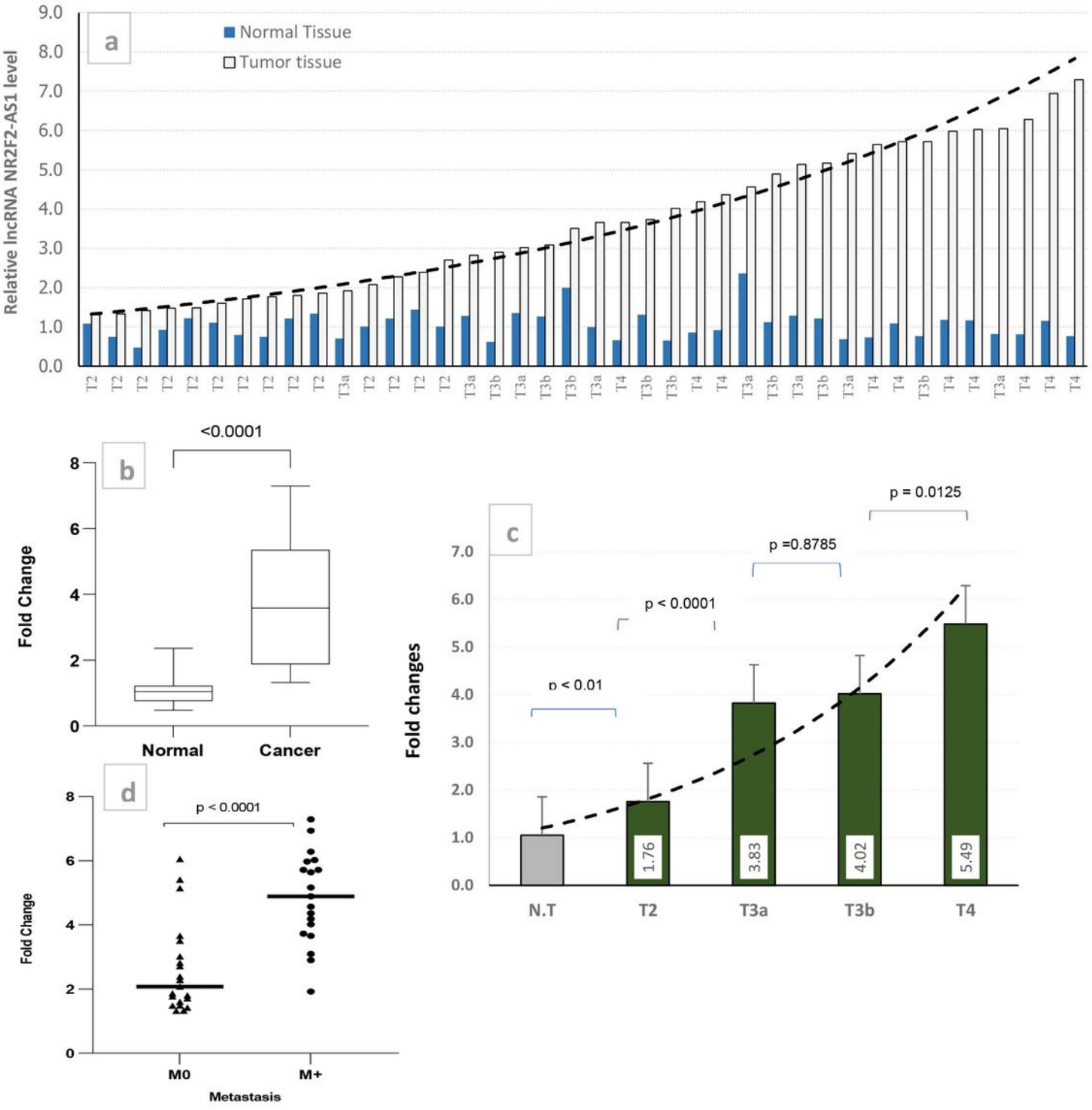

Figure 3

Relative LncRNA NR2F2-AS1 level in gastric tumor in comparison with adjacent normal tissues in each case **(a)** average of expression in all malignant tissues as compared to normal **(b)** in different stages **(c)** comparison between metastasis and non-metastasis cases **(d)**.

