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The Association between High Dose Radioactive Iodine Therapy and Telomeric and Chromosomal Damage in Thyroid Cancer Patients

갑상선암 환자에서 고용량 방사성 요오드 치료와 텔로미어 및 염색체 손상 간의 연관성

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Abstract

The Association between High Dose Radioactive Iodine Therapy and Telomeric and Chromosomal Damage in Thyroid Cancer Patients

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Radioactive iodine (RAI) therapy has been specialized to treatment of differentiated thyroid carcinoma during several decades, contributing the improvement of cancer survival. However, there were also concerns about internal radiation hazard from radioactive iodine, suggested by several epidemiologic studies that reported higher second malignancy rates in thyroid cancer patients with RAI treatment. Given these epidemiologic or experimental findings, cumulative doses for RAI treatment is recommended not to exceed over 600mCi; however, there were few studies about long term carcinogenic effects of RAI treatment. This study was designed to examine the changes of clinical outcomes and the incidence of second malignancies in thyroid cancer patients and to evaluate the association between high dose RAI therapy and telomeric and chromosomal damages. To

examine telomeric damages in thyroid cancer patient with or without RAI treatment, relative telomere length (RTL) and hTERT mRNA expression were measured and compared among RAI cumulative doses. Chromosomal damage was estimated by chromosomal fragility test, used in diagnosis of Fanconi disease that is characterized by easy chromosomal breakages and malignancy risk. In this study, during 4 decades, recurrence rate of thyroid cancer was decreasing with more favorable changes in pathological characteristics and increasing rate of RAI treatment. Compared with general population, the incidence of second malignancy after thyroid cancer treatment was significantly higher especially in patients with extremely high dose of RAI over 1000mCi. Of 505 cases used in analyses of relative telomere length, RTL showed trends of being shorter in patients with high dose of RAI treatment and was observed to be a significant correlation in multivariate analyses. This inversed correlation between RTL and RAI dose was more prominently observed in patients with higher RAI dose over 300mCi. Expression of hTERT mRNA did not show significant association with RAI doses. Chromosomal fragility was observed in patients with RAI treatment; however, the subgroup analysis by age groups showed that, only in older age groups over 40 years, corresponding trend was observed without statistical significances. In this study, a parameter of telomere length and a parameter of chromosomal fragility showed significant differences between two groups lower and upper than 100mCi and higher doses of cumulative RAI dose. However, in other parameters, there were significant differences at any cut-off doses. In summary, patients with higher dose RAI therapy tended to have shorter RTL, especially in patients group younger than 60 years old, and hTERT mRNA expression and chromosomal fragility were not associated with cumulative RAI dose. Despite limitation in interpretation, the results suggested the cut-off dose showing statistical significances from 100mCi.

Keywords : radioactive iodine, cumulative dose, thyroid cancer, second malignancy, telomere, hTERT, chromosomal fragility

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Introduction

Well differentiated thyroid carcinoma is rapidly growing endocrine tumors, with excellent survival outcomes in most patients. Improvement of survival rate would be attributed to early diagnosis using high resolution neck ultrasonography and standardized cytology report system. Additionally, effective treatment strategies, such as total thyroidectomy, postoperative radioactive iodine therapy, and active monitoring using thyroglobulin and ultrasonography, also might contribute improving cancer survival. The previous study demonstrated significant decreases in recurrence or cancer-specific mortality through the study periods (1). Especially, radioactive iodine (RAI) therapy is known to be beneficial to reducing recurrence rates and enabling early detection of recurrence in thyroid cancer patients (2). Several studies have reported the lower recurrence rate and cancer-specific survival in patients with RAI treatment (3-5).

Radioactive iodine doses used in thyroid cancer patients ranges from 30mCi to 200~300mCi, according to disease status of each patient. Internal radiation from administrated radioactive iodine make tissue damage by direct effect on DNA damage and indirect effect from inducing oxygen free radical and gradual tissue fibrosis (6). Although beta-ray from radioactive iodine has only weak penetration ability to be limited around thyroid follicular cells, there has been concerns about radiation hazard by repeated treatment with high dose RAI, supported by epidemiologic studies that the frequencies of second malignancies increased in thyroid cancer patients with RAI treatment than those without RAI treatment (7-9). In practice, clinical guidelines recommend the cumulative dose of RAI not to exceed 600mCi, in order to avoid excessive bone marrow suppression or hematologic malignancy (10). However, there were no evidences suggesting direct

association between internal radiation from RAI treatment and chromosomal damage, or the optimal cut off cumulative dose increasing hazard.

The association between external radiation and chromosomal damages and consecutive malignancy risk has been reported in previous studies (11-13). The adverse effects by RAI treatment also was reported in a few studies that peripheral white blood cells in patients with Graves' disease showed more frequent chromosomal breakages after RAI treatment, compared with before RAI treatment (14). Chromosomal damage after RAI treatment showed only in acute stage and recovered after several days or weeks, being attributed to the DNA repairing mechanisms. However, epidemiologic studies still showed significant risk elevation of second cancer of parotid gland, bladder, and bone marrow in patients with RAI therapy might suggesting the long-term effect by RAI treatment (15).

Based on the discrepancy between transient chromosomal damages and long-term risk effect, I hypothesized that there would be a certain residual damages in patients with high dose RAI treatment. Especially, the persistent residual damages might be observed in the level of chromosome or telomere, which were reported to be vulnerable to radiation. If the adverse effect from internal radiation would persist even several years after RAI treatment, damages on chromosome or telomere could be observed in patients with high dose RAI, with dose-response relationship. To measure the degree of damages of chromosomes and telomere, I planned to use chromosomal fragility and telomere length and hTERT mRNA expression.

Chromosomal fragility test has been used in diagnosis of Fanconi anemia, characterized by chromosomal instability and a consequent increase of malignancy risk (16). After white blood cells from both patients and healthy controls being exposed to cytotoxic radiation or chemicals, the proportion of the number of break point in patients'

sample are compared to the proportion shown in healthy controls. A previous study showed the increasing ratio of the chromosomal breakage counts in patients' blood sample after RAI treatment (17).

Telomere is the structure with repetitive DNA sequence at the terminal portion of chromosome, which plays a role of protecting chromosomes from inevitable loses of genetic information during cell divisions, finally followed by cell senescence or apoptosis. Several studies reported that chemotherapeutic agents and radiation therapies contributed to shorten telomere length and the effects persist even after the treatments (18-20). Furthermore, an epidemiologic study showed significantly short telomere length of peripheral white blood cell in Chernovl wokers than in general population, even three decades after the accident (21). To protect persistent shortening of telomere, cells are equipped with repairing machineries to compensate shortened length of telomere, of which telomerase compensates loss of telomere by synthesizing complementary DNA. In the situation of telomeric damage, the expression of human telomere reverse transcriptase (hTERT) gene increases to elevate activity of telomerase (22-24). Several studies reported increases of mRNA expression of hTERT gene, and the its association with tumor aggressiveness or clinical poor prognosis (25-27)

I aimed to examine the increases of second primary malignancy in patients with previous RAI treatment and to compare chromosomal or telomeric damages between patient with or without RAI treatment and groups, using chromosomal fragilities, hTERT gene expression, and shortening of telomere length as the parameters of DNA damages. Furthermore, I estimated possible cut-off level of RAI cumulative dose, at which statistical significances of telomere shortening or chromosome fragility start to be observed.

Methods

1. Subjects

Among the patients who underwent thyroidectomy with or without RAI treatment and followed in Seoul Nation University Hospital since 1962, 3147 patients with papillary thyroid cancer were used in analyses for secular changes in clinical characteristics and outcomes. Their clinical information including age at surgery and study enrollment, sex, cumulative doses of RAI and clinical outcomes were gathered through medical record review. Survival information was obtained using Korea Statistics Information System (KOSIS).

Telomere length was measured in 505 patients who were diagnosed as well differentiated thyroid carcinoma and underwent total thyroidectomy with or without RAI ablation in same hospital. With informed consents, clinical information including age at surgery and study enrollment, sex, cumulative doses of RAI and clinical outcomes was obtained. After obtaining their peripheral blood, genomic DNA for telomere length and RNA for hTERT RT-PCR were extracted within 24 hours after sampling. Of RNA samples of 92 patients, hTERT mRNA RT-PCR and quantification of the expression were measured in 62 samples which were suitable to perform PCR.

Chromosomal fragility was measured in 39 patients with consideration for matching age, sex, and cumulative RAI dose. All participants had given their written informed consent, and the Internal Review Boards approved the current study (IRB No. 1204-077-406).

2. Treatment and follow-up strategy and definition of recurrence

After thyroidectomy, postoperative RAI remnant ablation therapy was conducted in patients with high risk of recurrence, such as regional LN involvement, extra-thyroidal invasion, or distant metastasis. Patients without pathologically aggressive characteristics were treated repeatedly with 30mCi of RAI, until post-ablation whole body scan did not demonstrate iodine uptake. For patients with an aggressive pathology, including residual tumor lesions, gross invasions, lateral neck node metastases, or aggressive histological types predicting poor outcomes, higher doses of RAI of 100–200mCi were used selectively. Regular monitoring to detect recurrence or progression of thyroid cancer includes physical examinations, serum thyroglobulin and anti-thyroglobulin antibody titer, and periodic neck ultrasonography. For patients with a moderate to high risk for recurrence, patients underwent TSH suppression within undetectable levels of serum TSH during 5–10 years. In case of patients without evidence of recurrence, the dose of levothyroxine was adjusted to maintain a normal serum TSH level. For low risk patients, serum TSH levels were kept close to the lower margin of the reference range. Recurrence was defined as the cases that patients were pathologically confirmed to have recurrent disease by fine needle aspiration cytology or surgical specimens. Although pathologic confirmation was not made, highly suspicious lesions on imaging studies such as WBS, computed tomography, magnetic resonance imaging, or positron emission tomography were also defined as recurrence.

3. Reverse transcriptase PCR of hTERT mRNA expressions

RNA samples were extracted from peripheral white blood cells, which were obtained by centrifuge of whole blood within 24 hours after sampled from subjects. Total RNA was extracted by a commercial kit (RNeasy Mini Kit, Qiagen Inc.) from peripheral blood mononuclear cells which were stored at -80℃and extracted RNA samples were quantified spectrophotometrically at A260/280nm. In brief, after lysis buffer was added to each sample to make mixture of 350uL, the mixture was centrifused at 14,000 × g for 2 min at room temperature. After the supernatant was discarded, 70% EtOH remaining precipitate was added to remaining precipitate and well mixed until being aggregated. Then, after centrifused at 13,000 × g for 15 seconds, the supernatant was mixed with washing buffer and centrifused 13,000 × g for 15 seconds at three times. Finally, after RNAase free water 40uL was added and centrifused at 13,000 × g for 60 seconds, the precipitate was stored at -80℃.

