Research Paper

THRB Genetic Polymorphisms Can Predict Severe Myelotoxicity after Definitive Chemoradiotherapy in Patients with Esophageal Squamous Cell Carcinoma

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Abstract

Objective: Chemotherapy-related toxicities are difficult to predict before treatment. In this study, we investigated whether *thyroid hormone receptor beta* (*THRB*) genetic polymorphisms can serve as a potential biomarker in patients with esophageal squamous cell carcinoma (ESCC).

Methods: Forty-nine Japanese patients with ESCC who received a definitive chemoradiotherapy (CRT) with 5-fluorouracil and cisplatin in conjunction with concurrent irradiation were retrospectively analyzed. Severe acute toxicities, including leukopenia, stomatitis, and cheilitis, were evaluated according to 6 single nucleotide polymorphisms (SNPs) in the gene; the intronic SNPs of rs7635707 G/T, rs6787255 A/C, rs9812034 G/T, and rs9310738 C/T and the SNPs in the 3'-untranslated region (3'-UTR) of rs844107 C/T and rs1349265 G/A.

Results: Distribution of the 4 intronic SNPs, but not the 2 SNPs in the 3'-UTR, showed a significant difference between patients with and without severe acute leukopenia. Stomatitis and cheilitis were not associated with any of the 6 analyzed SNPs. Frequency of haplotype of the 4 intronic SNPs reached approximately 97% with the 2 major haplotypes G-A-G-C (73.4%) and T-C-T-T (23.5%).

Conclusions: *THRB* intronic SNPs can provide useful information on CRT-related severe myelotoxicity in patients with ESCC. Future studies will be needed to confirm these findings.

Key words: esophageal squamous cell carcinoma, thyroid hormone receptor beta, chemoradiotherapy, severe acute toxicity, prognosis.

Introduction

Esophageal cancer is a common gastrointestinal malignancy that continues to remain virulent. In Japan, 17,000 new cases of esophageal cancer are diagnosed each year, with poor prognosis [1]. The incidence of esophageal squamous cell carcinoma (ESCC) is higher than that of esophageal adenocarcinoma in east Asian countries, including Japan, whereas the incidence of esophageal adenocarcinoma is high in north America, the United kingdom/Ireland, and Australia [2]. The standard therapeutic approach for ESCC has traditionally been radical surgery with node dissection; however, recent advances in chemoradiotherapy (CRT) have been widely accepted as a promising modality in both neoadjuvant and definitive settings for patients with ESCC [3, 4]. A definitive CRT with 5-fluorouracil (5-FU) and cisplatin (CDDP) has been proposed as an organ-preserving treatment [5, 6], and clinical evidence supporting its efficacy has been collected in T4 and/or M1 lymph node [5, 6], and later stage II/III [7]. However, the effectiveness of cytotoxic chemotherapy is often limited by serious toxicities. In this definitive 5-FU/CDDP-based CRT, myelosuppression, stomatitis, and cheilitis are some of the most common toxicities [5-7], and severe acute leukopenia constitutes a life-threatening risk in more than a few patients. Identification of patients who can achieve a better treatment course would allow oncologists to maximize therapeutic benefits and minimize toxicities.

Genetic constitution is an important cause of individual variations in the tolerance to chemotherapeutic regimens. Because of the narrow therapeutic index of chemotherapeutic agents, pharmacogenomics has drawn increasing attention in the field of tailored medicine, ranging from gene expression profiles in tumor tissues to genetic signatures, including single nucleotide polymorphisms (SNPs) [3, 8, 9]. The application of SNP genotyping to anticancer therapy is an attractive approach, and serious toxicities have been explained by SNPs in well-known drug-metabolizing enzyme- or drug-transporter-related genes [3, 8, 9]. However, severe toxicities may have complicated associations with a large variety of genetic mutations; therefore, identification of novel markers using a different concept will be needed.

As a candidate gene approach, we focused on the *thyroid hormone receptor beta* gene (gene symbol: *THRB*, gene ID: 7068). Thyroid hormones (THs) play indispensable roles in differentiation, development, and metabolism in various tissues [10], and exert their actions by binding to thyroid hormone receptors (TRs) [10, 11]. TRs belong to the nuclear hormone re-