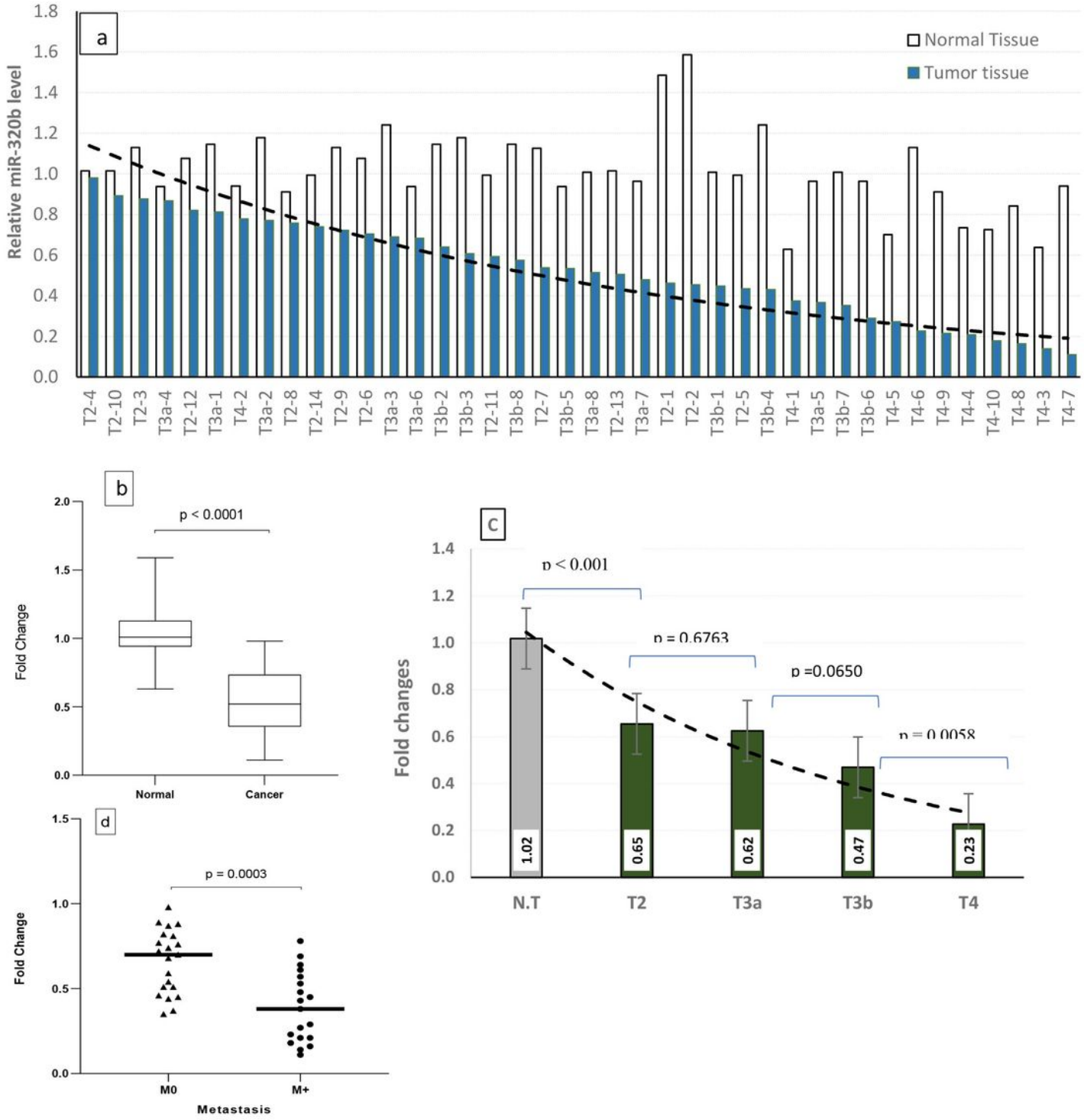

Figure 4

The expression of miR-320b in gastric tumor in comparison with adjacent normal tissues in each case **(a)** average of expression in all malignant tissues as compared to normal **(b)** in different stages **(c)** comparison between metastasis and non-metastasis cases **(d)**.