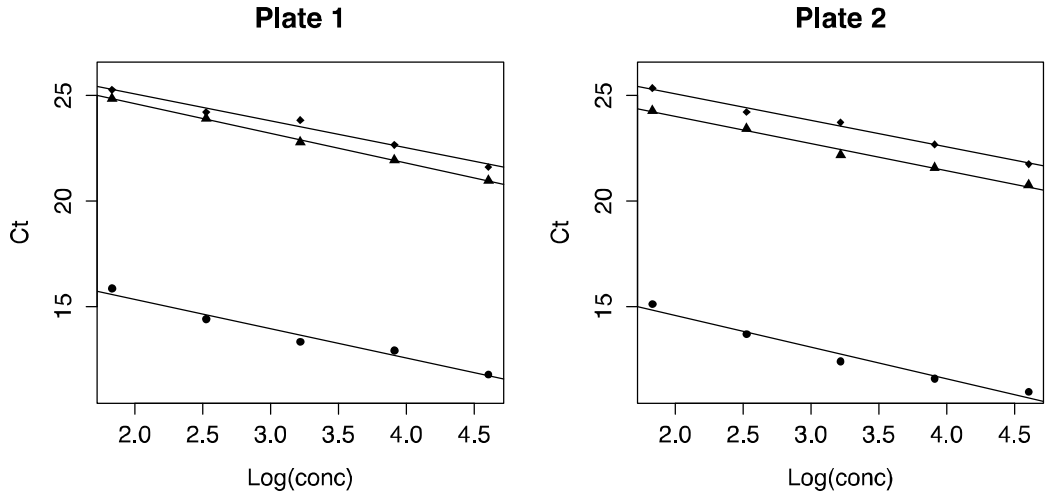
In each RNA sample, 0.25~0.5ug of RNA was diluted with DEPC solution and added with 5×buffer 5uL, 0.1M DTT 2.5uL, 10mM dNTP 1.25uL, oligo-dT 0.5uL, RNAase inhibitor 0.25uL and RTase 1uL, and final 25uL mixture was used in PCR for cDNA. Prepared cDNA was diluted in 1:4 and mixed with dNTP, 10×buffer, Tag polymerase, and forward and reverse primers, followed by PCR amplification. Primer and probes were designed, based on previously published study (28). The sequences for the hTERT primer were forward: 5'-AGAGTGTCTGGAGCAAGTTGC-3', reverse: 5'-CGTAGTCCATGTTCACAATCG-3'. This primer showed band at 183 bp and at the PCR condition of 53℃ and 40 cycles. GAPDH gene was used control to evaluate the quality of RT-PCR in each session. Quatification of hTERT mRNA expression were performed using densitometry by imaging analysis software (AlphaView, Santa Clara, CA, USA)

4. Measurement of Relative Telomere Length (RTL)

All DNA extractions were performed using the QIAcube and QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's protocol. DNA samples were centrifuged within 24 hours from whole blood samples and preserved in -70℃. RTL is a calculated ratio of copy number of repetitive telomeric DNA against copy number of single copy gene. The amount of target DNA was estimated by Ct value, the number of cycles required for the fluorescent signal to cross the threshold. Because telomere sequence repeats with TTAGGG, its copy number represent telomere length. Given that each blood sample has various cell number, total amount of telomere is needed to be adjusted for cell counts in each samples. For this purpose, the reference gene, known to be in a single copy in each cell, had been used to measure its copy number in every real-time PCR. I used two kinds of reference single copy genes, 36B4 and β-globin, to estimate mean content of telomeric DNA in each sample (29,30). The sequences of primer for telomere were forward: 5'-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3', reverse: 5'-GGCTTGCCTTACCCTTACCCTTACCC TTACCCTTACCCT-3'. The sequences of 36B4 gene were 5'-CAGCAAGTGGGAAGGTGTAATCC-3', reverse: 5'-CCCATTCTATCATCAACGGGTACAA
-3'. The sequences of β-globulin were forward: 5'-GCTTCTGACACAACTGTGTTCACTAGC-3', reverse: 5'-CACCAACTTCATCCACGTTCACC-3'. DNA concentrations of telomere and single copy genes in case samples were estimated using the linear regression equations from known concentration and their Ct values of standard samples in each PCR session (Fig 1). The ratios of telomeric DNA concentrations to single copy gene concentration were analyzed as

relative telomere length (RTL). Estimated concentration of telomere and two single-copy genes were measured two times and the mean values were finally used (Table 1). Two relative telomere lengths, ratios of telomere referred to 36B4 gene and β-globin gene, were analyzed.

Figure 1. Example of standard curves and regression models of Ct values according to concentration of standard samples in PCR of telomere and single copy genes. Concentrations of standard sample were 6.25, 12.5, 25, 50, and 100ng/mL, and used in the logarithm transformed values in regression. Circle indicates telomere gene, triangle 36B4 gene and diamond β-globin gene.



Gene	Plate 1	Plate 2
Telomere	-1.392 × Log(conc) + 18.127	-1.505 × Log(conc) + 17.586
36B4 gene	-1.404 × Log(conc) + 27.413	-1.280 × Log(conc) + 26.565
β-globin	-1.282 × Log(conc) + 27.642	-1.262 × Log(conc) + 27.609

Table 1. Estimation of concentration of telomere and single copy genes (Example)

	Plate 1						Plate 2						mean concentration			RTL	
	Ct			Estimated Concentration			Ct			Estimated Concentration							
Gene	telomere	36B4	β-globin	telomere	36B4	β-globin	telomere	36B4	β-globin	telomere	36B4	β-globin	telomere	36B4	β-globin	36B4	β-globin
Case 1	14.05	22.97	23.91	18.78	23.68	18.38	13.27	22.51	23.93	17.60	23.72	18.50	18.19	23.70	18.44	0.77	0.99
Case 2	13.72	22.94	23.57	23.67	24.28	23.92	13.08	22.56	23.61	19.99	22.81	23.70	21.83	23.54	23.81	0.93	0.92
Case 3	13.12	22.92	23.57	36.55	24.50	24.03	12.42	22.68	23.51	31.06	20.80	25.84	33.80	22.65	24.94	1.49	1.36
Case 4	13.55	22.58	23.26	26.72	31.17	30.63	12.90	22.21	23.33	22.55	30.03	29.71	24.63	30.60	30.17	0.80	0.82
Case 5	13.35	22.89	23.61	30.94	24.99	23.29	12.77	22.64	23.53	24.53	21.48	25.35	27.74	23.24	24.32	1.19	1.14
Case 6	13.86	23.24	23.86	21.39	19.56	19.18	13.19	22.94	23.81	18.58	16.99	20.26	19.98	18.28	19.72	1.09	1.01
Case 7	13.48	22.89	23.81	28.24	25.01	19.88	12.86	22.45	23.85	23.15	24.88	19.63	25.70	24.95	19.76	1.03	1.30
Case 8	13.55	22.94	23.82	26.87	24.22	19.67	12.94	22.85	23.87	21.93	18.16	19.38	24.40	21.19	19.52	1.15	1.25
Case 9	14.16	22.86	23.74	17.28	25.57	20.92	13.47	22.47	23.81	15.38	24.49	20.29	16.33	25.03	20.61	0.65	0.79
Case 10	13.58	23.02	23.58	26.17	22.88	23.85	12.80	22.71	23.54	24.08	20.39	25.07	25.12	21.63	24.46	1.16	1.03
Case 11	13.57	22.86	23.63	26.51	25.70	22.90	12.92	22.36	23.74	22.16	26.82	21.47	24.34	26.26	22.18	0.93	1.10
Case 12	13.19	22.67	23.73	34.63	29.23	21.13	12.52	22.62	23.78	29.06	21.80	20.75	31.85	25.52	20.94	1.25	1.52
Case 13	13.09	22.79	23.96	37.29	26.84	17.65	12.44	22.38	23.94	30.63	26.32	18.31	33.96	26.58	17.98	1.28	1.89
Case 14	13.62	23.35	24.40	25.41	18.13	12.59	12.98	22.93	24.45	21.28	17.18	12.27	23.35	17.65	12.43	1.32	1.88
Case 15	13.88	22.87	23.77	21.16	25.44	20.43	13.21	22.42	23.83	18.34	25.43	20.02	19.75	25.44	20.23	0.78	0.98
Case 16	13.44	22.83	23.48	29.08	26.22	25.70	12.96	22.50	23.40	21.67	23.89	28.02	25.38	25.05	26.86	1.01	0.94
Case 17	13.95	23.01	23.93	20.18	23.03	18.13	13.43	22.67	23.91	15.80	21.00	18.78	17.99	22.01	18.46	0.82	0.97
Case 18	14.03	23.13	23.63	19.05	21.08	22.93	13.40	22.82	23.62	16.12	18.72	23.59	17.58	19.90	23.26	0.88	0.76
Case 19	13.48	23.21	23.78	28.24	19.99	20.35	12.81	22.83	23.77	23.94	18.53	20.91	26.09	19.26	20.63	1.35	1.26
Case 20	13.86	23.06	23.45	21.42	22.24	26.39	13.15	22.74	23.43	19.12	19.93	27.47	20.27	21.08	26.93	0.96	0.75
Case 21	14.22	22.90	23.69	16.60	24.98	21.82	13.60	22.53	23.70	14.18	23.48	22.14	15.39	24.23	21.98	0.64	0.70
Case 22	13.47	22.86	23.70	28.36	25.61	21.65	12.72	22.32	23.61	25.41	27.52	23.84	26.89	26.56	22.74	1.01	1.18
Case 23	13.85	22.91	23.69	21.63	24.78	21.80	13.01	22.71	23.78	20.90	20.39	20.85	21.27	22.58	21.32	0.94	1.00
Case 24	13.98	22.77	23.80	19.62	27.38	20.05	13.55	22.53	23.73	14.57	23.45	21.57	17.10	25.41	20.81	0.67	0.82

RTL, relative telomere length (ratio of telomere concentration to concentration of each single copy gene)

5. Chromosomal Fragility test

To measure chromosomal fragility of subjects, I used the diagnostic test for Fanconi anemia, in which the affected cells generates more chromatid-hype chromosomal breaks after being treated with cytotoxic agents, as compared with the reference blood sample of sex-matched healthy individual. Two samples of both study subject and sex-matched healthy individual were obtained in heparinized tubes, respectively.

Peripheral blood culture of both samples were carried out under two conditions: without and with treatment of an alkylating agent, mytomycin-C (MCC). MCC was added at 24 hours of the culture, followed by incubation for 72 hours. To examine the hypothesis that higher RAI dose would be associated with more fragile chromosome to the cytotoxic agent, I compared the ratios of three parameters using observed counts of breakages compared with counts in sex-matched healthy volunteers; the ratios of cell number with aberrant chromosomes to total cell number (Ratio 1), the ratios of total counts of aberrant chromosome to total counts of chromosomes (Ratio 2), and the ratios of total counts of aberrant chromosome in cells with aberrant chromosomes (Ratio 3).

6. Statistics

Values are expressed as mean and standard deviation for continuous variables. Comparisons of continuous values among groups were made by ANOVA and ANCOVA. Correlations between two continuous variables were analyzed using Pearson's r or Linear regression models. Because β-globulin concentration showed left-skewed distribution, log-transformation was applied. Linear regression or ANOVA/ANCOVA analyses were

repeatedly performed by Python (version 2.7. Available at http://www.python.org) and R (R Foundation for Statistical Computing, Vienna, Austria. at http://www.R-project.org). P values less than 0.05 were considered significant.

Results

1. Secular trends of papillary thyroid cancer in Korea

During 4 decades, recurrent rates of thyroid cancer patients had been decreased, with more favorable changes in pathological characteristics, including smaller tumor size, decreasing proportion of LN involvement and extra-thyroidal extension. Additionally, RAI ablation rate also significantly increased, suggesting possible association with improvement in recurrence (Table 2).

2. Incidence of second primary malignancy and association with high dose RAI therapy

During median 7 years of follow-up in the patients, second primary malignancy (SPM) developed in 61 (2.5%), and there was no difference in history of RAI therapy between patients with and without SPM. However, cumulative RAI dose in patients with SPM was 540 ± 1032 mCi, being significantly higher 127 ± 324 mCi in patients without SPM (P=0.003). Most common SPM was breast cancer (n=17, 28%), followed by gastrointestinal cancer (n=14, 18%) and hematologic malignancy (n=11, 18%). Compared to cancer incidence in general Korean population (2005, Korea statistics), the CIR of SPM in thyroid cancer population was similar to in general population (Table 3). However, the CIR of SPM in patients with RAI treatment was higher than in patients without RAI treatment (309.1 vs. 243 per 100,000 person-year). Interestingly, among the patient with RAI treatment, the CIR of SPM began to increase with dose-response relationship at the RAI dose of 600mCi. Table 4 shows risk factors associated with increasing SPM in total subjects and subjects with RAI treatment. Compared to young age group under 40 years,

elderly patients showed increasing OR with statistical significances. Male sex also showed increasing risk for SPM. Despite the absence of significant risk effect of history of RAI treatment, extremely high dose over 1000mCi showed significant risk effect, in both total subjects and patients with RAI therapy.

