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ORIGINAL RESEARCH ARTICLE

Exon Sequence Analysis of the *ATG5, ATG12, ATG9B* Genes in Colorectal Cancer Patients During Radiotherapy

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Abstract Radiotherapy (RT) which is a treatment regime for cancer patients may cause genetic instability and side effects. Etiological associations exist amongst autophagy-related gene (ATG) mutation and cancer. RT increases the rate of autophagy previously proven in vitro. The aforementioned background diverted us to conduct exon mutation analysis for ATG5, ATG12, and ATG9B genes of colorectal cancer patients who were receiving neoadjuvant RT. Peripheral blood DNA from different time points (before/middle/after RT) of the same patients was isolated and most tandem repeat-containing exons of ATG5, ATG12, and ATG9B were polymerase chain reaction-amplified and examined for mutations by Sanger sequencing. CA19-9/CEA (Tumor marker of colorectal cancer/Carcinoembryonic Antigen) serum levels were retrieved from the clinic. No exon variations detected for ATG5 and ATG12 genes. However, 4 patients (17.4%) showed frameshift mutation for ATG9B gene. Exon variation analysis of 2 (8.7%) patients resulted in GGG deletion at 8G mononucleotide tandem repeat region of ATG9B. Assigning patients as before RT and after RT, CA19-9 levels in ATG9B (Mutation) patients were higher compared to ATG9B (Wild Type) patients. ATG9B is highly likely to mutate during RT and ATG9B mutation correlates to higher CEA and CA19-9 levels and patients show poor prognosis.

Keywords Mutation analysis · Autophagy · ATG9B · Radiotherapy

Introduction

According to the American Cancer Society, cancer is the second leading mortality cause in the United States. Statistics show that 18.4% of cancer cases are diagnosed with Digestive system tumors in both sexes. Colorectal cancer in the digestive system tumor group constitutes 46.3% of all cases [1].

Radiotherapy (RT) is the most commonly used treatment modality for cancer treatment by targeting ionizing radiation beams specifically on tumors but not on healthy surrounding tissues however current technology used in radiation oncology clinics targeting RT also on healthy surrounding tissues involuntarily [2]. Previous studies reviewed the side effect of this treatment by isolating peripheral blood from rectal cancer patients for checking markers involved in the endoplasmic reticulum (ER) stress and autophagy and concludes that ER stress markers and ATG gene expression levels detected by quantitative real-time polymerase chain reaction (QRT-PCR) changed after RT [3]. As well as RT is ameliorating some of the symptoms, some studies reveal that RT induces changes in the genome of the circulating blood cells in the patient [4]. Studies revealed that; there is a correlation between

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genetic sequence changes, serious side effects, and prognosis of patients [5–9].

Although applied dose for RT is optimized to patient benefit throughout the years, In clinics, fraction and dosimetry of RT are calculated for each patient's specifications individually [10]. RT is given as a treatment either adjuvant or neoadjuvant to alleviate the tumor burden of the patient and also RT targets either to shrink tumors before the operation or to eradicate tumors after the operation [11]. When the calculated dose is applied to the patient, 5-year follow-up results in < 5% side effect [8].

Autophagy is an evolutionarily conserved pathway throughout most organisms with residing homolog proteins. By activating autophagy; protein or damaged organelle whose lifespan is complete is recycled physiologically with the involvement of lysosome inside the cell [12]. No inflammation is caused by autophagy profoundly similar to apoptosis [13]. Genes that are involved in autophagy are called ATG (**A**utophagy Rela**T**ed **G**enes) and mutations occurring to ATG family genes lead to cancer and other metabolic diseases [14]. Especially, autophagy is accepted to play roles in cancer. Autophagy takes charge like a tumor suppressor in the early stages of tumorigenesis. Whereas it provides nutrients and energy to ensure survivor cancer cells in the advanced stage [15].

Conventional autophagy is initiated through Ulk1 (Unc5-like kinase) / ATG enzymes and Beclin-1 / Class III PI3K (phosphatidylinositol-3-kinase) complex containing ATG5, ATG7, and ATG1 proteins. Autophagosome formation is progressed through isolation membrane formation which is derived from omegasome regions on the cell membrane via PI3K and ATG6 (Beclin-1) [16, 17]. In the elongation step of autophagy, closure of isolation membrane is achieved through the recruitment of ATG5-ATG12, ATG8/LC3, and MAP-LC3 (ubiquitin-like conjugation pathway) [18]. In the gathering step, ATG9 constitutes phagophore formation with other ATG proteins and modulate turnover of autophagy pathway. In the last step, autophagosome and lysosome fuse for hydrolysis and degradation occurs [16]. ATG9 is a unique protein due to being the only transmembrane membrane protein that is essential for autophagy [19, 20]. ATG9 and ATG14 are unique proteins due to being degraded constitutively by proteosome in normal conditions but ATG9 level stays same at starving conditions [19].

In the alternative autophagy pathway, ATG5 and ATG7 proteins are included in the macroautophagy (sensu stricto autophagy) pathway but in a progressed stage, ATG9 and ubiquitin-like protein conjugation system proteins (ATG5, ATG7, and LC3) are not playing a role [21, 22]. Knocking out of ATG5 and ATG7 results in inhibition of phagosome formation which shows their essential role in this process [23].

Autophagic flux in the cell is controlled by 2 main elements: first model is, by the widely known protein conjugation systems (Atg5-Atg12/Atg16L and Atg7-Atg3-Atg8) results to LC3-II marker protein, second model is, Ser/Thr kinase Atg1 (mammalian Ulk1 and Ulk2) which is controlled by the upstream nutritional regulator mTOR (mammalian target of rapamycin) [24].

This study focuses on colorectal cancer patients who were treated with RT and investigates changes in genes involved in elongation and gathering step of autophagy namely: ATG5, ATG12, and ATG9B after image-guided radiotherapy. In this study, we report ATG5, ATG12, and ATG9B gene sequencing screening in 23 colorectal patients who received 25 fraction total 50.4 Gy radiation treatment in a time-dependent manner.