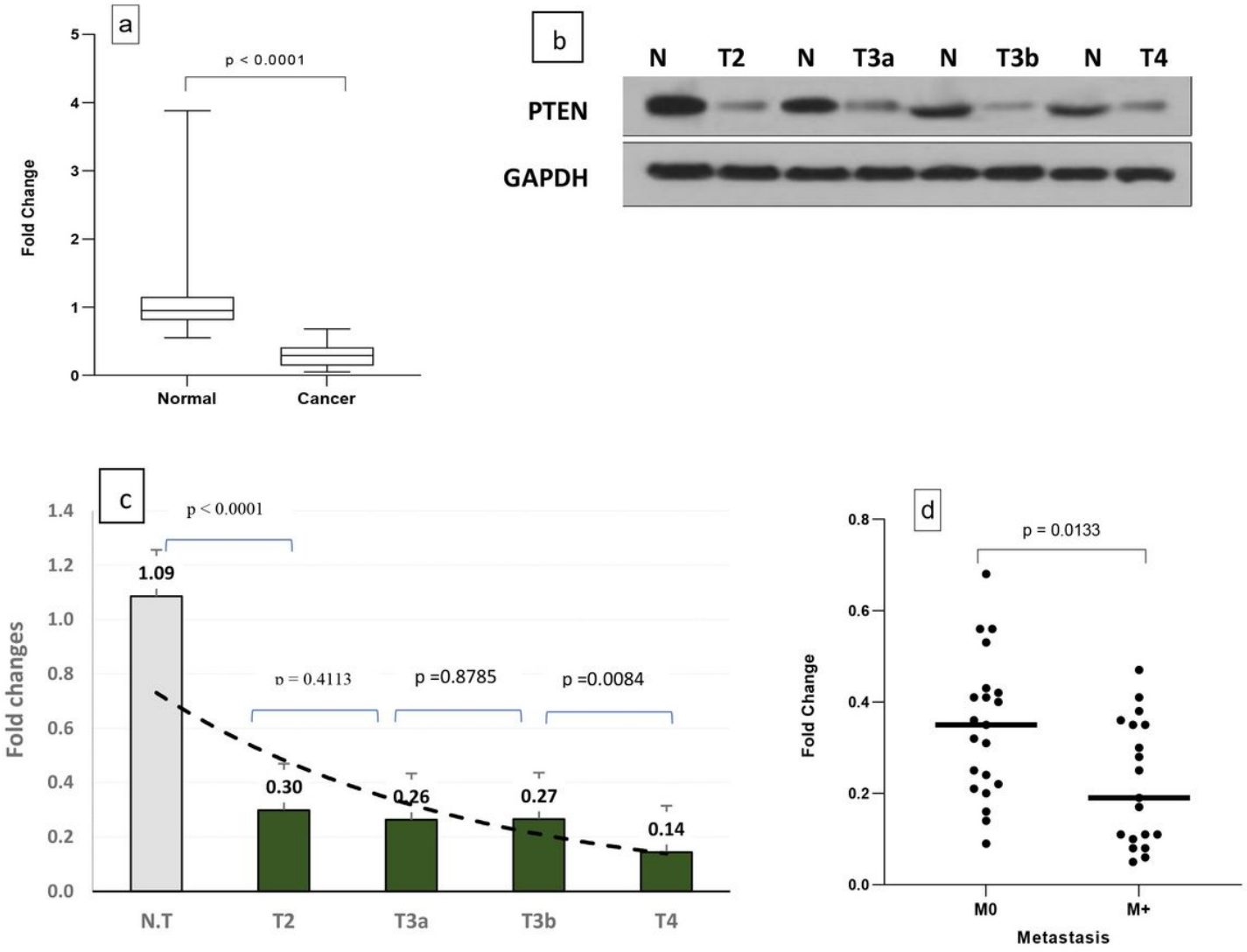

Figure 5

Relative PTEN level in gastric tumor in all malignant tissues as compared to normal **(a)** western blot analyzing of PTEN **(b)** in different stages **(c)** comparison between metastasis and non-metastasis cases **(d)**.