Table 2. Clinico-pathological characteristics and secular changes in papillary thyroid cancer (1962-2009)

	Total	Pre-1990	1990-1999	Post-1999	P
Cases	3147	278	840	2029	
Median Follow-up duration (years)	5.1 (1-43)	19.0 (1-43)	11.1 (1-20)	3.7 (1-10)	
Age (years)	46.5 ± 12.5	38.9 ± 12.8	44.5 ± 12.8	48.4 ± 11.8	<0.001
Male/Female	496/2651 (15.8%/84.2%)	37/241 (13.3%/86.7%)	115/725 (13.7%/86.3%)	344/1685 (17.0%/83.0%)	<0.001
Tumor size (cm)	1.6 ± 1.2	2.7 ± 1.7	2.3 ± 1.4	1.3 ± 0.9	<0.001
LN involvement	1200 (39.9%)	106 (49.3%)	341 (43.0%)	753 (37.6%)	<0.001
Extra-thyroidal extension	1643 (55.2%)	84 (37.2%)	445 (56.8%)	1124 (56.3%)	<0.001
RAI ablation treatment rate	2358 (55.7%)	132 (39.3%)	547 (51.4%)	1679 (59.3%)	<0.001
Recurrence rate					
Overall	418 (13.3%)	100 (36.1%)	164 (19.6%)	154 (7.6%)	<0.001
5 years	206 (6.6%)	29 (10.6%)	59 (7.0%)	118 (5.8%)	0.004
10 years	180 (16.2%)	53 (19.4%)	127 (15.2%)		0.097

P values were calculated by ANOVA for continuous variables and linearity test for dichotomous variables.

RAI, radioactive iodine; LN, lymph node

Table 3. Crude cancer incidence rate in general population and total study subjects according to age groups.

	Total		Age 40-59 years		Age ≥60 years	
	N	CIR	N	CIR	N	CIR
Korean population	48,683,049	300.5	13,404,623	390.8	6,222,658	1286.5
Total DTC	2,468	279.2	1,274	361.7	425	325.7
Without RAI	1,072	243.0	525	360.8	158	231.2
With RAI	1,396	309.1	749	362.3	267	377.1
Cumulative RAI dose						
30-150mCi	981	236.7	556	297.5	196	368.3
151-600mCi	302	189.6	149	191.0	51	288.2
601-999mCi	44	546.4	20	934.6	9	0
1000-mCi	69	1,314.2	24	1960.8	11	943.4

CIR, crude cancer incidence rate per 100,000 person-year; RAI, radioactive iodine; DTC, differentiated thyroid carcinoma

Table 4. Risk factors for second primary malignancy in thyroid cancer patients with or without RAI treatment

	Total patients		Patients with RAI	
	n	OR (95% CI)	n	OR (95% CI)
Age (years)				
<40	15 (2.0%)	Ref.	9 (2.4%)	Ref.
40-59	38 (3.0%)	2.71 (1.46-5.04) †	22 (2.9%)	2.83 (1.24-6.47) *
≥60	8 (1.9%)	2.51 (1.01-6.22) *	6 (2.2%)	3.66 (1.20-11.20) *
Female	46 (2.2%)	Ref.	25 (2.2%)	Ref.
Male	15 (3.8%)	1.92 (1.05-3.48) *	12 (4.9%)	2.75 (1.33-5.68) †
Without RAI	24 (2.2%)	Ref.		
With RAI	37 (2.7%)	1.14 (0.67-1.92)		
Cumulative RAI dose				
0 mCi	24 (2.2%)	Ref.		
30-150 mCi	18 (1.8%)	0.87 (0.47-1.62)	18 (1.8%)	Ref.
151-600 mCi	6 (2.0%)	0.67 (0.27-1.66)	6 (2.0%)	0.70 (0.27-1.78)
601-999 mCi	2 (4.5%)	2.04 (0.48-8.70)	2 (4.5%)	2.44 (0.56-10.61)
1000- mCi	11 (15.9%)	5.54 (2.64-11.63) ‡	11 (15.9%)	5.85 (2.64-13.07) ‡

*P<0.05, †P<0.01, ‡P<0.001

OR, Odds Ratio; CI, confidence interval; RAI, radioactive iodine

3. The association between RAI dose and relative telomere length

Of 505 patients whose relative telomere length were measured, the mean age at enrollment was 52.8 ± 12.7 years, and female subjects were 442 (87.5%). RAI therapy was performed in 366(72.5%) patients, and cumulative doses ranged from 60mCi to 3550mCi (Table 5). Median duration from surgery to enrollment was 6 years (up to 47 years). Papillary thyroid carcinoma was 444 (88.1%) and follicular thyroid carcinoma was 60 (11.9%). The mean tumor size was 1.7 ± 1.3 cm and small tumors less than 1cm comprised 41.7%. LN involvement, multiplicity, and extra-thyroidal extension were observed in 41.2%, 38.9%, and 51.8%, respectively. Figure 2 shows that RTL was inversely associated with age at enrollment, corresponding to a previous population—based epidemiologic study(31).

Cumulative dose of RAI showed inverse correlation with RTL (Fig 3). In subgroup analyses by age groups, this negative correlation between RAI dose and RTL was significant in the groups of younger than 40 years and between 40 and 60 years; however, in the group over 60 years, the correlation showed only insignificant trend (Fig 4). There results were similar to both RTL results, using 36B4 single-copy gene and β-globin gene.

Table 5. Clinico-pathological characteristics of subjects in each study

	Relative Telomere Length	hTERT mRNA RT-PCR	Chromosomal Fragility
Cases	505	62	33
Age (years)			
At surgery	43.8 ± 12.7	41.0 ± 13.6	53.6 ± 12.1
At enrollment	52.8 ± 12.7	53.8 ±12.8	41.0 ± 12.8
Sex (Male/Female)	63/442 (12.5%/87.5%)	6/56 (9.7%/90.3%)	2/31 (6.1%/93.9%)
Pathology			
Tumor size (cm)	1.7 ± 1.3	2.1 ± 1.5	2.7 ± 2.2
LN involvement	189 (41.2%)	23 (45.1%)	12 (44.4%)
Multiplicity	171 (38.9%)	11 (25%)	7 (29.2%)
Extrathyroidal	250 (51.8%)	32 (60.4%)	19 (67.9%)
Cumulative RAI dose			
None	138 (27.5%)	20 (34.5%)	6 (20.0%)
30 – 100 mCi	220 (43.8%)	14 (24.1%)	5 (16.7%)
101 – 150 mCi	48 (9.6%)	6 (10.3%)	1 (3.3%)
151 – 500 mCi	70 (13.9%)	7 (12.1%)	5 (16.7%)
501 – 1000 mCi	14 (2.8%)	5 (6.9%)	5 (16.7%)
1001 – mCi	12 (2.4%)	7 (12.1%)	8 (26.7%)
Unknown	3	2	3

Values are represented by mean ± SD or count (percent).

RT-PCR, reverse transcriptase polymerase chain reaction; RAI, radioactive iodine

Figure 2. Correlation between relative telomere length and age of enrollment

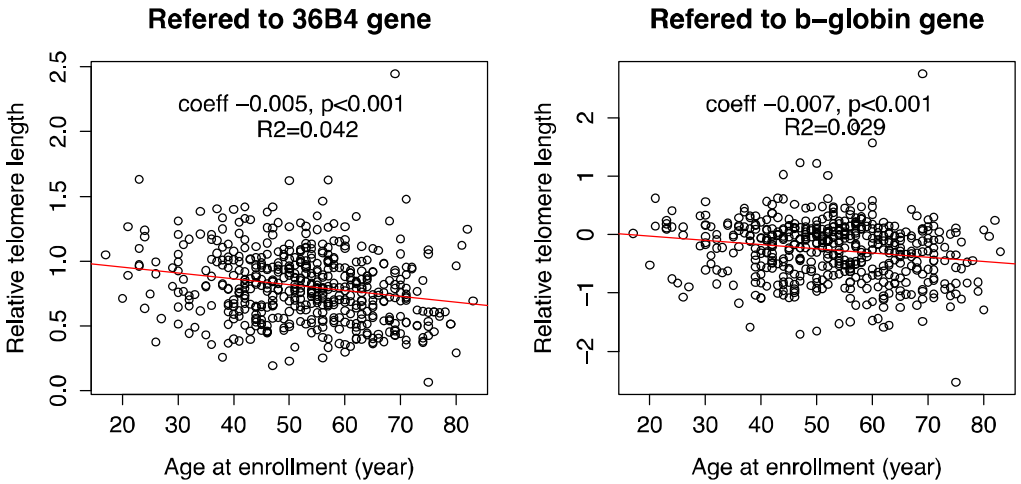


Figure 3. Correlations between relative telomere length and cumulative dose of RAI