Material and Methods

Patients

Approval for this study was obtained from the Istanbul University ethics committee chair. The study followed the Declaration of Helsinki and Good Clinical Practice guidelines. The study included patients aged 18 years or older with the histologically or cytologically proven, locally advanced, or metastatic gastrointestinal tumor that needs to be treated with long-term radiotherapy. Cancer patients were selected for participation in this study were followed throughout the RT treatment fractions and all required patient information was gathered in detail in Table 1. Three blood drawing intervention was done. Blood samples and patient data were obtained following the informed consent of the patients.

PCR Amplification

Genomic DNA was extracted from EDTA blood (Jena Bioscience DNA isolation kit-PP-213L). A set of 6 PCR primer pairs were used to PCR-amplify all 3 exon regions of the ATG5, ATG12, and ATG9B genes from each patient sample at 3 timepoints. PCR amplifications were performed with 100 ng of genomic DNA using 2X mastermix (Jena Bioscience Crystal Taq Master).

All primers listed below: ATG5 Forward:5'-ACAATG GGCTGTTTTTGCCA-3', ATG5 Reverse:5'- CCACTG CAGAGGTGTTTCCA-3', ATG12 Forward:5'-TGCTGG AGGGGAAGGACTTA-3', ATG12 Reverse: 5'- CCTCCT TCAAAGAGCTCGCA-3', ATG9b Forward: 5'- AGGGAT TTGGCCTCAGGTTG -3', ATG9b Reverse: 5'- GAGAGC ACTGTGGCAAGACT -3'.

Table 1 Patient information regarding to chemotheraphy, radiotheraphy fraction and dose

Patient ID	Chemotherapy	RT dose (Daily)	Total RT dose	Number of Fraction	Diagnosis	ATG9B Mutation Status
Patient-1	4 cycles of 5-FU	230 cGy	46 Gy	20 fr	Rectum Ca	Phase I, II, III Frameshift Mutation
Patient-2	5 cycles of 5-FU	230 cGy	46 Gy	20 fr	Rectum Ca	Phase I, transition mutation, Phase II and III Wildtype
Patient-3	4 cycles of capecitabine	200 cGy	50.4 Gy	25 fr	Rectum Ca	Phase I, transition mutation, Phase II and III Wildtype No Mutation detected
Patient-4	4 cycles of 5-FU	230 cGy	46 Gy	20 fr	Rectum Ca	No Mutation detected
Patient-5	5 cycles of 5-FU	230 cGy	46 Gy	20 fr	Rectum Ca	Phase I, II, III Frameshift Mutation
Patient-6	4 cycles of 5-FU	230 cGy	46 Gy	20 fr	Rectum Ca	No Mutation detected
Patient-7	5 cycles of FOLFOX	180 cGy	45 Gy	25 fr	Rectum Ca	No Mutation detected
Patient-8	5 cycles of 5-FU	200 cGy	50.4 Gy	25 fr	Rectum Ca	No Mutation detected
Patient-9	4 cycles of capecitabine	180 cGy	45 Gy	25 fr	Rectum Ca	No Mutation detected
Patient-10	5 cycles of 5-FU	230 cGy	46 Gy	20 fr	Rectum Ca	No Mutation detected
Patient-11	5 cycles of 5-FU	180 cGy	45 Gy	25 fr	Rectum Ca	No Mutation detected
Patient-12	5 cycles of 5-FU	180 cGy	50.4 Gy	25 fr	Rectum Ca	No Mutation detected
Patient-13	4 cycles of 5-FU	230 cGy	46 Gy	20 fr	Rectum Ca	No Mutation detected
Patient-14	4 cycles of capecitabine	180 cGy	45 Gy	25 fr	Rectum Ca	No Mutation detected
Patient-15	4 cycles of capecitabine	180 cGy	45 Gy	25 fr	Rectum Ca	No Mutation detected
Patient-16	4 cycles of 5-FU	230 cGy	46 Gy	20 fr	Rectum Ca	No Mutation detected
Patient-17	4 cycles of capecitabine	180 cGy	45 Gy	25 fr	Rectum Ca	No Mutation detected
Patient-18	4 cycles of capecitabine	180 cGy	45 Gy	25 fr	Rectum Ca	No Mutation detected
Patient-19	4 cycles of capecitabine	180 cGy	45 Gy	25 fr	Rectum Ca	Phase I, II, III Frameshift Mutation
Patient-20	4 cycles of capecitabine and oxaliplatin	180 cGy	45 Gy	25 fr	Rectum Ca	No Mutation detected
Patient-21	4 cycles of capecitabine	180 cGy	45 Gy	25 fr	Rectum Ca	No Mutation detected
Patient-22	4 cycles of 5-FU	180 cGy	45 Gy	25 fr	Stomach Ca	No Mutation detected
Patient-23	Declined to take chemotheraphy	180 cGy	45 Gy	25 fr	Esophagus Ca	Phase I, II, III Frameshift Mutation

Cycle Sequencing

For cycle sequencing, purified PCR product were used in each sample. Cycle sequencing was done at initial denaturing step for 3 min at 95 °C followed by 30 cycles of amplification with denaturation for 10 s at 95 °C, annealing for 5 s at 50 °C, and chain elongation for 2 m 30 s at 60 °C. Samples were purified in a filtered 96-well microplate by using Sephadex G-50 DNA Grade Fine (Sigma-Aldrich). Same primers as for PCR also were used for sequencing. Cycle sequencing was performed on both strands using dye-terminator chemistry (BigDye™ Terminator 3.1, Thermo Scientific).

Sanger Sequencing

For automated sequencing, sample denaturated then sequence program (Perkin Elmer) is used and files uploaded by using Applied Biosystems/Hitachi 3130 (XL) instrument. The data was assembled, compared to a prior to RT genomic sequence of each patient to after RT. Results analyzed by using GeneStudio™ Pro 2.2.0.0 and BLAST performed (https://blast.ncbi.nlm.nih.gov/).

Bioinformatic Analysis

For the Kaplan Meier plots (kmplot.com/analysis/) website was used. ROCplot shows significance of chemotherapy/ radiotherapy responding status of patient group for a specific gene [23]. UALCAN database used to retrieve gene expression level in normal vs tumor samples also individual cancer stage/ TP53 mutation status/Patients weight data were used from TCGA database [25, 26].