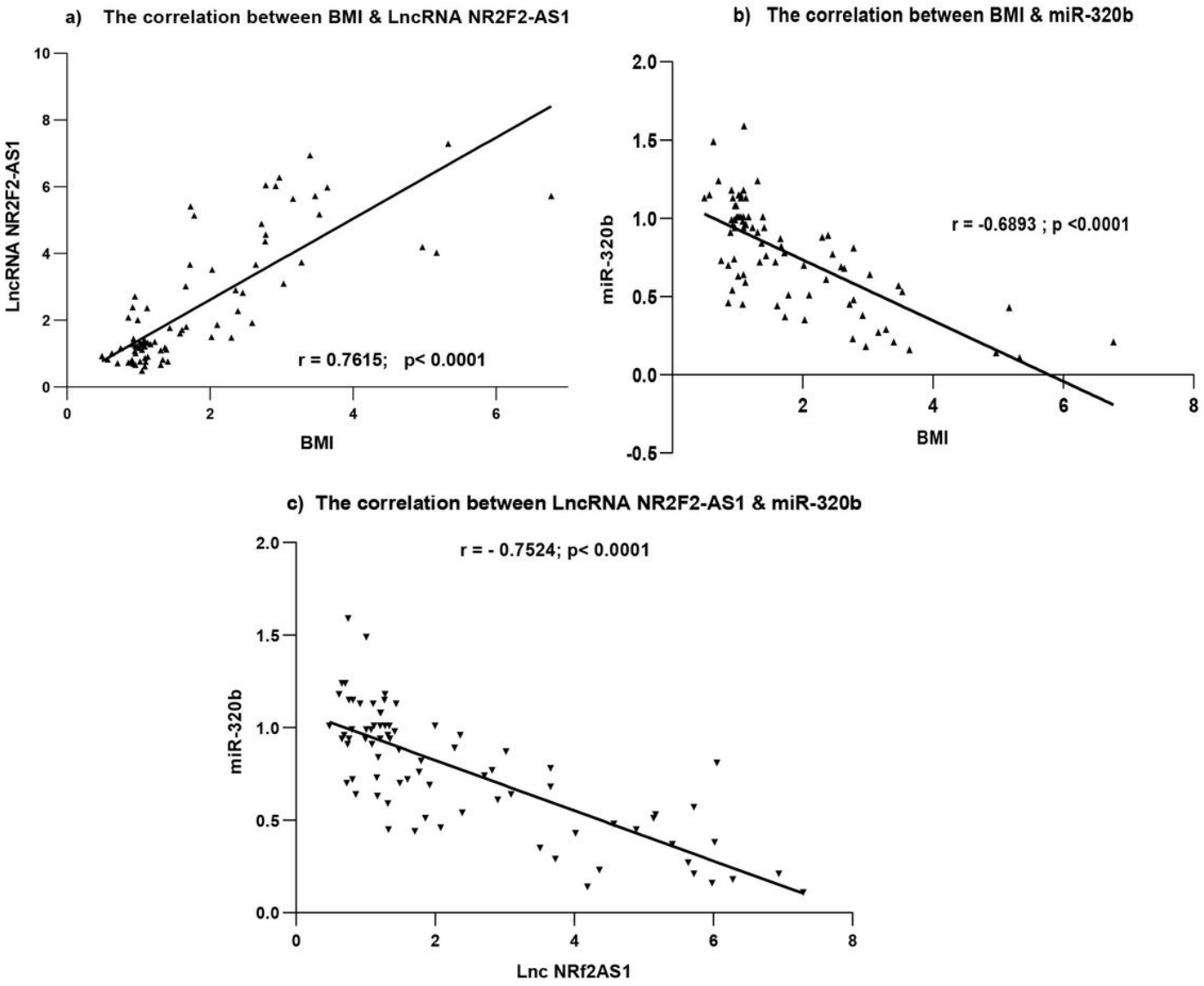

Figure 6

Correlation analysis between lncRNA NR2F2-AS1 and BMI1 **(a)** between BMI1 and miR0320b **(b)**, and between lncRNA NR2F2-AS1 and miR-320b **(c).**

