Author's response to reviews

Title: p16INK4a hypermethylation and p53, p16 and MDM2 protein expression in Esophageal Squamous Cell Carcinoma

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Author's response to reviews: see over
Dear Editor,

I and all the authors of this manuscript appreciate your reviewer’s careful reviews of our manuscript. We have prepared a revised manuscript addressing the reviewers’ questions and comments. In addition, we have also addressed every one of the reviewer’s comments below separately. Detailed description of all the performed changes is mentioned at the end of the cover letter. I believe that considering our answers to the reviewer’s comments and all the revisions made in our manuscript, the new revised manuscript should be acceptable for publication. I appreciate your time and consideration of this matter.

Reviewer #1:

1) Reviewer’s comment: The figures are numbered 1-5, however the figure legend is labelled differently.

Author’s Response: The labeling was corrected and the figures are resubmitted accordingly.

Reviewer #2:

1) Reviewer’s comment: The manuscript is too descriptive and needs to be condensed.

Author’s Response: A total of 22 lines, in addition to one table, were omitted from the body of the text including Backgrounds, Materials and Methods, Results and Discussion in order to condense the manuscript.

2) Reviewer’s comment: The language errors need to be corrected.

Author’s Response: In addition to a second look by the authors, the manuscript was literally revised by a native expert.

3) Reviewer’s comment: The authors have mentioned few other studies from Iran so they need to clarify how their study is different from the existing reports and what new information is gained from this study.

Author’s Response: Few studies were conducted in Iranian population mentioned in the text. One study has focused on p16 methylation in esophageal squamous cell carcinoma in the northeastern Iran although the sample size was smaller in that study and they did not study p16 IHC. Another study reported p53 IHC in ESCC. No study has reported MDM2 IHC in this population. What is difference and unique in the study, is the concomitant analysis of p53 IHC and p16 hypermethylation and the correlation is the first report. On the other hand, the cross talk between p16 pathway and p53-MDM2 pathway has not been reported. Although some overlaps exist between these reports, the main results and conclusions of this study are novel.
4) Reviewer’s comment: The authors quote Age standardized rates of incidence of Esophageal cancer that are not published, so they need to mention the source of this data. Esophageal cancer can also be adenocarcinomas, but no mention has been made about their incidence in Iran.

Author’s Response: That source of these local data is based on the results of an ongoing active surveillance and population-based cancer registry; a joint effort between Digestive Disease Research Centre (DDRC) of Tehran University of Medical Sciences and the International Agency of Research in Cancer (IARC, France).

The data reported from the published data mentioned below (Reported in Farsi):


The predominant form of esophageal cancer in northeastern (as well as other parts) is ESCC and Adenocarcinoma has a low incidence. (Islami et al, 2004 – 3rd reference of the manuscript)

5) Reviewer’s comment: Data on MSP should be shown in a figure.

Author’s Response: A figure representative of the data of MSP analysis is included as "Figure 2" to show the methylation status of p16 gene in ESCC tissue.

Reviewer #3:

1) Reviewer’s comment: Minor Essential Revisions; such as in Table 3, I guess the "no 1 2 3" were represented of "a b c d ".

Author’s Response: The details of the table were corrected as “"a b c d ".

Detailed description of all the performed changes is mentioned below:

Abstract

• Line (1): “Backgrounds: Tumor suppressor……” ----changed to------ “ Background: Tumor suppressor…..”
• Line (2, 3): “......as the essential G1 cell cycle regulatory genes that loss of their function......” ----*changed to*---- “...... to be essential G1 cell cycle regulatory genes whose loss of function......”

• Line (3, 4): “.....We assessed aberrant....” ----*changed to*---- “......We assessed the aberrant.....”

• Line (4): “.....of *p16* gene .....” ----*changed to*---- “...... of the *p16* gene......”

• Line (5): “.....expressions in the patients with.....” ----*changed to*----“...... expressions in patients with......”

• Line (6): “......Golestan province, northeastern......” ----*changed to*----“...... Golestan province of northeastern......”

• Line (6, 7): “......the highest cancer incidence, well above.....” ----*changed to*----“...... the highest incidence of cancer, well above.....”

• Line (10): “......status of the *p16*........... proteins were detected.....” ----*changed to*--
  “...... status of *p16*........... proteins was detected.....”

• Line (12, 13): “......and *p53*, and not *MDM2*, were significantly......” ----*changed to*----
  “...... and *p53*, but not *MDM2*, was significantly......”

• Line (15): “....methylation of *p16*<sub>INK4a</sub>” ----*changed to*------“....... methylation of the *p16*<sub>INK4a</sub>”

• Line (18): “......as well as overexpression.....” ----*changed to*------“...... as well as the overexpression.....”