Statistical Analysis

All data are expressed as mean ± %SEM. Correlation, three-way analysis of variance was conducted using SigmaPlot version 12.0 (Systat Software, Inc., San Jose, CA, USA). One-way analyses of variance and *t-test* were conducted

using GraphPad Prism version 8 (GraphPad Software, Inc., La Jolla, CA, USA). The effect of Frameshift mutation on CA 19-9 and CEA was examined by using a student *t*-test.

Results

In our study group, neoadjuvant chemotherapy/radiotherapy was given to 23 patients 20–25 fractions at dose of 180–230 cGy daily dose and completing 45–50.4 Gy total radiation dose. Patients were treated with various chemotherapy including, 5-FU, capecitabine, FOLFOX, oxaliplatin. Only one patient declined to take chemotherapy but continue to take RT (Table 1). Our study group comprised of 91.3% colorectal cancer and 8.7% other (esophagus and stomach cancer) types. Tumor site for colorectal cancer patients is distal rectum at 62.9% and Metastatic patient rate was 21.74% (Table 2). Blood was drawn from patients in 3 separate phases. Phase I represents a reference for the gene that will be sanger sequenced and it is drawn before radiotherapy. Phase II represents the middle timepoint for radiotherapy which is after 15 days of the RT. Phase III represents the end timepoint for the radiotherapy which is the end day of the treatment (Day 25 or 28) and each phase was taken for each patient individually.

Table 2 Patient statistics regarding to the tumors

Demographic and clinical parameters	Patient n (%)
Gender	
Female	5 (21.7)
Male	18 (78.3)
Gastrointestinal system tumor	23
Colorectal cancer	21 (91.3)
Others	2 (8.7)
Tumor site in colon cancer	
Distal Rectum	13 (61.9)
Mid Rectum	5 (21.74)
Proximal Rectum	3 (14.3)
Local Invasion	
T2	4 (17.4)
T3	17 (73.9)
T4	2 (8.7)
Lymph node involvement	
N0	5 (21.74)
N1	7 (30.43)
N2	9 (39.13)
N3	1 (4.35)
N4	1 (4.35)
Metastasis	
Met	5 (21.74)
Non-Met	18 (78.26)
Histologic grade	
High grade	7 (30.43)
Intermediate grade	11 (47.83)
Low grade	5 (21.74)

ATG5 Gene not Showing Exon Variations in any Phases of Radiotherapy

Clinicopathological role of ATG5 in colorectal cancer is paradoxical. Patients with sporadic colorectal cancer has strongly low expression level of ATG5 gene however, lymphovascular invasion of tumor relates to higher ATG5 gene expression [27]. The Kaplan–Meier survival analysis showed that as ATG5 gene expression increases, cancer survival probability also increases to 85.6 months from 24.9 months (Fig. 1A). ROC plotter with settings of any chemotherapy and RT showed that ATG5 gene expression did not change non responder vs responder patients (Fig. 1B). There were no exon alterations detected for ATG5 genes for Phase I, II, and III in our study group (Fig. 1C) which is parallel to the ROC plot. UALCAN database showed lower expression of ATG5 for rectum cancer that corresponds to the KM plot (Fig. 1D). Among different stage of rectum cancer, there is not significant change between other stages (Fig. 1E).

ATG12 well Conserved in All Phases During Radiotherapy

Many studies pinpoint ATG12 downregulation enhances radiosensitivity for colorectal cancer [28, 29]. Survival analysis demonstrates ATG12 gene expression level is not significantly associated with survival (Fig. 2A). Additionally, any type of chemotherapy /radiotherapy receiving patients also didn't show differentiated gene expression level for ATG12 gene in responder vs non-responder group (Fig. 2B). Colon adenocarcinoma samples had a higher gene expression level compared to healthy tissues however protein expression level does not differ among normal vs tumor samples (Fig. 2D-E). ATG12 expression for different stages of rectum cancer, did not vary for other stages (Fig. 2F).

ATG9B Gene Revealed Exon Variations During Radiotherapy

Delicate balance exists between physiology to pathology for autophagy. Recent RNAseq analysis for colorectal cancer patients also showed ATG9B is upregulated significantly compared to adjacent tissue [30].

ATG9B low level of gene expression correlates with high survival rate (107 months vs 32.1 months) in KM plot (Fig. 3A). Also chemotherapy/RT receiving group has lower ATG9B gene expression in good prognosis (responsive to