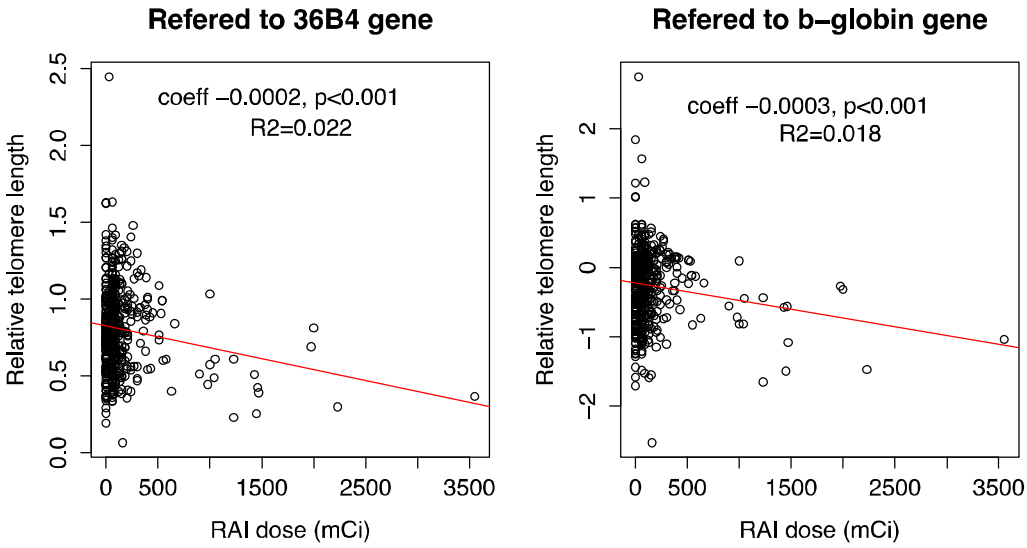
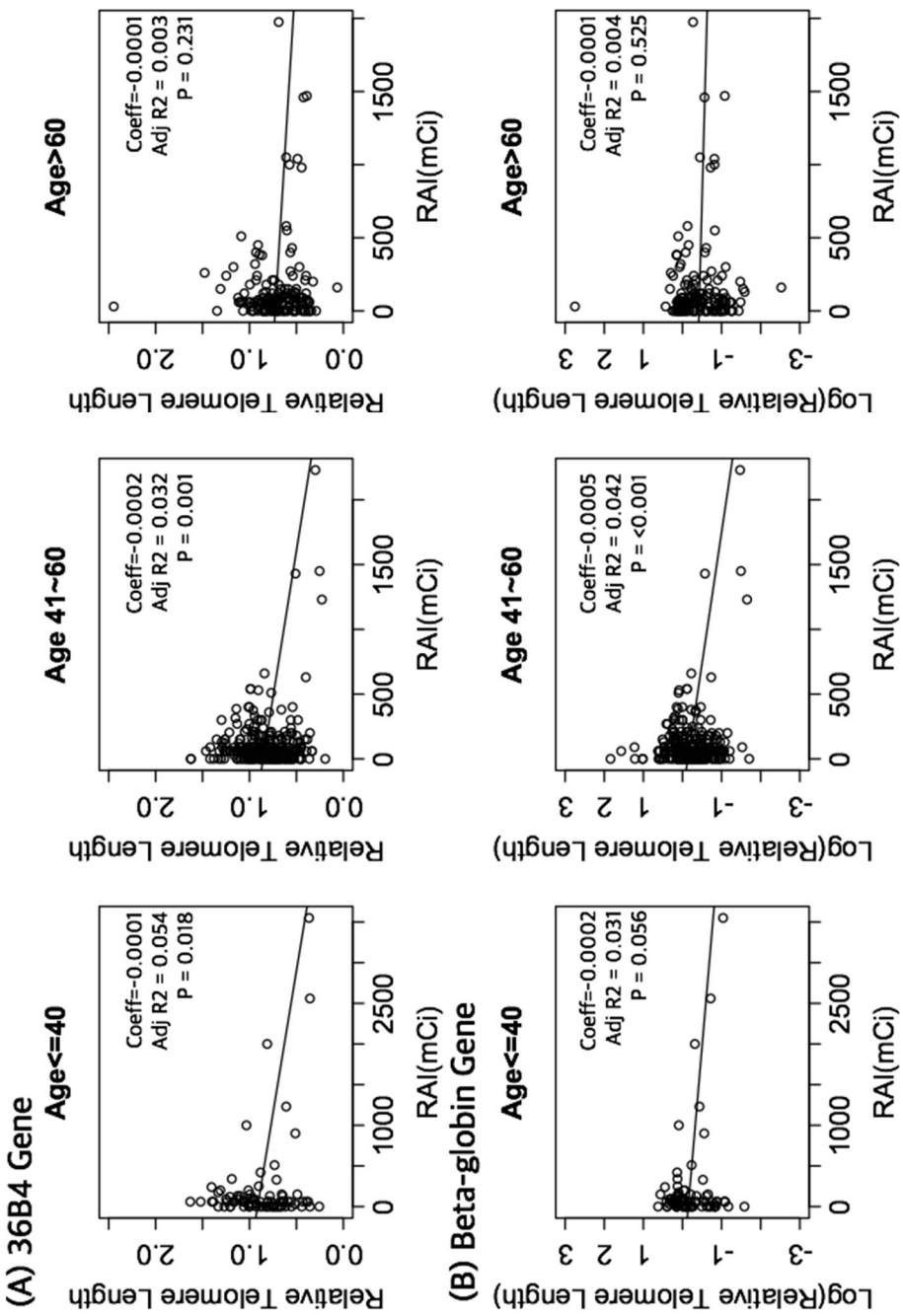


Figure 4. Association between RTL and RAI dose, according to subgroups by age at enrollment



Given that protection mechanism against radiation hazard would be working well in low dose of radiation, it was possible that radiation effect would be prominent over the certain threshold dose of radiation. In fact, figure 3 also shows that linear correlation between RAI dose and RTL was more reliable in patients with higher RAI dose. In figure 5, the regression model between RAI dose and RLT showed better fitness in patients with RAI dose higher than 300mCi, in both of 36B4 gene-based RLT and β-globin-based RLT. Subgroup analyses by enroll age also showed same results; RLT showed negative correlation with cumulative RAI dose, especially in patients with RAI dose higher > 300mCi. However, in subgroup for high dose RAI>300mCi, all age groups showed meaningful correlation between RAI dose and RTL (Fig 6).

Figure 5. Correlation between RTL and RAI dose in patients with higher doses than each cut-point

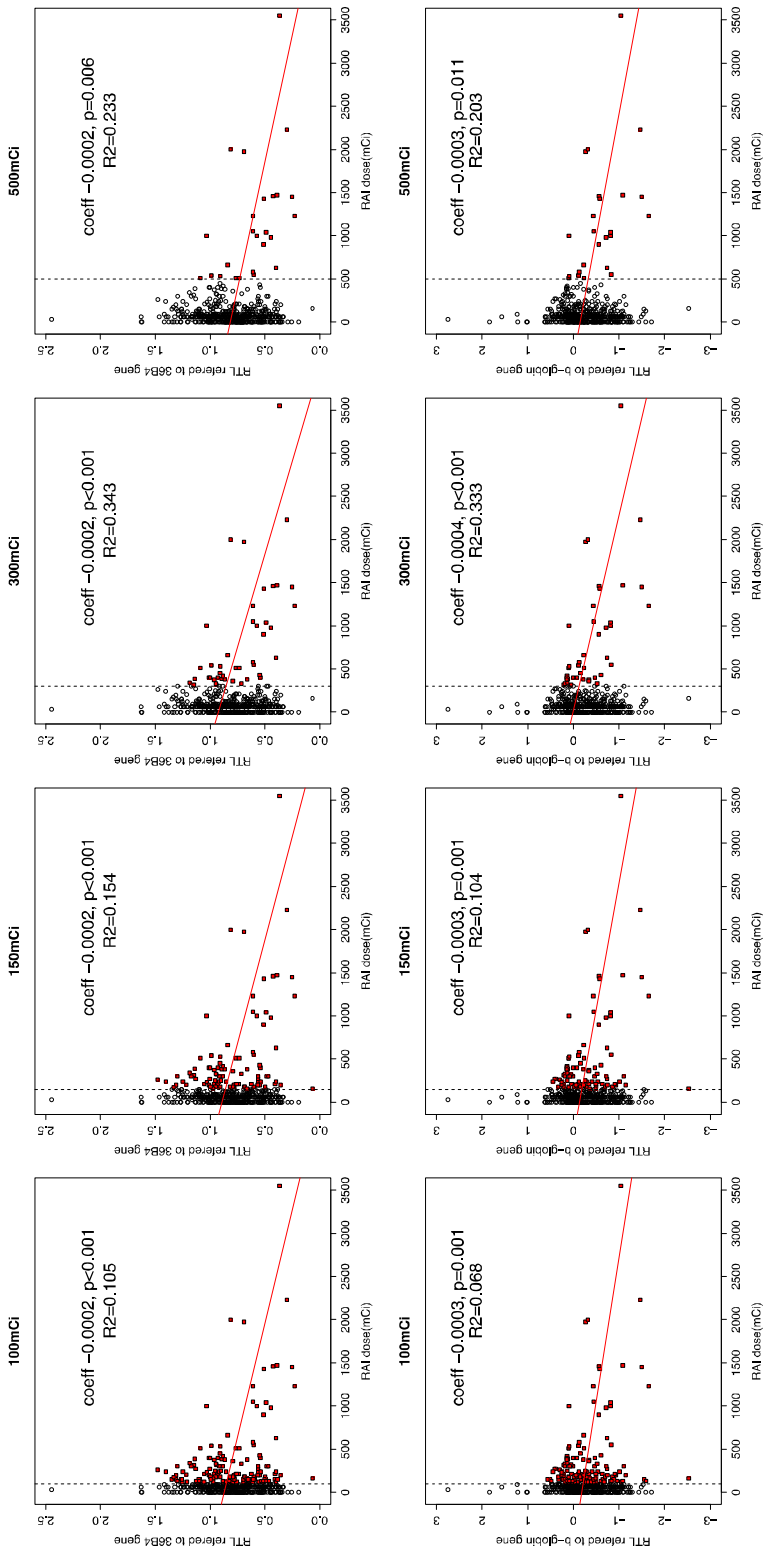
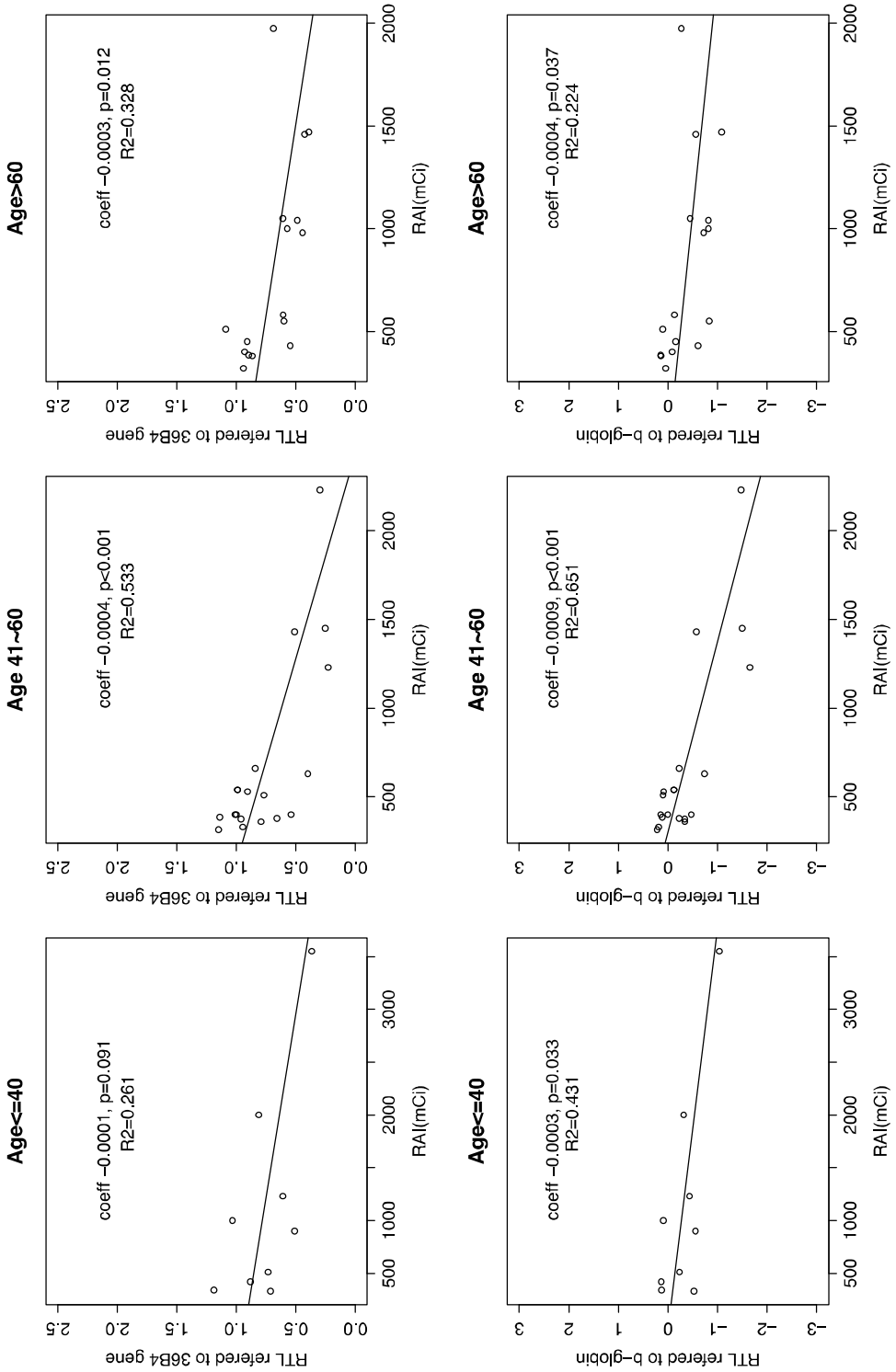


Figure 6. Association between RTL and RAI dose, according to subgroups by age at enrollment in patients with RAI doses > 300mCi



Given that insignificant results in elderly patients might be attributed to the longer duration from time at surgery (Fig 7), the multivariate analyses needed to include the duration from surgery to enrollment. Table 6 shows that the duration between surgery and enrollment did not show significant effect on RTL in univariate and multivariate analyses. Instead, cumulative RAI dose and patients' age at enrollment showed significant negative correlation with RTL, although male sex was not associated with RTL. In subgroup of patients with higher RAI dose over 300mCi, RAI dose showed persist significance, although age, duration between surgery and enrollment and male sex showed significant association with 36B4 gene based RTL, but not in β-globin gene based RTL (Table 7).