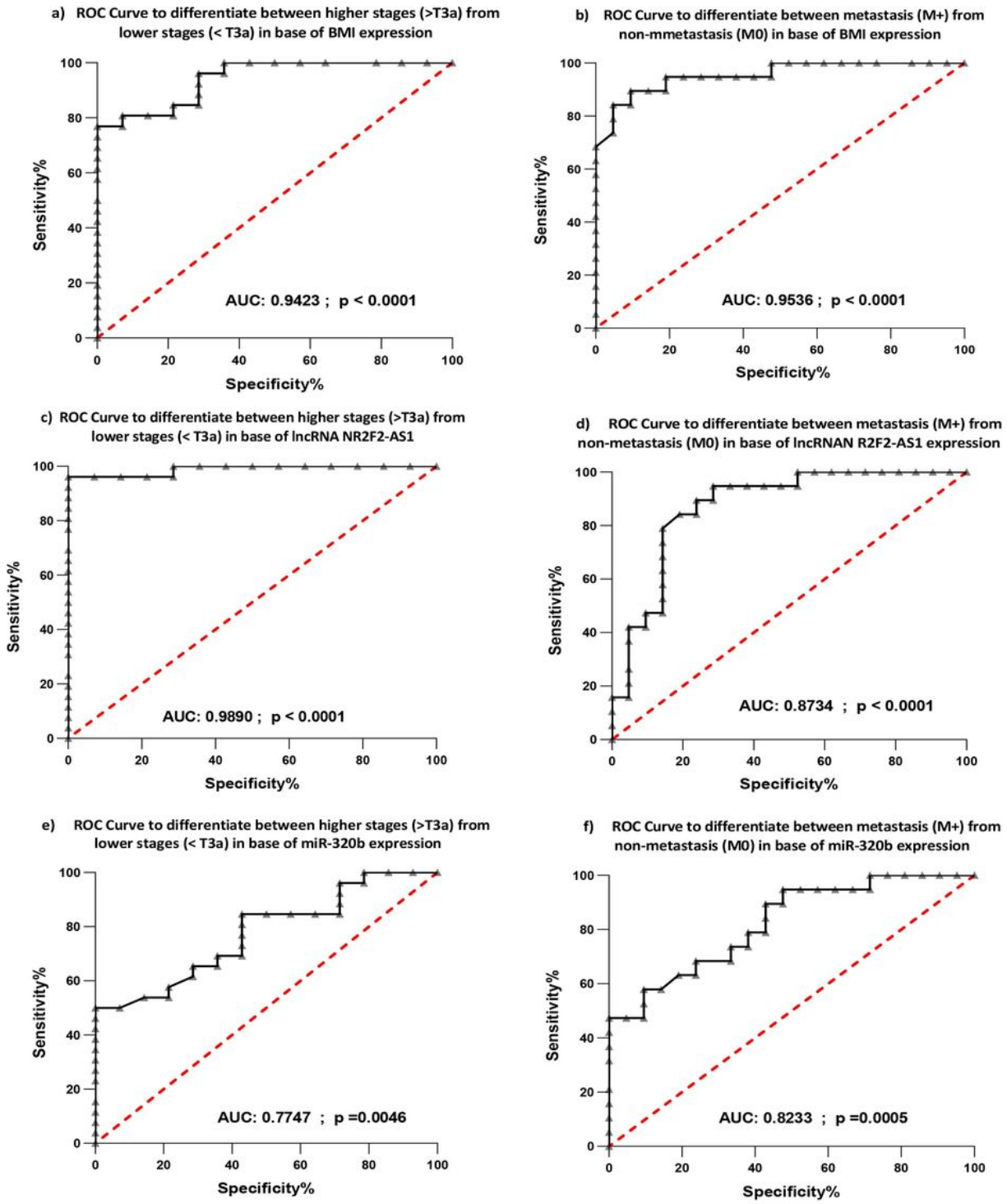

Figure 7

ROC carve assessment revealed that BMI1 **(a & b)**, lncRNA NR2F2-AS1 **(c & d)**, and miR-320b **(e & f)** can serve as a marker to differentiate the higher stage from the lower stages as well as metastatic from non-metastatic.