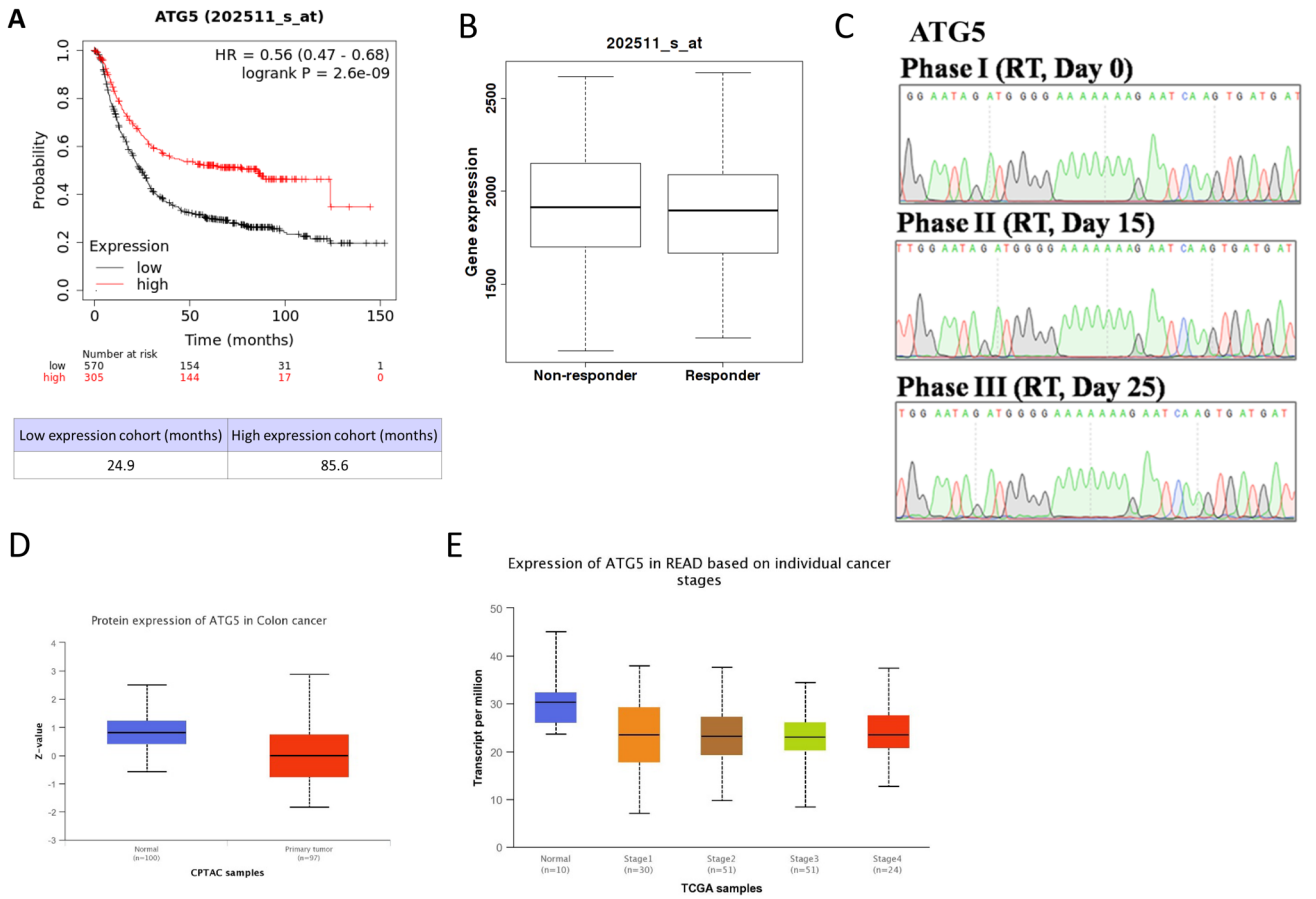


Fig. 1 ATG5 gene analysis. Kaplan Meier Plot for ATG5 gene (**A**). RT/CT response for ATG5 gene (**B**). A representative figure of sanger sequencing of 23 patients for ATG5 genes (**C**). Protein expresssion level for ATG5 gene in Colon cancer (**D**). Expression pattern according to stages of cancer (**E**)

therapy) group and AUC and p value is significant for separation of false negative vs false positives (Fig. 3B).

We evaluated blood samples from 23 to 17 patients did not show any type of insertions or mutations and their genome in blood cells are not effected from RT (Fig. 3C). ATG9B gene expression level is higher in primary tumor of colorectal adenocarcinoma patients (n = 286) compared to normal (n = 41) tissue (Fig. 3D). ATG9B gene expression level was significantly higher in stomach cancer, esophagus cancer and rectum adenocarcinoma (Figure S1A).

Individual patient staging data showed that as stage increases ATG9B transcript level in parallel increases in transcript number when compared to normal or previous stage (Fig. 3E). Evaluation of stomach cancer, esophagus cancer and rectum adenocarcinoma for ATG9B gene expression in different stage of patients also showed a trend in as stage increases ATG9B gene expression increases additionally (Figure S1B).

p53 which is guardian of the genome gene mutation status also gave a significant finding which is p53 mutation positive samples have significantly higher ATG9B gene expression compared to normal tissue in colorectal cancer (Fig. 3F), stomach cancer, esophagus cancer and rectum adenocarcinoma (Figure S1C). Patient weight data versus ATG9B gene expression correlation showed that obese and extreme obese patients have significantly higher gene expression level in colorectal cancer, esophagus cancer and rectum adenocarcinoma patients. Data for stomach cancer patients was not available so we could not make a correlation for ATG9B versus body weight.

A total of six (n = 6) exon mutations (26.1%) in the sequence of ATG9B gene were detected in the study group (n = 23) during RT. The sequence aberrations included a frameshift mutation (FS) in four patients (17.4%) in two different regions on the genome in phase I, phase II, and phase III (Fig. 4A) and transition mutation in two patients (8.7%) in the same region on the genome in phase I (Fig. 4B).

When the patient's data is evaluated, patients having a frameshift mutation compared to wildtype gene includes T insertion at position 3870 nucleotide (nt) occurred and 8G tandem repeat region has 3G deletion after 5th Guanine in Phase I. In phase II, the 8G mononucleotide tandem repeat