This cross-sectional study had fundamental limitation that previously absorbed radiation doses in each individual could not be evaluated. Instead, given that stimulated serum TSH level would represent absorbed radiation from radioactive iodine, simulated TSH levels at last RAI treatment were analyzed, indicating insignificant association with RTL in both low dose group and high dose group (Fig 8, 9).

Figure 7. Correlation between age at enrollment and duration between surgery and study enroll

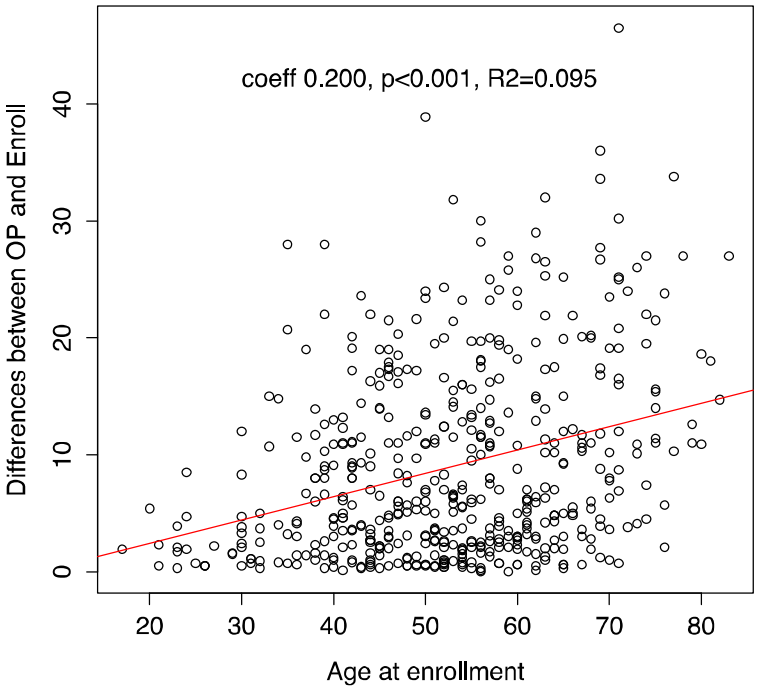


Table 6. Analyses of effects of risk factors on relative telomere length

Reference Gene	Factors	Univariate		Multivariate	
		Coefficients	P	Coefficients	P
36B4	RAI (mCi)	-0.0001	<0.001	-0.0002	<0.001
	Age at surgery (years)	-0.0045	<0.001	-0.0048	<0.001
	Duration (years)	-0.0011	0.462	0.0025	0.121
	Male	-0.0621	0.091	-0.0448	0.215
β-globin	RAI (mCi)	-0.0003	0.001	-0.0003	<0.001
	Age at surgery (years)	-0.0072	<0.001	-0.0077	<0.001
	Duration (years)	-0.0011	0.703	0.0048	0.120
	Male	-0.1255	0.074	-0.0948	0.174

* Correlation coefficients and P values were calculated by linear regression, with or without adjustment for age at both of surgery and enrollment and male sex.

RAI, radioactive iodine

Table 7. Analyses of effects of risk factors on relative telomere length in patients with cumulative RAI dose higher than 300mCi

Reference Gene	Factors	Univariate		Multivariate	
		Coefficients	P	Coefficients	P
36B4	RAI (mCi)	-0.0002	<0.001	-0.0003	<0.001
	Age at surgery (years)	-0.0037	0.180	-0.0075	0.002
	Duration (years)	-0.0003	0.944	0.0141	0.001
	Male	0.0515	0.637	0.2108	0.026
β-globin	RAI (mCi)	-0.0004	<0.001	-0.0005	<0.001
	Age at surgery (years)	-0.0045	0.378	-0.0081	0.0898
	Duration (years)	-0.0083	0.328	0.0123	0.146
	Male	0.0707	0.725	0.1822	0.345

* Correlation coefficients and P values were calculated by linear regression, with or without adjustment for age at both of surgery and enrollment and male sex.

RAI, radioactive iodine

Figure 8. Distribution of stimulated serum TSH level at last RAI treatment.

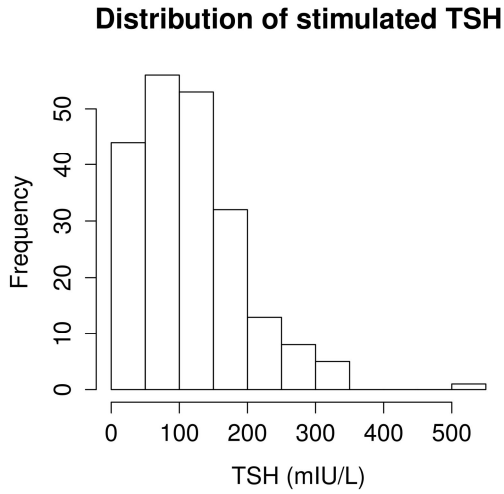
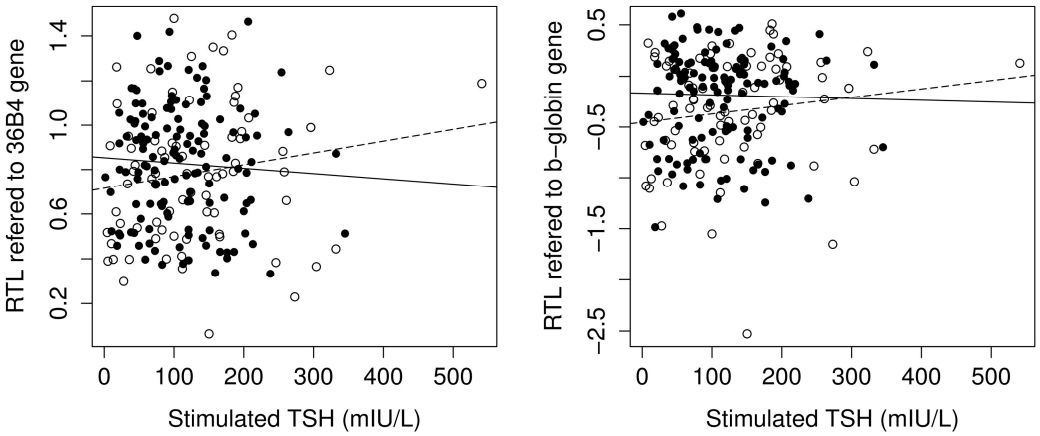


Figure 9. Correlation between stimulated serum TSH levels and RTL, according to subgroups of cumulative RAI dose. Open circle and solid line represent cases with RAI dose less than or equal to 100mCi and the linear regression line, and closed circle and dashed line represent cases with RAI dose over 100mCi.



4. The association between RAI dose and hTERT mRNA expression

Of 62 subjects used in analyses of hTERT mRNA PCR, mean age at enrollment was 53.8 ±12.8 and female consisted of 90.3% (Table 4). Quantitative densitometry of expression does not show significant associated with cumulative RAI doses (Fig 11). Figure 11 also showed insignificant correlations in subgroups of patients with RAI dose higher than 100mCi and 300mCi, respectively. With adjustment for male sex, age at enrollment, and duration from surgery to enrollment, significant correlations were not observed in all factors. In subgroup analyses by patients' ages, there were no consistent results with statistical significance (Fig 12).

Figure 10. hTERT gene mRNA expression in study subjects

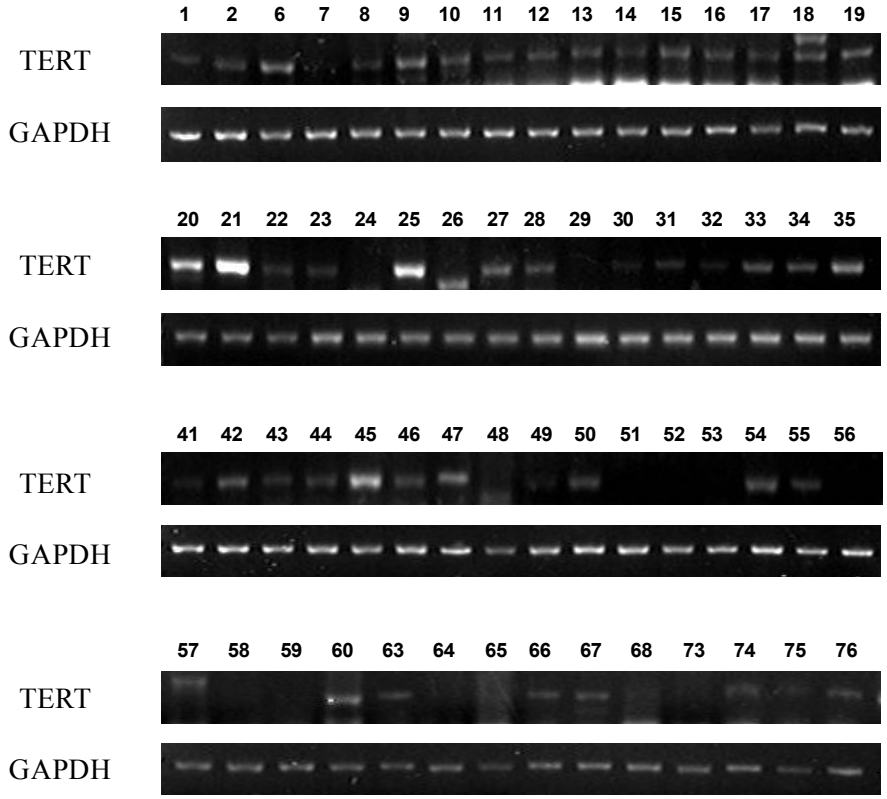


Figure 11. Correlation between cumulative doses of RAI and expression of hTERT mRNA in both all and subgroups with higher doses than 100mCi and 300mCi. Close circles mean the cases with RAI doses higher than 100mCi and 300mCi, respectively.