ceptor superfamily of transcription factors, which generate multiple isoforms by alternative promoter usage and splicing [12]. TRs are widely expressed in different organs and tissues, and TR expression is tissue-dependent and developmentally regulated [13, 14]. THs are thought to be associated with myeloid cell proliferation, given their influence on bone marrow function [15]. Extensive investigations have provided new insights into hematopoietic regulation by THs and TRs [16-18]. TRs are encoded by 2 genes, *THRA* and *THRB,* which are located on chromosomes 17 and 3, respectively [19]. Recent studies have revealed their unique functions in radiosensitivity, hepatic proliferation, and renal function [20-24]. In cancer patients receiving radiation therapy with or without chemotherapy, leukopenia and oral mucositis are the commonly detected severe toxicities [25, 26]. Collectively, the information led us to postulate that *THRB* polymorphisms could have predictive value for CRT-related toxicities, and in this study, we retrospectively investigated whether *THRB* SNPs can predict severe acute toxicities after a definitive 5-FU/CDDP-based CRT in Japanese patients with ESCC.

Materials and Methods

Ethics statements

A series of studies has been performed to evaluate the effects of genetic polymorphisms on clinical response 1 month after or severe acute toxicities during treatment with a definitive 5-FU/CDDP-based CRT in Japanese patients with ESCC [27-30]. These studies were conducted with the authorization of the institutional review board (IRB) and followed the medical research council guidelines of Kobe University. All the patients analyzed agreed to the studies and preservation of genomic DNA for future investigations, and additional studies were again authorized by the IRB and followed the guidelines of Kobe University. Written informed consent was obtained from all participants prior to genotyping.

Patients

The subjects were recruited from the patients diagnosed with ESCC and treated with a definitive CRT at Kobe University Hospital during 2003–2006. Throughout this period, the treatment regimen was standardized at our institution as described below, and was employed for those who were medically unfit for an operation or refused an operation.

In the present study, 49 patients analyzed were on the basis of the following criteria: 1) clinical stage T1 to T4, N0 or N1, and M0 or M1 according to the

International Union Against Cancer tumor-node-metastasis (TNM) classification; 2) age less than 85 years; 3) an Eastern Cooperative Oncology Group performance status of 0 to 2; 4) adequate bone marrow, renal, and hepatic function; 5) no prior chemotherapy; 6) no severe medical complications; 7) no other active malignancies (except early cancer) and 8) availability of written informed consent, as described above. The tumors were histologically confirmed to be primary, and no patients with recurrence were included in this study.

Protocol

A course consisted of the continuous infusion of 5-FU at 400 mg/m²/day for days 1-5 and 8-12, the infusion of CDDP at 40 mg/m²/day on days 1 and 8, and the radiation at 2 Gy/day on days 1-5, 8-12, and 15-19 (a total dose of 60 Gy in 30 fractions). This schedule was repeated twice every 5 weeks (Fig. 1). When detecting grade 3/4 hematological toxicities, chemotherapy was withheld until the blood cell counts recovered beyond the critical level, and then a reduced dose was resumed. For fever higher than grade 2, chemotherapy was withheld pending improvement. For renal toxicity, the subsequent dose of CDDP was reduced depending on the degree of toxicity. Radiotherapy was also withheld during the time when the chemotherapy was withheld for severe hematological and/or non-hematological toxicities. In principle, the total radiation dose was not reduced.

Severe acute toxicities

Leukopenia, stomatitis, and cheilitis, which are the commonly detected severe acute toxicities, were evaluated using criteria defined by the Japan Clinical Oncology Group. These criteria are based on the National Cancer Institute Common Toxicity Criteria. Toxicity was assessed on a 2- to 3-day basis during the CRT and a subsequent hospitalization period and on every visit after the completion of CRT. Episodes of leukopenia, stomatitis, and cheilitis during the first 2 courses and subsequent 2 weeks (until day 70) were recorded as acute toxicities and those of grade 3 or higher as severe acute toxicities.