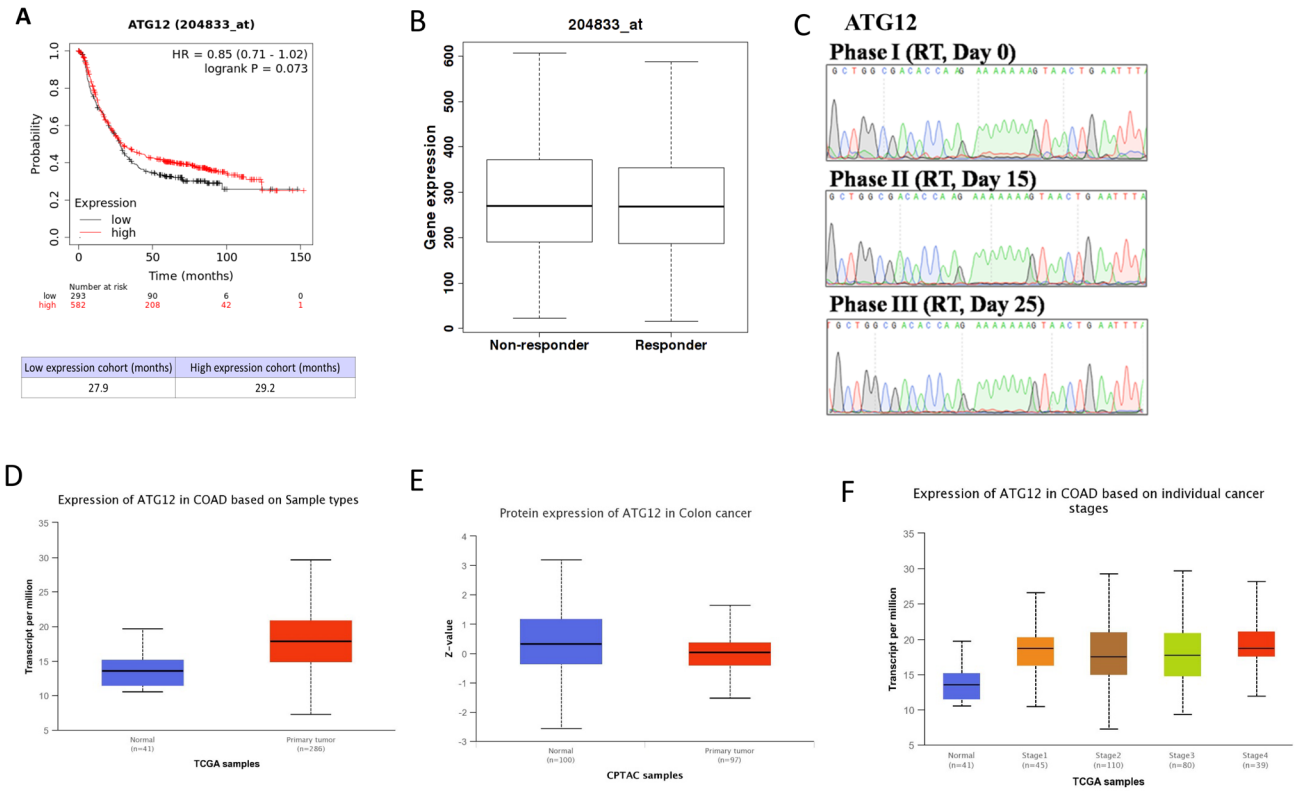


Fig. 2 ATG12 gene analysis. Kaplan Meier Plot for ATG12 gene (**A**). RT/CT response for ATG12 gene (**B**). A representative figure of sanger sequencing of 23 patients for ATG12 genes (**C**). Gene expression level for ATG12 gene in Colon cancer (**D**). Protein expression level for ATG12 gene in Colon cancer (**E**). Expression pattern according to stages of cancer (**F**)

region still has the same 3G deletion after 5th Guanine. In phase III, GTG codon deletion occurred at position 3890-3893nt and then T insertion at position 3961 nt and lastly 3G deletion after 5th Guanine of 8G repeat region. In a summary, this patient group has the most diverse deletion compared to other patients.

All mutations (n = 6) was detected in exon 1 of ATG9B. Patients having transition mutation compared to wildtype gene, resides in 8G mononucleotide tandem repeat region in the genome and results in GGG trinucleotide transition in phase I, however, that mutation was eliminated and genome of the patient reestablished to wildtype in phase II and phase III (Fig. 4B). All mutations occurring were found to be heteroallelic mutations. If each patient's phase I genome status is compared to phase II and phase III, there is a 3G transition mutation at position 3978 nt that occurred in the 8G mononucleotide region of ATG9B.

Cancers with microsatellite instability prevent the success of treatment and genes containing mono or dinucleotide repeats are more susceptible to mutations [31]. In our study, there were mutations in the mononucleotide (monoguanine) region of the ATG9B gene detected and only the ATG9B gene has 8 Guanine repeats whereas ATG5 and ATG12 have Adenine repeats. The ATG5 and ATG12 genes are located far away from the satellite regions on the chromosome, and may not be mutated because they are well preserved or located within the heterochromatin region, and contain monoadenine repeats(7A) (Fig. 4C).

Biogrid database also showed that ATG9B physically interacting with KIF3A which interacts with XRCC4 one of the radiation activated gene (Fig. 4C).

Protein Translation Characteristics of Mutated ATG9B Gene

FS mutation occurring in 4 patients results in a truncated protein (146 amino acids) which is 37 amino acids shorter compared to wild-type protein (183 amino acids) homolog due to the formation of the early stop codon (nonsense) in genetic code. Protein characteristics changed from positive non-polar to negative polar. GTG and GGG codons refer to valine and glycine amino acid subsequently. Deletion of these amino acid means deletion of hydrophobic and non-polar amino acids in the ATG9B protein sequence. These deletions affect the hydrophobicity of the ATG9B protein. After frameshift R, M, G, W, G, G, R amino acids are converted to L, N, G, L, G, E, K amino acids. Loss of valine