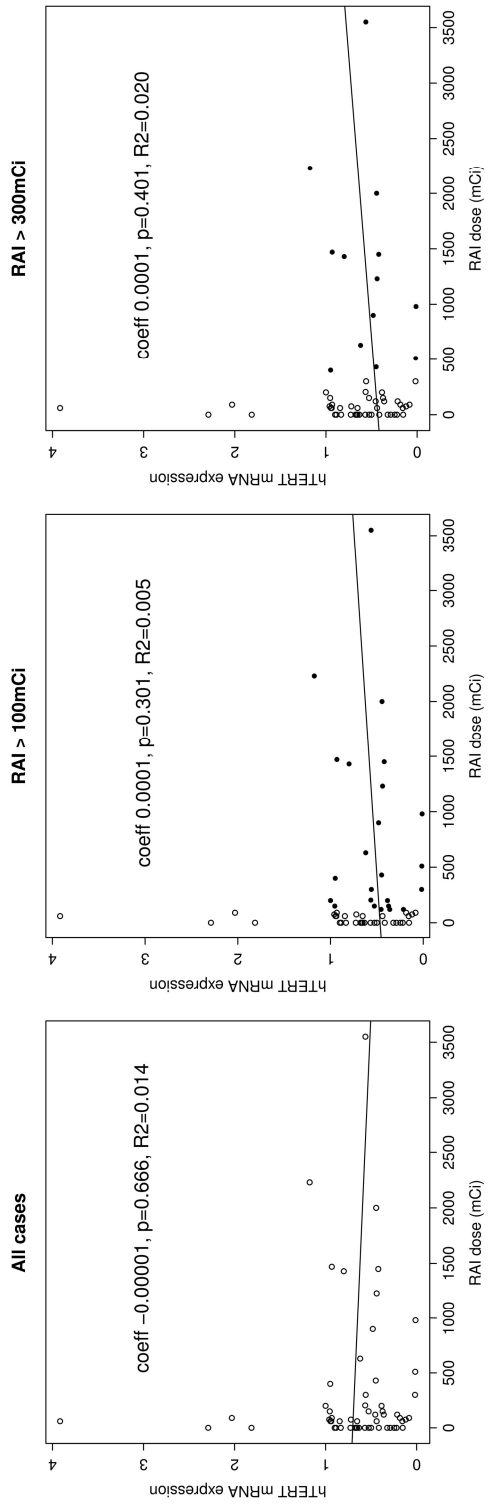
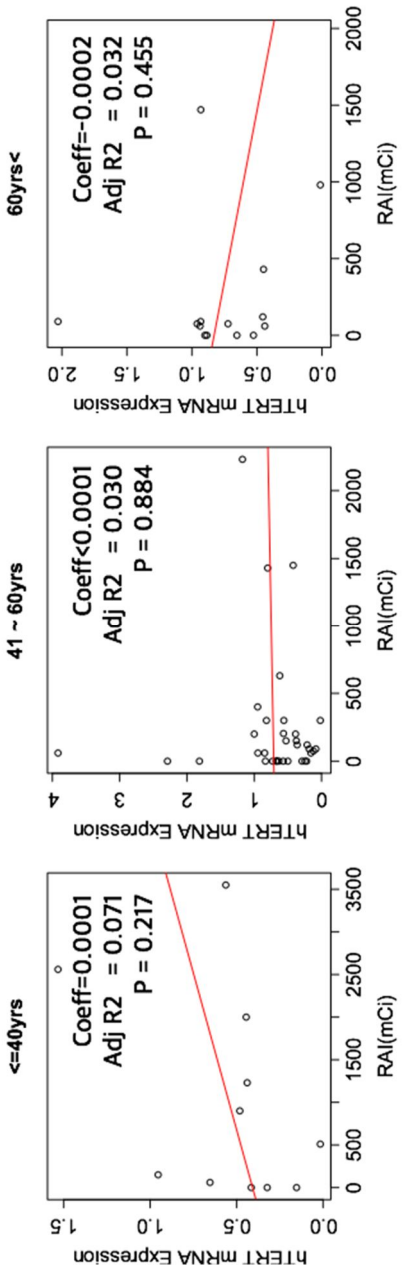


Figure 12. Correlation between cumulative doses of RAI and expression of hTERT mRNA, in each subgroup by age at enrollment



5. The association between RAI dose and chromosomal fragility

Figure 13 shows representative images of chromosomal breakages after treatment with mitomycin-C during cell culture in the course of chromosomal fragility test. In figure 14, of chromosomal fragility parameters, Ratio 3 showed significant positive correlation with RAI dose and Ratio 2 showed marginally increasing trend without significance, although Ratio 1 did not show meaningful association with RAI dose. However, subgroups analyses for age (≤40, 41—60, 60<) did not significant or consistent results, especially in younger age group under 40 years old (Fig 15).

Figure 13. Example of results obtained from chromosomal fragility test

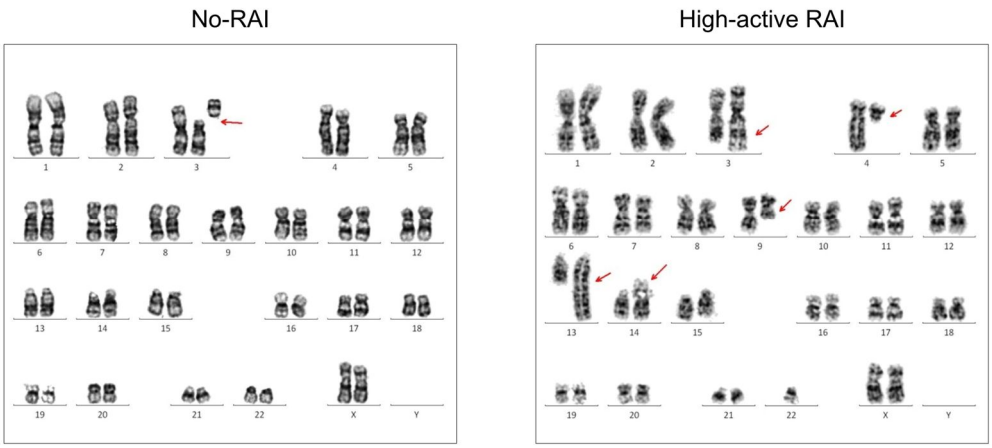


Figure 14. Correlation between cumulative doses of RAI and three parameters representing chromosomal fragility

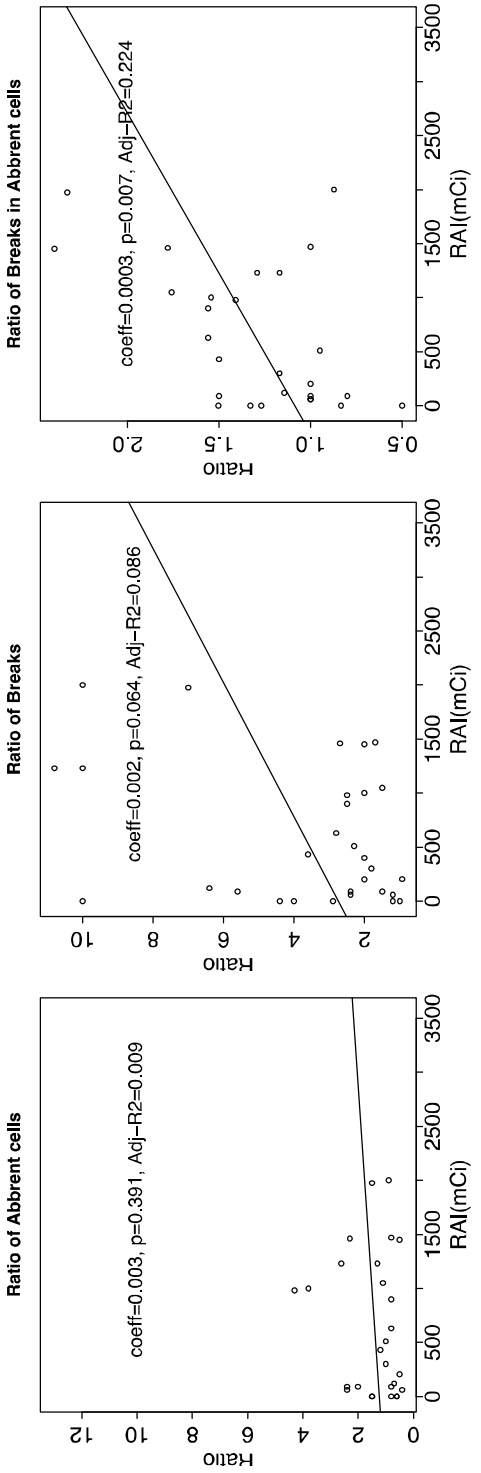
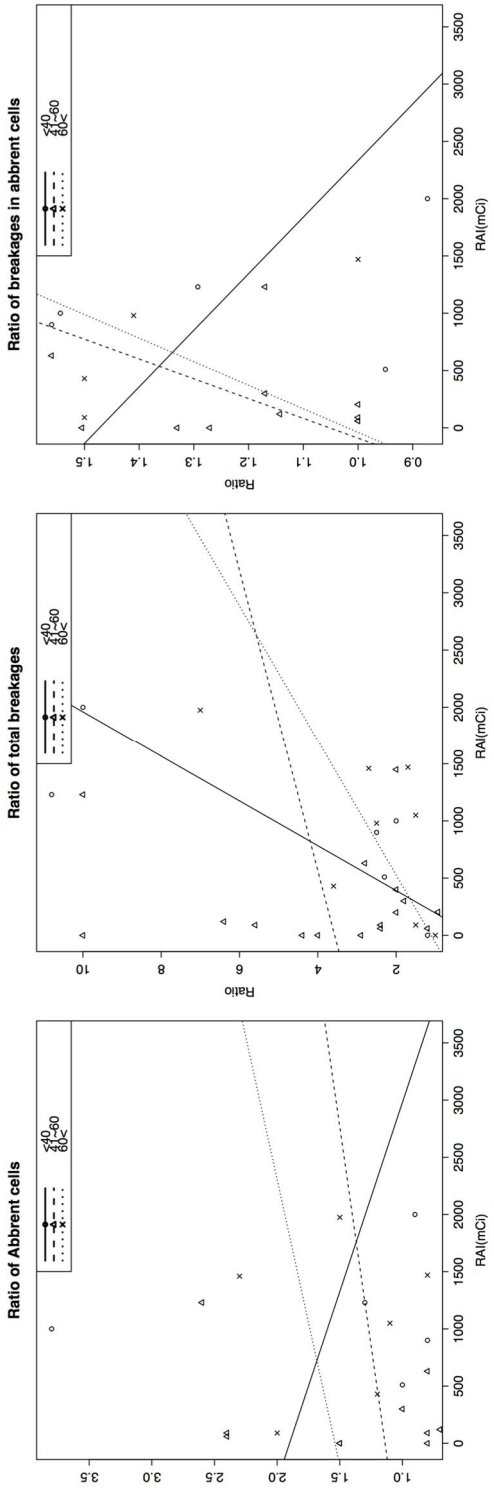


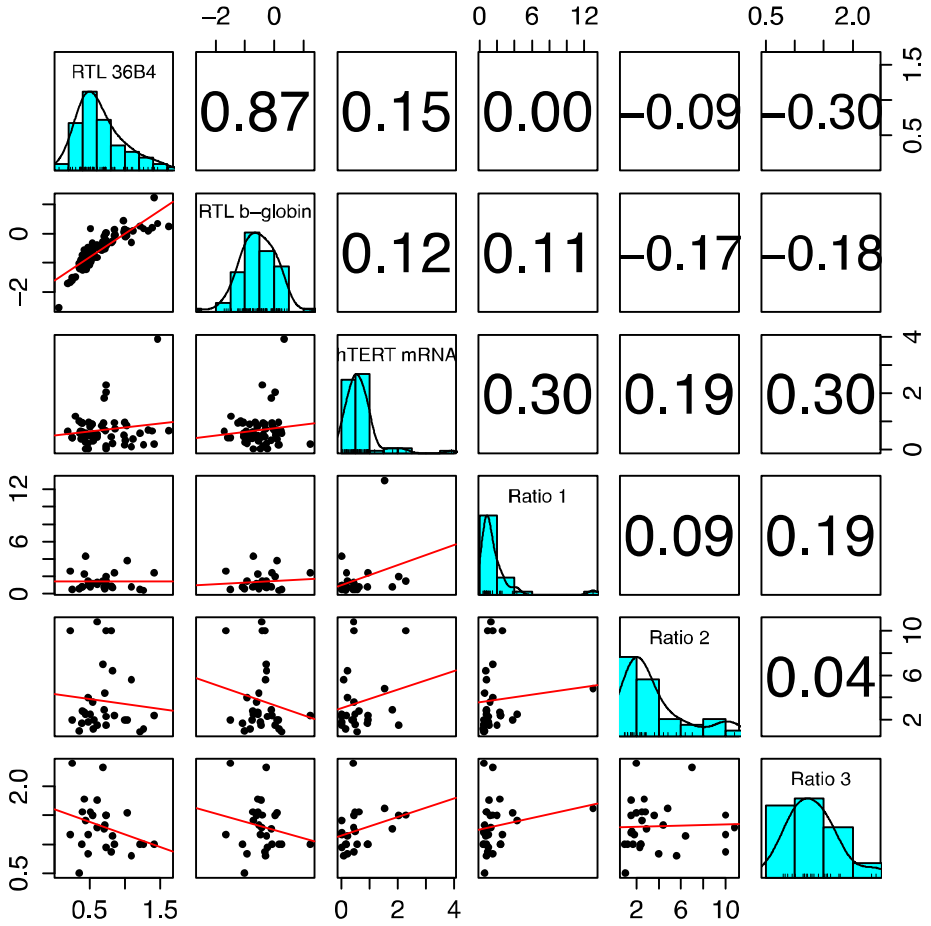
Figure 15. Correlation between RAI cumulative doses and three parameters representing chromosomal fragility, in each subgroup by age at enrollment



6. Correlations among three parameters representing telomeric and chromosomal damages

Telomere plays a role in protection of terminals of chromosome from being recognized as breakage point. Previous studies reported the association between chromosomal breakage and telomere shortening. Three parameters in this study – RTL, expression of hTERT mRNA, and chromosomal fragility – also showed corresponding correlation (Fig 16). Based on patterns of correlations among three results in this study, I inferred that persistent effects of high dose RAI treatment might result in telomeric shortening and chromosomal fragility and consequent activation of hTERT gene expression as a compensatory mechanism. However, any correlations among the parameters did not show statistical significances and I did not perform experimental studies for association among these factors, explanation about the mechanisms are limited.