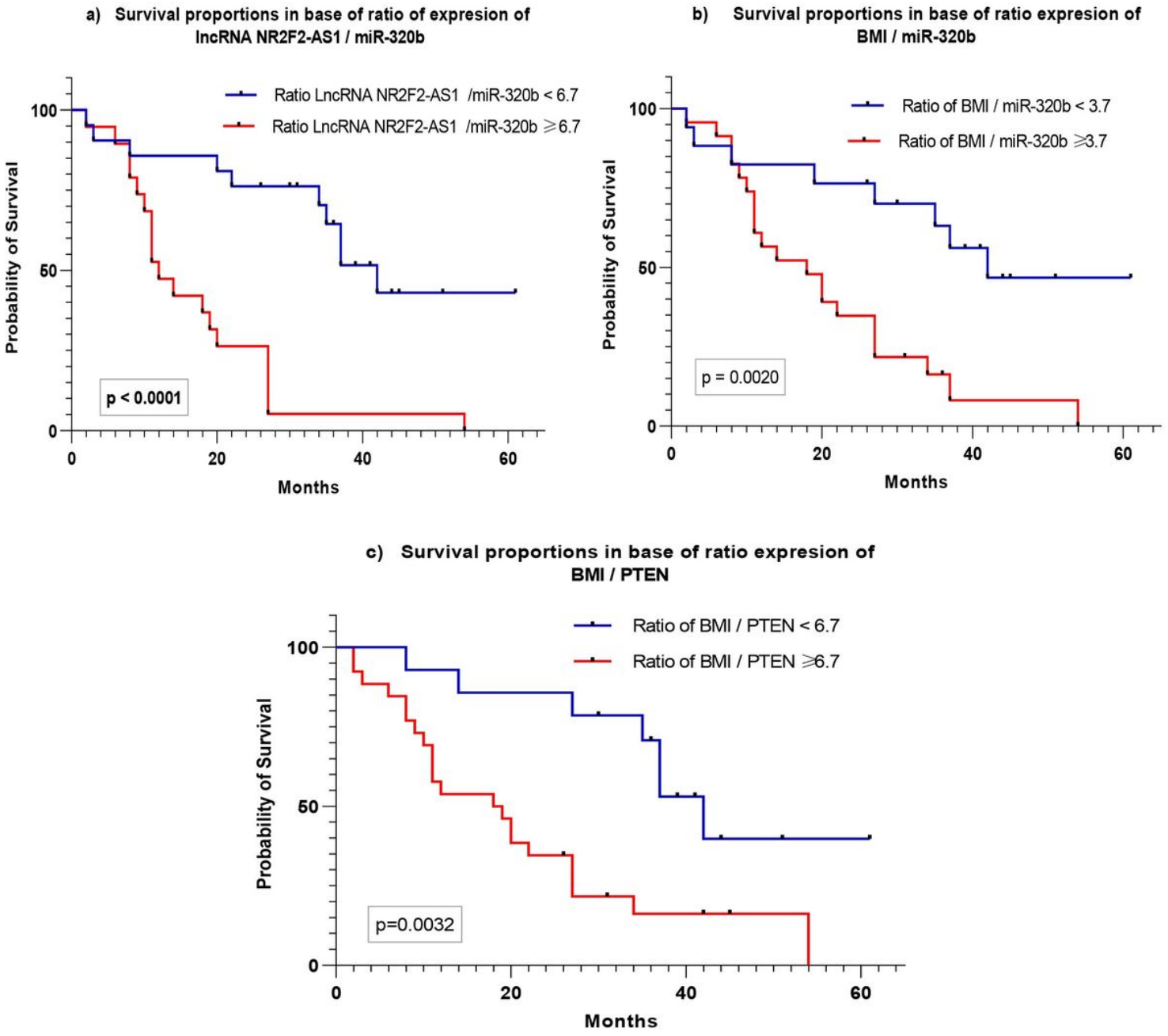

Figure 8

Survival analysis in a base of the ratio of NR2F2-AS1 to miR-320b **(a)**, BMI1 to miR-320b **(b)**, and BMI1 to PTEN **(c)** indicate that high ratio of lncRNA NR2F2-AS1 to miR-320b and as well as the high ratio of BMI1 to miR-320b and PTEN correlate with reduced survival.