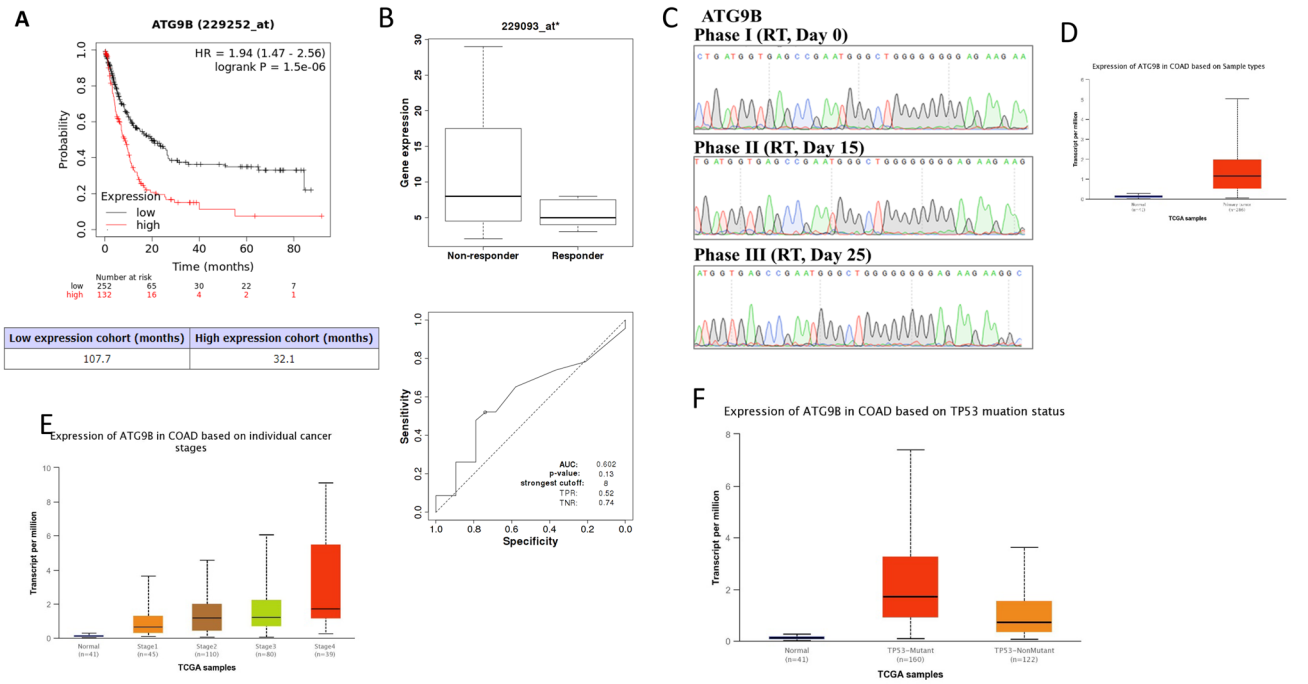


Fig. 3 ATG9B gene analysis. Kaplan Meier Plot for ATG9B gene (**A**). RT/CT response for ATG9B gene (**B**). A representative figure of sanger sequencing of 23 patients for ATG9B genes for no exon variation groups (**C**). Gene expresssion level for ATG9B gene in Colon cancer (**D**). Gene expression pattern according to stages of cancer (**E**). Gene expression level classified according to TP53 mutation status (**F**)

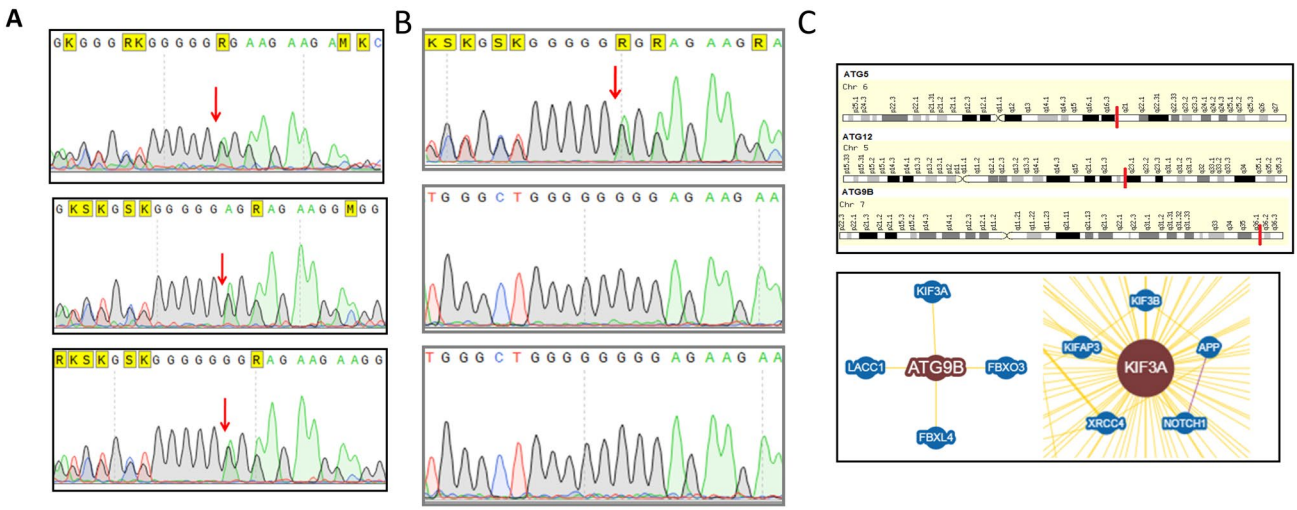


Fig. 4 Sanger sequencing results of all 4 patients for ATG9B gene frameshift mutation. Yellow highlights represent the location of changes detected in the genome. K stands for G or T, R stands for G or C, M stands for A or C, S stands for C or G nucleotide. The red arrow shows the changes in tandem repeat in the ATG9B gene (**A**). Sanger sequencing results for 2 patients who has ATG9B transition mutation in tandem repeat region and red arrow represents the location of transition occurring in the genome. Transition mutation occurred as GGA and AGG in 8 tandem repeat of Guanine of ATG9B gene. K stands for G or T, R stands for G or C, S stands for C or G nucleotide (**B**). Physical map of ATG5, ATG12 and ATG9B gene and Biogrid protein interaction analysis for ATG9B gene (**C**)

amino acid in phase III of 4 patients affects hydrophobicity of the ATG9B protein. All 6 patients have transition mutation in GGG codon which leads to valine deletion in a protein sequence.

Examination of CEA and CA 19-9 Levels in ATG9B Gene Aberration Bearing Patients

Patients having an abnormal level of CEA is 5 (21.7%), patients having a normal range of CEA is 18 (78.3%). Patient having an abnormal level of CA 19-9 is 3 (13.04%) patients having normal range of CA 19-9 is 20 (86.96%). The number of a patient having a high level of CA 19-9 and CEA together is 2 (8.7%). The number of patients who have ATG9B^{Mut} with local recurrence is 1 (%16.7) and distant recurrence is also 1 (%16.7). Patients have neither local nor distant recurrence is 4 (%66.7).

When the patient's histologic grade and ATG9B^{Mut} correlated, the number of patients having well-differentiated tumor is 1 (16.7%) and patients having poor-differentiated tumor is 5 (83.3%). There is no undifferentiated tumor-bearing patient in the ATG9B^{Mut} group.

Colon cancer patients bearing ATG9B^{Mut} have retention at distal colon with 2 (33.3%), at proximal colon with 2 (33.3%), and middle colon location with 2 (33.3%).