• Line (20): “......important role in the ESCC development.....”----*changed to*------
  “...... important role in ESCC development......”

• Line (21): “.........associated to *p53* protein......... influence accumulation........”
  ----*changed to*------ “...... associated with *p53* protein ................. influence the accumulation......”
Introduction

- Line (1): The word “Backgrounds”-----changed to-----“ Background”
- Line (2): “……the 5th leading cause…….”-----changed to-----“ …… the fifth leading cause……”
- Line (3): “……and it is considered the…..”-----changed to-----“…… It is also considered the…..”
- Line (4): “…….Golestan province, northeastern…….”-----changed to-----“……. Golestan province of northeastern…….”
- Line (5): “……10^5 persons-year) (not published…….”-----changed to-----“……10^5 persons/year) (unpublished…….”
- Line (7): “…….Iran with high incidence…..”-----changed to-----“……. Iran, where there is a high incidence…..”
- Line (8,9): “……alterations are not well clarified in this region…….”-----changed to-----“…… alterations have not been well defined for this region…….”
- Line (10): “…….involved in the esophageal…….”-----changed to-----“……. involved in esophageal…….”
- Line (14): “…….proto-oncogene, Murine…..”-----changed to-----“……. proto-oncogene MDM2 (Murine…..”
- Line (15): “…….Minute 2 (MDM2), are considered as the essential G1…….”-----changed to-----“……. Minute 2), are considered to be essential G1…….”
- line (16): “…….genes that loss of their function…..”-----changed to-----“……. genes and whose loss of function…….”
The phrase “In response to DNA damage, p53 protein ….” was added.

Line (19): “…..arrest or apoptosis in response to DNA damage [10].
Abnormalities…..” changed to “……. arrest or apoptosis [10].
Abnormalities……”

Line (20, 21): “…….lead to loss of the regulation ………. apoptosis, which are…….” changed to “……. lead to the loss of regulation ………. apoptosis, all of which are…….”

Line (24): “…resulting in abrogation of p53…..” changed to “…resulting in the abrogation of p53…..”

Line (25): “…….consequently deregulation ………. into tumor……” changed to “……. consequently the deregulation ………. which leads to tumor…….”

Line (27, 28): “…..the cell cycle in G1/S phase in a p53-dependent manner [14, 15].” changed to “…..the cell cycle in the G1/S phase in a p53-dependent pathway [14, 15].”

Line (30, 31): “…….p16^{INK4a}, is an important mechanism for inactivation of this gene and commonly…..” changed to “…….p16^{INK4a}, is a critical mechanism for their inactivation and is commonly…….”

Line (32): “…….transcription, leading to development……” changed to “……. transcription which promotes the development…….”

Line (34): “…..mechanism for inactivation…..” changed to “…..mechanism for the inactivation…..”

Line (36, 37): “…..northeastern Iran [19, 20]. These investigators also reported the aberrant p16 hypermethylation as a possible…..” changed to “…..northeastern Iran [19, 20]. Aberrant p16 hypermethylation is also suggested as a possible…….”

Line (39): “….., including mutation…..” changed to “….., including the mutation…..”
Materials and Methods

- Line (2, 3): “….recruited from patients referred…..”----*changed to*----“….. recruited from May 2006 to June 2007, from among patients referred…..”

- Line (4): “…centers in the north eastern…..”----*changed to*----“…… centers in northeastern…..”

- Line (5, 6, 7): “……oncology hospital of the northeastern Iran, from May 2006 to June 2007. We estimate that ……”----*changed to*----“….. oncology hospital in northeastern Iran. It is estimated that …..”

- Line (8): “….. *The* patients did not…..”----*changed to*----“….. Patients did not…..”

- Line (9, 10): “Their follow up was done from 6 months to 24 months.”----*changed to*-----“ Follow up carried out 6 to 24 months afterwards.”
• Line (11, 12, 13): “…..including cigarette, hookah and Nass use or consumption (………, and Lime) [4] was obtained…………. interviewers based on a standard………”---- changed to ----“…….., including the smoking or consumption of cigarettes, the hookah and nass (a mixture of tobacco, ash, and lime) [4], were obtained ……… interviewers using a standard……..”

• Line (14): “…..GI endoscopy, and tissue samples…..”---- changed to ----“…….. GI endoscopy. Tissue samples…..”