Figure 16. Correlations among the parameters representing telomeric or chromosomal damages



7. Cut-off levels of RAI cumulative doses associated with telomere shortening or chromosomal fragility

After categorizing cumulative doses of RAI into six groups – none, under 100mCi, 100~149mCi, 150~299mCi, 300~499mCi, 500~999mCi and over 1000mCi, RTL and chromosomal fragility were compared among these subgroups. Among the subgroups by RAI doses, there were no significant differences of RTL and chromosomal fragility parameters in ANOVA and following post-hoc analyses; although, groups with higher RAI dose tended to be associated with shorter RTL and more frequent breakage counts (Fig 17, 18). Comparing those parameters between two groups at each cut-off level, I could observe the cut-off levels of 100mCi from which there were significant differences in RTL by 36B4 single gene and breakage count ratio in the cells with aberrant chromosomes (Ratio 3) (Fig 19, 20). These differences were also observed in multivariate analyses with adjustment for age at enrollment and male sex (Table 8, 9). Other parameters also showed correspond trends of cut-off levels of cumulative RAI dose; however, statistical significances were not observed.

Figure 17. Comparisons of mean RTL among the subgroups by cumulative RAI doses

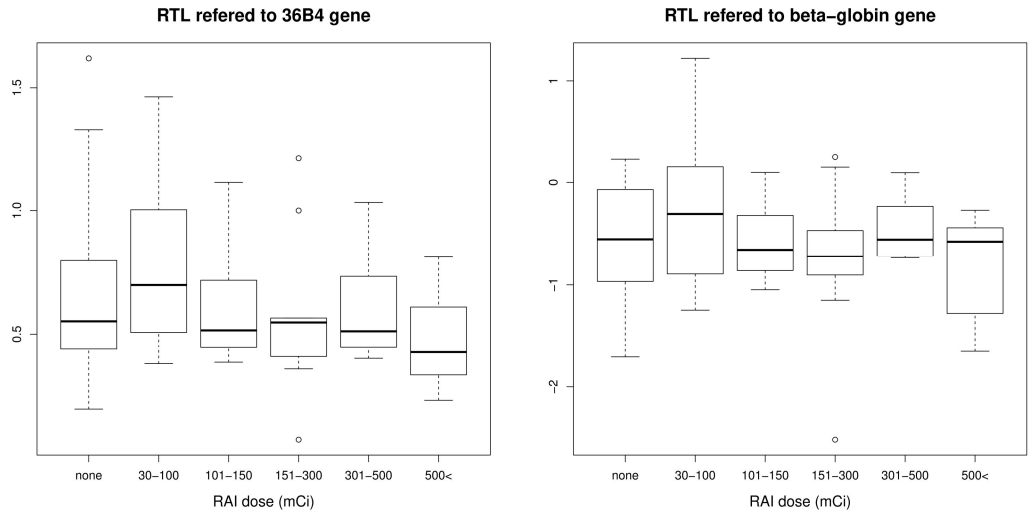


Figure 18. Comparisons of mean ratios of chromosomal breakages among the subgroups by cumulative RAI doses

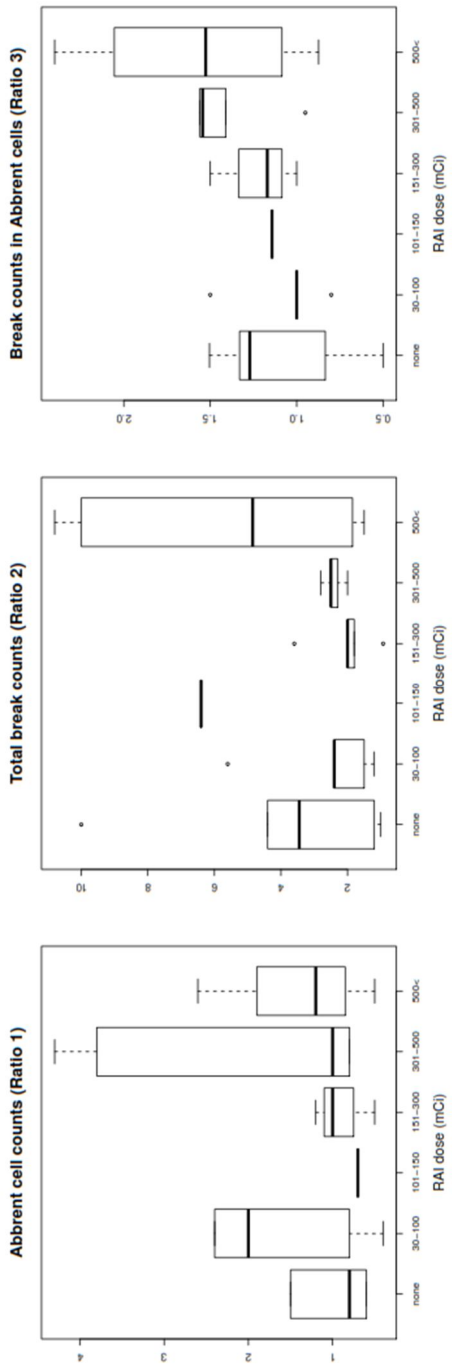


Figure 19. Comparisons of mean RTL at each cut-off levels of cumulative RAI dose

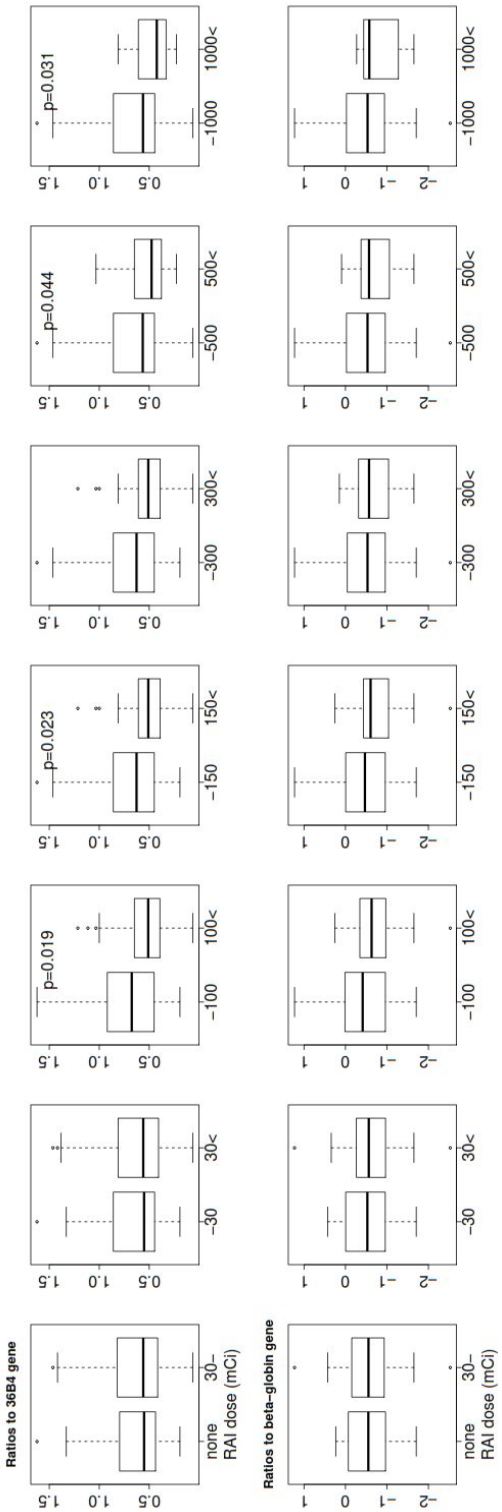


Figure 20. Comparisons of mean ratios indicating chromosome fragility at each cut-off levels of cumulative RAI dose

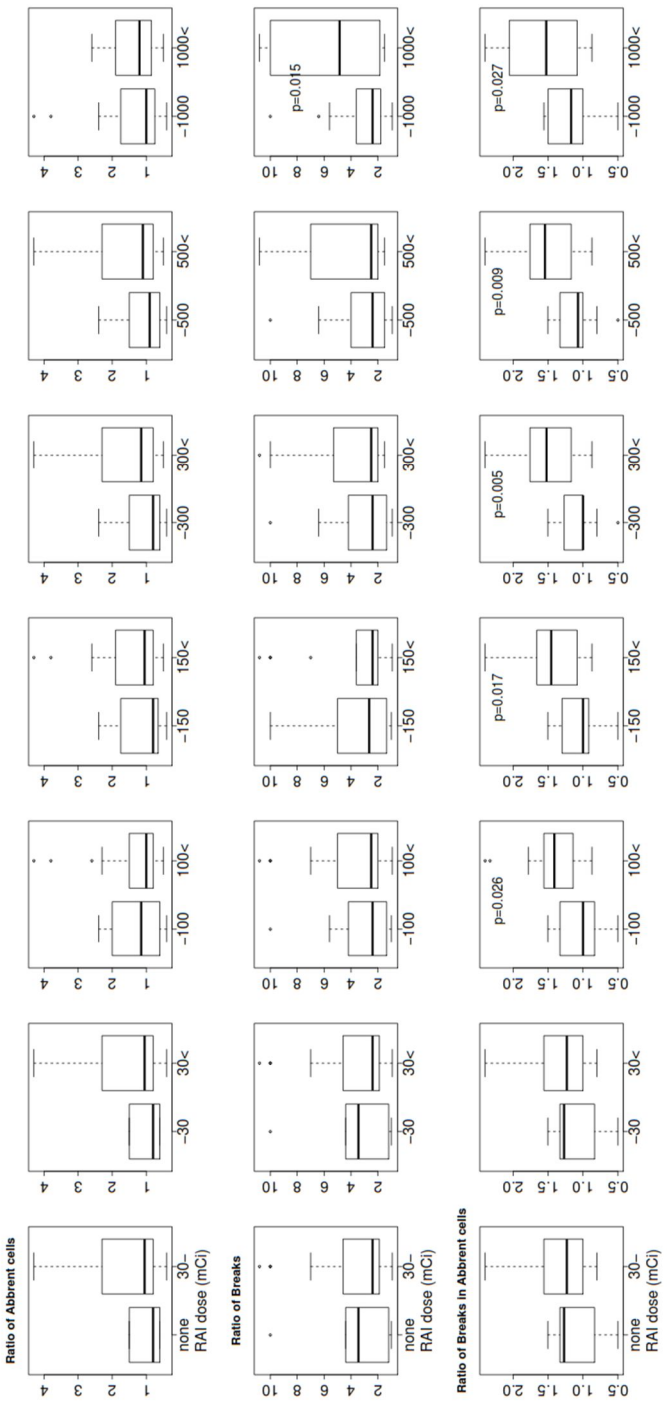


Table 8. Statistical significances of differences of mean RTL between patients with lower and higher RAI doses at each cut-off dose

	36B4 gene		β-globin gene	
Cut-off dose (mCi)	Univariate P	Multivariate P	Univariate P	Multivariate P
0	0.909	0.903	0.752	0.741
30	0.805	0.793	0.710	0.698
100	0.026	0.019	0.088	0.076
150	0.031	0.023	0.077	0.066
300	0.108	0.087	0.378	0.356
500	0.059	0.044	0.220	0.198
1000	0.041	0.031	0.077	0.065

* P values are calculated by ANOVA or ANCOVA, with adjustment for age at enrollment and male sex.