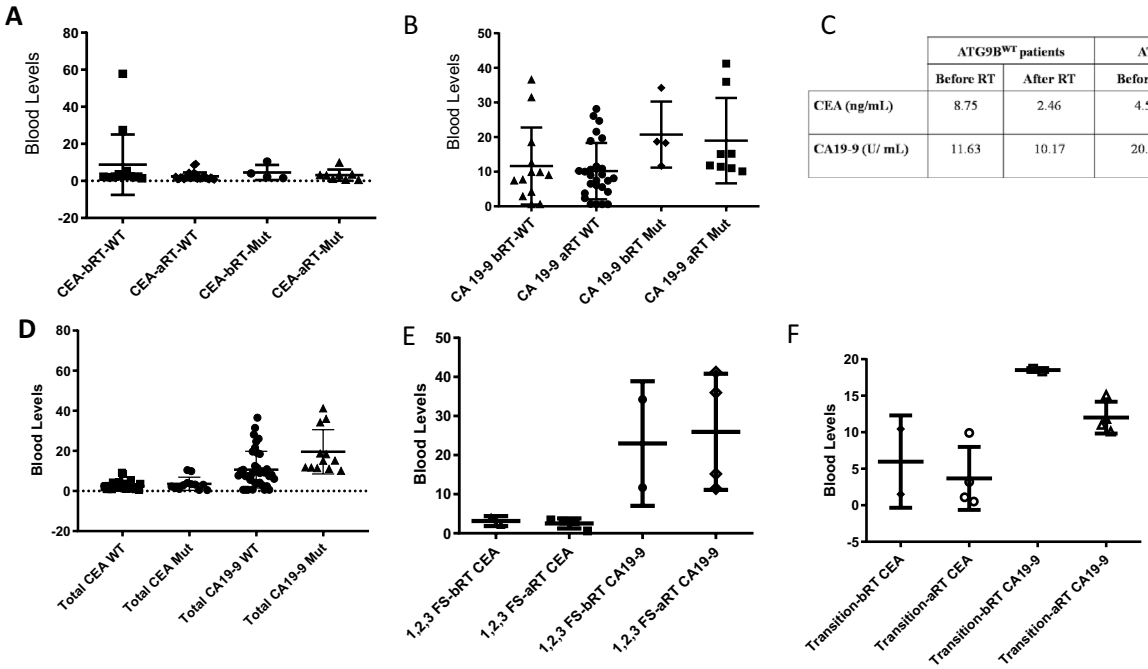
ATG9B^{Mut} gene and tumor grading correlation show us; 16.7% (n = 1) of patients have T2 grade, 66.7% (n = 4) of patients have T3 and 16.7% (n = 1) of patients have T4 grade tumor. There is no patient with ATG9B^{Mut} at T1 grade in the study group.

Relationship between ATG9B gene and lymph node invasion shows that, ATG9B^{Mut} bearing patients at N0 is 33.3% (n = 2), at N1 is 50% (n = 3) and at N2 is 16.7% (n = 1). There is no patient with ATG9B^{Mut} at N3 and N4 grade in the study group.

When metastasis and ATG9B^{Mut} correlated, only 1 (16.7%) patient has metastasis, others, (n = 5, 83.3%) have no metastasis diagnosed.

Chemotherapy status and ATG9B^{Mut} relationship show that patients take only capecitabine with the ratio of 33.3% (n = 2), 50% (n = 3) takes only 5-FU and 16.7% (n = 1) refused to take chemotherapy.

Basal level of CEA in ATG^{WT} for before RT group was higher. When before (bRT) and after RT (aRT) groups are compared by means of ATG9B^{Mut} and ATG9B^{WT}, patients bearing ATG9B^{Mut} respond to the RT poorly. Patients who carry ATG9B^{WT} have experienced dramatic decay in CEA values after RT when compared to ATG9B^{Mut} (Fig. 5A).



	ATG9B^{WT} patients		ATG9B^{Mut} patients	
	Before RT	After RT	Before RT	After RT
CEA (ng/mL)	8.75	2.46	4.55	3.09
CA19-9 (U/ mL)	11.63	10.17	20.73	18.97

G

	ATG9B^{Mut} 3G Del, 2,3N patients		ATG9B^{Mut} 1,2,3, FS patients	
	Before RT	After RT	Before RT	After RT
CEA (ng/mL)	5.97	3.67	3.13	2.52
CA19-9 (U/ mL)	18.51	12.00	22.96	25.95

Fig. 5 Representation of CEA before (bRT) and after RT (aRT) in ATG9BMut and ATG9BWT patients (**A**). Representation of CA 19-9 before (bRT) and after RT (aRT) in ATG9BMut and ATG9BWT patients (**B**). Quantitative data for Fig. 5B (**C**). FS data analysis for CEA and CA 19 9 levels (**E**). Transition mutation data analysis for CEA and CA 19 9 levels (**F**). Quantitative data for Fig. 5F (**G**)

$ATG9B^{WT}$ started with low CA19-9 values, aRT values did not significantly change and $ATG9B^{WT}$patients indicate a good response to RT through CA19-9 values. When the initial level of CA19-9 is compared between groups of $ATG9B^{Mut}$ and $ATG9B^{WT}$, CA19-9 values of $ATG9B^{WT}$ patients were 50% lower than $ATG9B^{Mut}$ patients. RT lowered CA19-9 levels of each group however didn't alter the level significantly (Fig. 5B and C).

Blood levels of CEA for $ATG9B^{Mut}$ for phase I, phase II, and phase III subsequently are; 4.55, 3.5 and 2.68 ng/ml whereas CA19-9 mean values for the same group subsequently are 20.73, 20.1, and 17.84 U/ml.

Blood panel result of $ATG9B^{Mut}$ bearing patient at phase I, phase II, and phase III indicates that total CA19-9 mean values is higher when compared to $ATG9B^{WT}$ (Fig. 5D).

4 patients in the group of $ATG9B^{Mut}$ frameshift mutation groups' mean values of CEA levels bRT and aRT are 3.13 and 2.52 whereas mean values of CA19-9 levels are 22.96 and 25.95 (Fig. 5E and F).