• Line (15, 16): “……normal tissue from the site distant to tumor…..”---- changed to ----“………. normal tissue from a site, remote from the tumor,……”

• Line (18, 19, 20): The sentence “Tumors were histologically verified as esophageal squamous cell carcinoma and sub-typed based on the grade of differentiation as well differentiated, moderately differentiated or poorly differentiated.” Was deleted.

• Line (20, 21): “The research ethics committees of Tehran University……”---- changed to ----“ The Research Ethics Committees of Tehran University……”

• Line (22): “……study design and all eligible…..”---- changed to ----“…….. study design. All eligible…..”

• Line (23): “……..signed written ……consent prior to participation in the study”------ changed to ------“…… signed a written………….. consent prior to participating in the study”

• Line (27): “…….. purified by phenol/chloroform……”------ changed to ------“…….. purified by the phenol/chloroform……..”

• Line (28, 29): “……in ethanol, as described[20].”------ changed to ------“………. in ethanol, as previously described[20].”

• Line (34): “Briefly, two microgram of DNA……”------ changed to ------“ Briefly, 2 µg of DNA…..”
• Line (35, 36, 37): the phrase “by adding a 500µl of a freshly prepared bisulfite solution (2.5M sodium bisulfate and 125mM hydroquinone) following to an incubation for 12 h at 50º.” *Was added.*

• **Line (37, 38, 39, 40, 41): the phrase** “30µl of hydroquinone 10 mM, (Sigma Chemical Co, Germany) and 520 µl of sodium bisulfate solution (Sigma Chemical Co, Germany) (pH 7.0, concentration, 3 M), which was freshly prepared and added to each sample. The final solution (final volume 615.5µl) was incubated at 50°C for 16 h at dark. After incubation, the…..” *was deleted.*

• **Line (41): “…..samples was desalted…..”----*changed to----“……. samples were desalted…..”

• **Line (42): “…….Promega), and then treated……”----*changed to----“……. Promega) afterward, and then treated…..”

• **Line (43, 44, 45): the phrase** “….. followed by precipitation. The pellet was washed with 1/10 volumes ethanol, dried and then re-suspended in optimum de-ionized water stored at -70° until used for MSP.”------*changed to----“……. followed by precipitation with 75µl ammonium acetate (5M). The pellet was washed with 2.5 volumes of ethanol, dried, and re-suspended in 20µl tris (pH 8.0, 5mM), then stored at -70° until used for MSP. “

• **Line (48): “The sense and anti-sense primer sequences of the p16 gene for methylated and unmethylated DNA are shown in Table1” was deleted.**

• **Line (50): “(previously described sequences[20])” was added**

• **Line (55): “…..at 72˚C), with a final 10…..”----*changed to----“……. at 72˚C), and a final 10…….”

• **Line (55, 56): “…….at least two times. In addition of a negative control,…….”----*changed to----“……. at least twice. In addition to a negative control,…….”

• **Line (58): “…….product was loaded on 6% poly…..”----*changed to----“……. products were loaded on 2.5% agarose gel and 6% poly…….”

• **Line (60, 61, 62): the phrase** “In this study, we also performed PCR amplification with p16 unmethylated specific primers for all bisulfate treated DNA samples to
control bisulfate modification process. Amplification of all samples confirmed successful bisulfite modification.” **Was deleted.**

- Line (67): “......and boiled in Target Retrieval......” **changed to** “...... and boiled in the Target Retrieval......”
- Line (71): “.......Calif., USA) (IgG2a.......” **changed to** “....... CA, USA) (IgG2a.......”
- Line (74): “....MDM2 we used (IgM mouse monoclonal; clone 1B10; caRboxy terminus......” **changed to** “....MDM2, IgM mouse monoclonal clone 1B10 (carboxy terminus......”
- Line (75): “....MDM2 (Novocastra.....” **changed to** “........ MDM2) was used (Novocastra.........”
- Line (78): “.....was revealed using......” **changed to** “........ was detected using......”
- Line (81): “......concentration alcoholic.....” **changed to** “........ concentrations of alcoholic......”
- Line (85): “....The cutoff levels for abnormal......” **changed to** “....... The cutoff values for abnormal.......”

**Statistical Analysis**

- Line (3): “......Fisher’s exact test......” **changed to** “...... Fisher’s exact tests......”
- Line (5): “.....compared survival of.....” **changed to** “........ compared the survival of......”
- Line (6): “.....considered as significant statistical.....” **changed to** “........ considered as the significant statistical......”

**Results**
• Line (1): “…… 83 years; the mean age was …..”---- changed to ----“ ….. 83 years; with the mean age of …..”

• Line (3,4): “. The tumor size ranged ….., and the mean diameter……”---- changed to ---“. Tumor sizes ranged ….., with a mean diameter…….”