Genomic DNA extraction and *THRB* genotyping

Genomic DNA was isolated from whole blood using a Taq-Man® Sample-to-SNP™ kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's directions, and stored at -80°C until analysis. Genetic polymorphisms of *THRB* were determined in a TaqMan® MGB probe-based polymerase chain reaction (PCR) using the StepOne™ real-time PCR system (Applied Biosystems) and TaqMan® SNP genotyping assays (Applied Biosystems). The PCR was carried out according to the manufacturer's protocol. For each set of reactions, the negative controls containing H_2O instead of DNA were added to detect contamination.
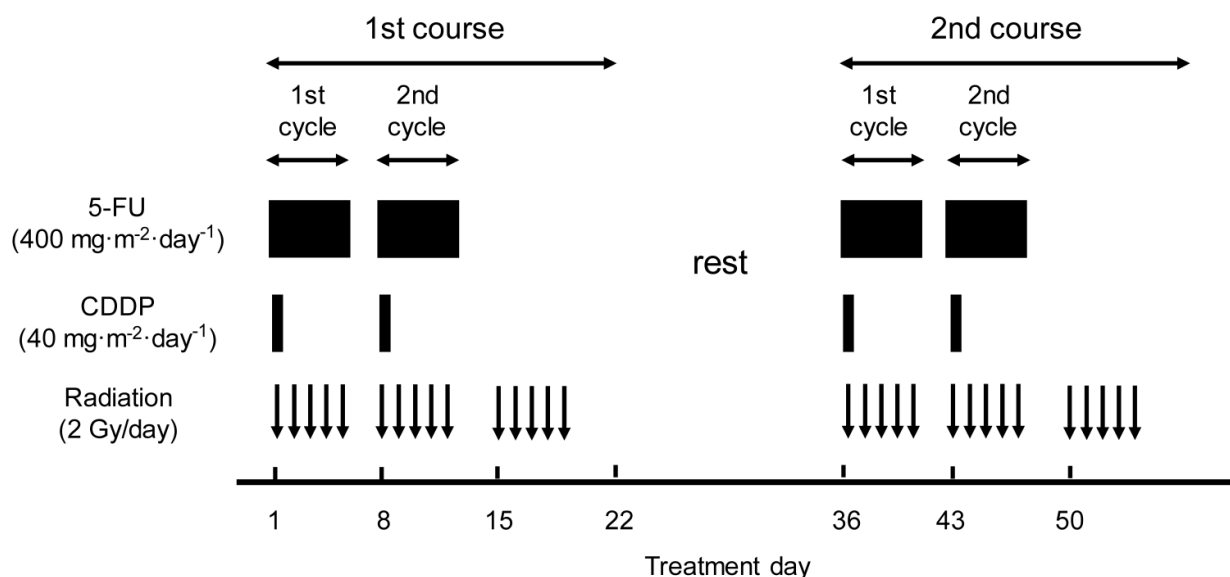

Figure 1. Treatment schedule of a definitive 5-FU/CDDP-based chemoradiotherapy. 5-FU, 5-fluorouracil; CDDP, cisplatin.

SNP selection

The human *THRB* gene expresses 2 isoforms, TRβ1 and TRβ2, in developmental and tissue-specific patterns by alternative splicing [12]. TRβ1 and β2 differ only in their N termini. TRβ2 exon 1 is located in intron 4 of TRβ1 with the other exons being identical. The 2 isoforms share a common DNA-binding domain and ligand-binding domain. TRβ2 is restricted to the central nervous system, whereas TRβ1 is widely expressed. Because only a little is known about the mechanisms underlying their expression, we constructed a genetic concept based on TRβ1. To date, 4 TRβ mRNA variants have been identified, and all encode a common protein and have a common non-coding exon 2 (Fig. 2), which led to the hypothesis that SNPs in introns adjacent to exon 2 may influence TRβ mRNA and protein expression. Therefore, candidate SNPs were selected according to the following criteria: 1) Minor allelic frequencies of the SNP were >40% (the frequency of chemotherapy-induced myelotoxicity) in the National Center for Biotechnology Information (NCBI) variation database (dbSNP) [31], and 2) the SNP was located in the adjacent intron, within 10 kb from the 5'- or 3'-end of the common exon 2. The allele frequency was related to genetic variations of the SNPs studied and the reference sequences were adopted on the basis of the NCBI dbSNP database. Four SNPs, reference SNP (rs) 7635707, rs6787255, rs9812034, and rs9310738, met the selection criteria described above. The SNPs rs7635707 and rs6787255 are located in intron 1, and rs9812034 and rs9310738 in intron 2. Considering a possible role in TRβ mRNA stability, additional candidate SNPs in the 3'-untranslated region (3'-UTR), rs844107 and rs1349265, were also selected. The positional relationships of the 6 SNPs analyzed are shown in Figure 2.

Haplotype analysis

Haplotype frequencies of the SNPs of rs7635707 G/T, rs6787255 A/C, rs9812034 G/T and rs9310738 C/T were estimated. The haplotypes were statistically inferred with a fair degree of precision using an algorithm based on Bayesian inference, PHASE version 2.1. Haplotypes were inferred by running PHASE 10 times, and the relative standard deviation (SD) of their frequencies was 0.1% or less of the mean value [32, 33]. The estimated haplotype frequencies are expressed as the average population haplotype frequencies for the whole sample, as described previously [34, 35].