$ATG9B^{Mut}$ 1,2,3, FS patients having a mutation in each phase, their response to RT treatment is not as good as $ATG9B^{Mut}$ transition mutation in phase I but $ATG9B^{WT}$ in phase II and phase III patients. The former group's CA19-9 values increased, and CEA values decreased at aRT however the latter groups both blood levels of CEA and CA19-9 declined at aRT.

Discussion

Autophagic cell death definition came to the literature less than a decade however, an increasing body of evidence indicates that aberration in the autophagy signaling pathway leads to some abnormalities such as neurodegeneration, microbial infection, cancer [32]. In this paper, the relationship between autophagy gene mutations and gastrointestinal system tumors was managed to be documented. Our study contains variation analysis of ATG5, ATG12, ATG9B genes by using the Sanger sequencing technique to documents an association between radiotherapy and gene mutation. This study is one of the first conducted in radiogenomics focusing autophagy.

One of the most resembling studies to this project is done by Kang and his colleagues; proving that autophagy-related genes (ATG2B, ATG5, ATG9B, and ATG12) having a 7 or more nucleotide repeats can have a mutation detected by single-strand conformation polymorphism (SSCP) analysis in cancer patients. The number of mutation detected in gastric and colorectal patients with microsatellite instability compared to normal tissue of the same individual are 2,13,0 for ATG5, ATG9B, and ATG12 respectively [33, 34]. In our study also, we checked the same gene mutations and we have similar results by detecting no mutation for ATG12 and detecting some mutations for the ATG9B gene. ATG5 and ATG12 proteins act accordingly together in the cell and none of them have a mutation is not an unexpected result for ATG5 and ATG12 genes. However, literature has shown that ATG12 deficiency results in intracellular glutamine depletion, abrogation of tumor hypoxia and a favorable prognosis in cancer [35]. Recently, a study reported that ATG5 activation plays a role in the metastasis of esophageal squamous cell carcinoma, and ATG5 upregulation predicts a poor clinical outcome [15].

The frameshift mutation resulting from T nucleotide insertion in 4 patients in the ATG9B gene leads to the loss of amino acid loss (truncated mutant protein), which in turn can lead to changes of the autophagy pathway.

It has been determined in the previous studies that nucleotide repeat regions likely to have mutation during synthesized from DNA polymerase during the replication, in some cases, it causes mutations in cases where it jumps or slips from the template due to the repetitive regions which are mutation hotspot points [36]. This indicates that the mononucleotide repeat region of the ATG9B gene containing 8G is a hotspot mutation site.

In the cell, there are two DNA repair pathways for double-strand break repairs; homolog recombination and non-homologous-end-joining [37]. NHEJ repair system is activated when the HR mechanism is halted, in the instance of gene mutation which is involved in the HR repair mechanism. Genes that take a role in the NHEJ mechanism are MRN complex (MRE11-RAD50-NBN, PARP1, XRCC1, and LIG1 or LIG3) [38]. DNA repair, Wnt signaling pathway, and apoptosis pathway were inactivated by frameshift mutations in cancer patients with microsatellite instability [39]. If we examine the proteins that interact with ATG9B protein physically (protein–protein interaction), ATG9B is closely related to DNA repair enzymes. The ATG9B protein shows physical interaction with the KIF3A protein and the KIF3A protein interacts with XRCC4 protein which is involved in the NHEJ ligation step in DNA repair mechanism (Fig. 4C).

Microsatellite instability is present in 10% to 30% of patients diagnosed with gastric and colon cancer [40]. The ATG9B gene is localized in very close proximity to the satellite regions of the chromosome on the physical chromosome map, in which case due to the ATG9B gene location, occurrence of instability and loss of function may be speculated (Figure-5B).

CA 19-9 is a tumor marker that is detected in colorectal cancer patients with advanced stages. In the study conducted by Forones and Tanaka, they found that risen levels of preoperative CA19-9 values had a prognostic value in patients followed for 30 months. CEA was increased in 42% of patients and CA 19-9 was increased in 35%. CEA or CA 19-9 levels increased in 73% of patients with recurrence. In

the first year of recurrence, 93% of the patients had elevated CEA and/or CA 19-9 values [41]. In patients who have not undergone surgery, CEA and/or CA 19-9 values are markers of increased mortality until the cancer is controlled and indicate that these patients are at high risk [42]. In our study, according to our analysis, patients with $ATG9B^{Mut}$ had high levels of CA 19-9, before starting RT besides there was no decline after RT. In patients with $ATG9B^{WT}$, lower values of CEA and CA19-9 were detected before RT and CEA levels significantly decreased after RT.

Genetic mutations: occurring as nucleotide repeats insertion, deletion, frameshift are generally acquired mutations and causing amino acid sequence change in resulting proteins. ATG9B can be a potential biomarker for radiosensitivity because Xiao et al., recently published a study showing chloroquine and ATG9B inhibitor sensitized hepatocellular carcinoma cells to ^{125}I radioactive particles in vitro and in vivo [43]. Moreover, recent a study reported that ATG9B is upregulated and is positively associated with poor prognosis in CRC patients [44]. However, some clinical databases regarding gastrointestinal tumors showing that ATG9B gene is overexpressed in those tumors and CT/RT responsive group lowering this gene expression to have good prognosis. Gil et al., recently also showed in RNA seq analysis only ATG9B and ULK4 is significantly upregulated in tumor samples and 38 CRC cell lines [30].

Further functional studies are required with a larger sample-size to see how the truncation mutants of ATG genes contribute to tumor development, progression, and therapy response. Future studies may be planned as the determination of involvement of the autophagy-related marker genes before and after radiotherapy, in tumor tissues, or by repeating this study with a larger population for different gene investigation. In addition to an exon, intron mutations can also be examined by the NGS method.

Conclusions

An accomplishment achieved with this study is detecting genome mutagenesis by the effect of radiotherapy. And this can be a permanent or temporary effect on the genome. The most likely effect is temporary due to chemotherapy and radiotherapy.

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Declarations

Conflict of interests The authors declare no conflict of interests.

Ethical Approval Istanbul Training and Research Hospital Ethics Committee (No.: 2015/2060).

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