RTL, relative telomere length; RAI, radioactive iodine

Table 9. Statistical significances of differences of mean ratios of chromosomal fragility between patients with lower and higher RAI doses at each cut-off dose

Cut-off dose (mCi)	Cell counts		Total breakage count		Breaks in cells	
	Uni-P	Multi-P	Uni-P	Multi-P	Uni-P	Multi-P
0	0.315	0.328	0.850	0.849	0.230	0.210
30	0.315	0.328	0.850	0.849	0.230	0.210
100	0.665	0.675	0.608	0.606	0.034	0.026
150	0.484	0.496	0.860	0.860	0.025	0.017
300	0.230	0.242	0.347	0.342	0.008	0.005
500	0.199	0.210	0.241	0.238	0.015	0.009
1000	0.905	0.908	0.024	0.015	0.032	0.027

* P values are calculated by ANOVA or ANCOVA, with adjustment for age at enrollment and male sex.

Uni-P, p values by univariate analysis; Multi-P, p values by multivariate analysis; RAI, radioactive iodine

Discussion

Radioactive iodine therapy is established treatment to improve the prognostic outcomes by ablation of remnant thyroid tissue or occult residual tumors after total thyroidectomy. Well differentiated thyroid cancer, as well as normal follicular thyroid cell, also has Na-I symporter, which makes highly concentrated radioactive iodine within thyroid tissue. Beta-ray from concentrated radioactive iodine has only weak penetration ability to be limited around target follicular cells; however, there have been concerns about radiation hazard on other tissues with Na-I symporter, such as bone marrow, bladder, breast mammillary tissue, or salivary glands (32). Several epidemiologic studies reported increasing risk of second malignancies of these organs in thyroid cancer patients who were treated with RAI (8,15,33-35). Recently, in Korea, a national–wide study showed considerable increases of hematologic malignancy risk among the RAI treated thyroid cancer patients in the manner of dose-response relationship with total cumulative doses (7).

The carcinogenic effects from telomeric damages and chromosomal instabilities were suggested by several mechanisms, in the light of failure of mechanisms of DNA repairing and cell senescence of damaged cells (36). Telomere are located at the end of DNA strands with repetitive sequences, having potential possibility to be recognized as a DNA breakage unless their typical protective three dimensional structure of the T-loop and associated telomeric proteins. However, as telomeres become shorter in the course of repetition of cell divisions, their structures become too short to maintain their specific structure for protection of end point of telomere, followed by being recognized as breakage point and activation of DNA repairing mechanisms and cell senescence. Therefore, telomere shortening is closely associated with chromosomal instability (37). Some

carcinogenic mechanisms include down regulation in the pathway from telomere shortening and inducing apoptosis. One could infer that progressive telomere shortening by repetitive or persistent radiation hazard would induce DNA repairing mechanism, resulting in chromosomal instability and finally elevating risk of carcinogenesis. In fact, several studies reported the association between telomere damages and radiation and the persistent elevation of expression of RNA or protein for telomere synthesis (18,21). This study did not include patients with second malignancy after RAI treatment, although a recent meta-analysis reported the significant association between short telomere length and risk of second malignancy (38).

Prospective cohort studies of workers at Chernobyl nuclear accident showed results that, in blood sample in workers at that time, telomere length was significantly shorter than radiation unexposed residents or minimally exposed workers, with dose response of radiation exposure (21). Despite these epidemiologic results, experimental studies showed only time-limited effects of radiation on chromosomal damages within several days of weeks. This study aimed to demonstrate significant persistent changes of telomere or chromosomes even in the patients who received RAI therapy at least one year ago. In this study, high dose RAI treatment was associated with shorter telomere length and the relationship was significant in young age group less than 60 years. Elderly patients group over 60 years showed only insignificant trends, might be attributed to profound correlation between age and telomere length. The longer time interval between RAI treatment and sampling could attenuate the RAI effects; however, multivariate analyses did not show its significant effect. However, because of the clinical of a cross-sectional study, the result did not support any causal explanation, warranting experimental and prospective studies in the future.

hTERT is the encoding gene of the reverse transcriptase in telomerase, which is known to be activated in case of decreased telomere length. Previous studies reported that increased expression of hTERT gene was associated with short telomere length and malignancy risk (28,39,40). Consequently, I expected increased expression of hTERT RNA in patients with high cumulative RAI dose, only to observe inconsistent results. Given that telomerase was observed mainly in germ cells, not in somatic cells, the method using hTERT gene expression might have potential limitation (41). Furthermore, we used hTERT gene expression as a parameter of telomerase activity rather than telomerase enzymatic activity itself. In the future, to demonstrate the correlation between radiation hazard and telomeric damage, further studies using telomerase enzymatic activity itself.

I used chromosomal fragility test in assessing susceptibility to external stimulation on chromosomes, which used in diagnosis of Fanconi disease. Fanconi disease is characterized by susceptibility to chromosomal breakage and high prevalence of hematologic malignancy. Breakages on DNA strand activate repairing mechanisms, such as homologous recombination or nonhomologous end joining. Although most breakages are repaired not to affect genetic function, a part of processes could make translocation or dicentric chromosomes, leading to inter–chromosomal bridges or further breakages. Propagation of chromosomal breakages would induce more profound chromosomal instability. In general, damaged cells with aberrant chromosome are led to apoptosis or senescence through the pathways of p53 or Cdc25A; however, repetitive chromosomal damages and persistent instability could contribute carcinogenesis. In fact, several studies reported increased risk of second malignancies after radiation exposure (42). In this study, ratios of chromosomal breakage showed increasing trend with higher RAI dose, without

statistical significance. Considering long term periods from last radiation, further prospective study might demonstrate clinically meaningful results.

In the light of cut-off levels associated with RTL shortening and more fragile chromosome, despite not all parameters, I observed that cut-off point of 100mCi from which RTL by 36B4 gene was shorter and ratio of breakage counts in the cells with aberrant chromosomes increased. Given that cumulative dose of 100mCi is widely used doses in post-operative remnant thyroid ablation, I could not rule out the possibility of radiation hazard by even low dose RAI therapy. In fact, recent epidemiologic studies also suggested higher incidence of leukemia in patients with RAI therapy, from even 100mCi of cumulative doses (7). Furthermore, several previous epidemiologic studies also showed increasing incidence of second malignancy from even low dose of RAI (9,34). Of course, the results did not show significant cut-off levels in all parameters; however, trends in each parameters were consistent at least. Considering long survival duration resulted from excellent cure rate and insidious progression, possible long term effects from even low dose RAI should be evaluated by well-designed prospective studies.

In this study, I used human samples to examine the association between internal radiation from RAI and telomeric or chromosomal hazard, finally being potential carcinogenic stimuli. Mostly the results did not show significant effects of radiation dose on telomere length, hTERT gene expression and chromosomal instability, except inversed correlation between telomere length and RAI dose in younger patient group. However, despite statistical insignificance, these parameters showed corresponding changes according to RAI dose, suggesting the its potential effects and concern of further carcinogenic effect. To explicit the potential effects of RAI and guide safe usage in clinical practice, further epidemiologic or experiment studies are valuable.

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국 문 초 록

수 십년간 분화갑상선암의 치료에 사용해 온 방사성 요오드 치료는 갑상선암의 생존률을 개선하는데 기여해 왔다. 하지만 투여된 방사성 요오드에서 방출되는 방사선으로 인한 피폭과 이차암 발생의 위험성에 대한 우려가 있어 왔으며, 몇몇 역학 연구에서는 의미있는 결과를 보고한 바 있다. 이러한 연구결과등을 바탕으로 현재 분화갑상선암의 진료지침에서는 투여되는 누적 방사선량이 600mCi 를 초과하지 않도록 권고하고 있으나, 이차암 발생의 위험을 증가시킬 수 있는 염색체 손상과의 연관성에 대해서는 시행된 연구가 드문 실정이다. 이번 연구는 갑상선암 환자의 임상적 특징과 예후의 변화, 이차암 발생률 분석 및 위험인자를 분석하고, 방사성 요오드 치료 및 투여 누적선량과 텔로미어, 염색체 취약성 등간의 연관성을 분석하고자 하였다.

총 3147 명의 갑상선 유두암 환자의 의무기록자료 분석을 통해, 지난 40 년간 갑상선암의 병리학적 특징의 호전 및 방사성 요오드 치료율 증가와 함께 갑상선암의 예후가 유의하게 개선되어 왔음을 관찰하였다. 국내 인구집단에서의 암 발생률과 비교하였을 때 갑상선암 환자에서의 이차암 발생률에는 전체적인 증가양상이 보이지 않았으나, 1000mCi 이상의 고용량의 방사성 요오드 치료를 시행받았던 환자에서는 이차암 발생률이 유의하게 증가함을 관찰하였다. 상대적 텔로미어 길이를 측정한 505 명의 환자에서는 투여된 방사성 요오드의 양이 많을수록 텔로미어의 길이가 짧아지는 경향을 보였으며, 이러한 경향성은 60 세 미만의 연령군에서 유의하게 나타났다. 하지만 300mCi 이상의 고용량의 방사성

요오드 치료를 받았던 환자군에서는 연령에 상관없이 누적 요오드양과 텔로미어 길이 간에 유의한 상관관계를 나타내고 있었다. hTERT 유전자의 메신저 RNA 발현정도는 기존에 투여된 방사성 요오드의 양과 유의한 상관관계를 나타내지 않았다. 염색체의 취약성은 고용량의 방사성 요오드 치료를 투여 받았던 환자에서 높게 나타나는 양상을 보이고 있으나, 연령군으로 나누어 분석한 결과에서는 일관된 결과를 확인할 수 없었다. 방사성 요오드의 누적 투여량이 100mCi 를 초과한 지점부터 텔로미어 길이와 염색체 취약성과 관련된 일부 파라미터에서 유의한 차이를 나타내고 있었으나, 그밖의 파라미터에서는 통계적으로 유의한 결과를 확인할 수 없었다.

요약하면, 고용량의 방사성 요오드 치료를 받았던 환자에서 텔로미어의 길이가 짧은 양상을 보였으며, 이는 주로 60 세 미만의 연령군에서 유의하였으나, 300mCi 를 초과하는 고용량 치료군에서는 모든 연령대에서 유의한 결과를 보였다. hTERT 메신저 RNA 의 발현 정도와 방사성 요오드 투여량간의 유의한 연관성은 보이지 않았으며, 염색체 취약성은 방사성 요오드 누적용량이 높은 환자에서 다소 증가하는 것으로 보였으나 통계적으로 유의하지 않았다. 일부의 파라미터에서, 방사성 요오드 용량 100mCi 를 초과한 지점 부터 텔로미어 길이와 염색체 취약성의 유의한 차이가 나타나고 있어, 텔로미어 단축 및 염색체 취약성 증가와 관련한 방사성 누적용량 100mCi 의 가능성을 제시하였다.

주요어: 방사성 요오드, 누적용량, 갑상선암, 이차암, 텔로미어, hTERT 유전자, 염색체 취약성