• Line (5): “table 2” ---- changed to ----“ table 1”

• Line (6): “Methylation status …..” ---- changed to ----“ The methylation status …..”

• Line (9): “….. status of p16 gene ….. studied factors……”---- changed to ----“ …. status of the p16 gene …. the factors studied, …..”

• Line (10,11, 12): “…..tumor site and size, opium use …..,(…..,hookah use) (Table 2).”---- changed to --------- tumor site and size, and opium and tobacco use ….. (cigarette and hookah smoking, Nass chewing) (Table 1). Figure 1 represents MSP analysis of p16 gene.”

• Line (13, 14): “Immunohistochemical staining

IHC staining ….. figure 1. “---- changed to ----“ IHC staining ….. figure 2.”

• Line (15): “a) Immunohistochemical staining of p16”---- changed to ----“ a) Immunohistochemical staining of p16, p53 and MDM2”

• Line (16, 17): “Negative expression of p16 …..(14%) of normal tissues …..”---- changed to -------“ The negative expression of p16 ….. (14%) normal tissues …..”

• Line (17, 18, 19, 20): The paragraperh “With respect to histological subtype, loss of p16 immunoreactivity was found in 16/25 (64%) well differentiated, 6/13 (46.2%) moderately differentiated and 1/7 (14.3%) of poorly differentiated tumors with no statistically significant difference. (P = 0.06)” was deleted.

• Line (21): the sentence “b) Immunohistochemical staining of p53 and MDM2” was deleted.
• **Line (22, 23):** “Positive expression ……… proteins were detected in the nuclei in 31/50 (62%)……..” ***changed to*** “The positive expression ……… proteins in the nuclei was detected in 31/50 (62%)……..”

• **Line (24):** “……… (60%) of the normal esophageal tissues……….” ***changed to*** “……… (60%) normal esophageal tissues……….”

• **Line (26, 27):** The sentence “The histological grade of differentiation was not associated with the IHC staining of p16, p53 and MDM2.” ***Was added.***

• **Line (27, 28, 29, 30):** The paragraph “With respect to histological subtype, p53 immunoreactivity was found in 15/25 (60%) well differentiated, 8/13 (61.5%) moderately differentiated and 3/7 (42.9%) of poorly differentiated tumors, with no statistically significant difference.” ***Was deleted.***

• **Line (31):** “c) Concomitant p16, ………” ***changed to*** “b) Concomitant p16,……….”

• **Line (32):** “Overexpression of MDM2 ……… associated to abnormal……..” ***changed to*** “The overexpression of MDM2 ……… associated with the abnormal……..”

• **Line (33, 34):** “…… no significant correlation……. and p53 and also p16 and………” ***changed to*** “……. neither significant correlation……. and p53, nor between p16 and…….”

• **Line (35):** “Concomitant abnormal accumulation of p53………. ” ***changed to*** “Concomitant abnormal accumulation of the p53………. ”

• **Line (37):** “……. compared to normal; p16-/p53+……. ” ***changed to*** “……. compared to normal tissue; p16-/p53+……. ”

• **Line (38):** “……. in 20/50 (40%) of tumors……..” ***changed to*** “….. in 20/50 (40%) tumors…..”
Discussion
• The first sentence “Several genetic and epigenetic alterations are thought to play crucial role in ESCC carcinogenesis and many of them have yet remained to be clarified” was deleted.

• Line (3): “…… in the northeastern Iran …… ESCC, well above ……” ------
  changed to ------“…… in northeastern Iran …… ESCC, which is well above…….”

• Line (4): “…………aspects of complex…” ------ changed to ------” …… aspects of the complex…”

• Line (7, 8, 9, 10): The sentence “The main targets were p16$^{INK4a}$, MDM2 and p53 proteins. Recent evidences show that transcriptional silencing due to hypermethylation is an important mechanism in the p16 tumor suppressor gene inactivation, leading to carcinogenesis.” Was deleted.

• Line (10, 11): “Thus, …… hypermethylation, its impact on ……..” ------
  changed to ------“Thus, …… hypermethylation, as an important mechanism of gene silencing, its impact….”

• Line (14, 15): “methylation of ……… may have played an important role in the ……….” ------
  changed to ------“methylation of ……… may play a critical role in the ……….”

• Line (18, 19): “critical role ……. in the ESCC development in this high-incidence region.” ------
  changed to ------“critical role ……. in the development of ESCC in this high-incidence region.”

• Lines (21, 22): “……of ESCC patients in the Fareast……. and showed the p16 gene ……….” ------ changed to ------“……among ESCC patients in the Far East, ………and detected the p16 gene ……….”