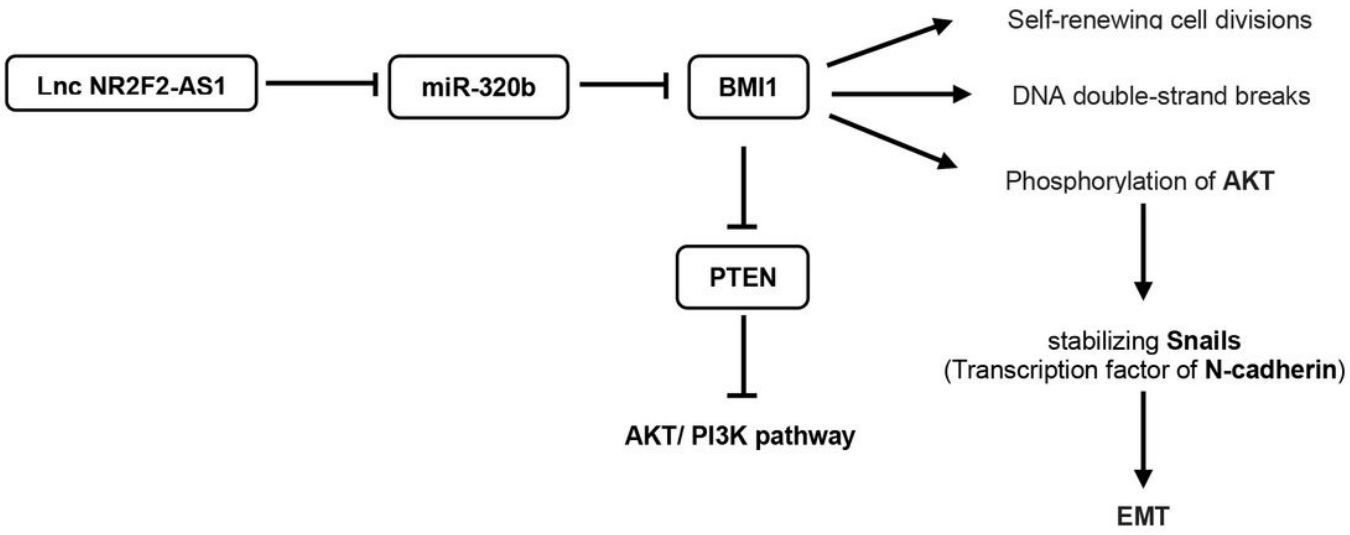

Figure 9

The proposed model centered on imbalancing of lncRNA NR2F2-AS1 / miR-320b in the progression of gastric adenocarcinoma

Supplementary Files

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