• Line (26): “88% ………. Abbaszadegan reported …….” ------ changed to ------“88% ……… Abbaszadegan et al reported ……”

• Line (28): “In support ……. the important role ……..” ------ changed to ------“ In support ……. the critical role ……..”

• Line (30, 31 , 32): “……. examined the P16 …….. adjacent tumor to clarify ……… existed in the ……. in 2 ESCC patients ……..” ------ changed to ------“
………… examined p16 methylation ……… adjacent to tumor, in order to clarify
………… occurred in the ……… in two ESCC patients, ………”

- Line (34, 35): “………… expressed as well.”----changed to----“ ………
…………………… expressed either.”
- Line (35, 36): “Hibi …… reported one case ………”----changed to----“ Hibi ……
presented one case ………”
- Line (39): “……. reported the evidence ………”----changed to----“ ………
provided evidence ………”
- Line (40, 41): “p16 methylation ……… reported by Esteller et al [31]as well and
Guan et al [32]……..”----changed to----“ p16 methylation ……… was also shown
by Esteller et al [31].Guan et al [32]reported………..”
- Line (42): “We could speculate that ………”----changed to----“ We can speculate
that ………”
- Line (47, 48, 49): “Environmental …… such as exposures ……… factors, as
previously reported to influence aberrant hypermethylation[34-37], along ………”--
changed to-----“ Environmental …… previously reported as influencing
aberrant hypermethylation, such as exposure ………… factors, [34-37], along
………..”
- Line (54): “environmental ……… in methylation status ………..tissue.”----
changed to----“ environmental ……… on the methylation status ………..
tissue.”
- Line (57): “(25%) …… tissue with p16 immunostaining negative suggested
that………..”----changed to-----“(25%) ……… tissues with negative p16
immunostaining. This suggested that………..”
- Line (59): “may …… to the p16 ………”----changed to----“ may …… to p16
………..”
- Line (61): “This was possibly explained by ……… of p16 gene or it may reflect
low detection sensitivity threshold”----changed to----“ This can possibly be
explained by ……… of the p16 gene. It may also reflect the high sensitivity”
- Line (63): “methylated DNA, indicating that …………..”----changed to-----“
methylated DNA, which would indicate that ………..”
• Line (64, 65): “positive … while they may ……”-----changed to-----“positive … even though they may ……”
• Line (70, 71): “we observed the ESCC tumors …… inactivation were more often haRbored increased …….”-----changed to-----“we observed that ESCC …… inactivation more often harbored increased …….”
• Line (78, 79): “DNA …… in the neoplastic ……having a p53 …… p53, assessing promoter ……”-----changed to-----“DNA ……. neoplastic …….. with a p53 …… p53, when assessing the promoter …,,,”
• Lined (81): “…… associated to the p53 ……. …….associated with p53 ……”
• Line (87, 88): “…… associated with more accumulation ……”-----changed to-----“…… associated with a larger accumulation ……”
• Line (94): “…… not been yet ruled out”-----changed to-----“…… not yet been ruled out”
• Line (95): “level …… hydrocaRbons may ……”-----changed to-----“level …… hydrocarbons may ……”
• Line (99): “However …… a lack of precise ……”-----changed to-----“However … the lack of a precise …,,,”
• Line (104): “. It …… to p53 protein…..”-----changed to-----“. It ……. with p53 protein…..”
• Line (107, 108): “Considering this finding, in addition to the impact of p16 hypermethylation ……”-----changed to-----“The impact of p16 hypermethylation …,,,,”
• Line (109): “……in the ……. along with observing concomitant ………”-----changed to-----“……in the ……. along with the observed concomitant ………”
• Line (111): “suggests a ……. in the ESCC development…….”-----changed to-----“, suggests a ……. in ESCC development…….”
• Line (113): “……a large sample size along with the assessment ………”-----changed to-----“a large sample size, accompanied by the assessment ………”
• Line (115): “…… in the ESCC carcinogenesis…….”-----changed to-----“…… in ESCC carcinogenesis…….”
“Figure 1: MSP analysis of p16 gene in ESCC patients.

DNA from H1299 cells served as a positive control for hypermethylated DNA and L132 as a positive control for unmethylated DNA. Patient 17 (p17) and patient 25 (p25) were hypermethylated, which revealed 150 bp bands (M) with hypermethylated primers. Patient 12 (p12) and patient 9 (p9) were not methylated, having only unmethylated band (U).”

Was Added

‘Figure 1'----changed to----“Figure 2”

Sincerely yours,

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