Data analysis and statistics

All values represent mean ± standard deviation (SD). The associations between clinical characteristics, including each genotype in *THRB*, and severe acute toxicities were analyzed with Chi-square test or Fisher's exact test. *P*-values <0.05 (2-tailed) were considered significant.
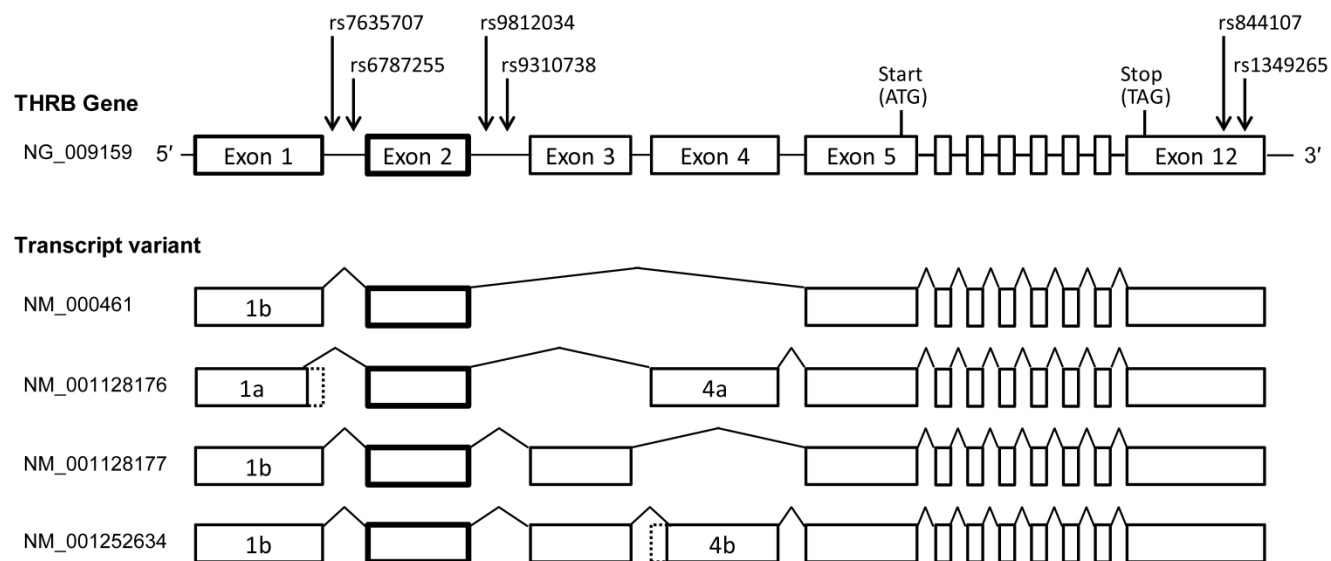

Figure 2. Genomic organization and transcript variants of human *THRB* genes. The 4 intronic SNPs and 2 SNPs in the 3'-UTR tested are indicated by arrows.

Results

Patient characteristics

Patient characteristics are presented in Table 1. The mean age was 64.5 ± 7.4 years (median age, 65 years; range, 48–78 years). Twenty-four, 20, and 4 patients showed ECOG PS of 0, 1, and 2, respectively. The PS of 1 patient was not precisely known; however, the PS of this patient was considered to be 0 or 1, based on the daily activity information from medical records. None of the subjects exhibited clinical manifestations of hypo- or hyperthyroidism that would necessitate medication, although precise laboratory data, including free triiodothyronine (T3), free thyroxine (T4), and TH-stimulating hormone (TSH), were not routinely examined.

Table 1. Demographic and clinicopathologic characteristics of 49 patients with esophageal squamous cell carcinoma.

Age (y)	64.5 ± 7.4 (48-78)[a]
Height (cm)	163.5 ± 6.6 (150-180) [a]
Weight (kg)	56.1 ± 9.6 (33-79) [a]
Sex	Male/Female = 46/3
Performance status	0/1/2/unknown = 24/20/4/1
Tumor differentiation	Well/moderate/poor/unknown = 7/28/8/6
TNM [b]	T1/T2/T3/T4 = 16/6/15/12
	N0/N1 = 22/27
	M0/M1 = 41/8
Stage	I/II/III/IV = 12/10/19/8

[a] Values represent mean (±SD), with the range in parentheses.
[b] TNM, tumor node metastasis.

Genotypes, allele frequencies, and haplotypes

Genotype and allele frequencies are shown in Table 2. The genotype frequencies of wild-type homozygotes, heterozygotes, and homozygote variants of the SNPs rs7635707, rs6787255, rs9812034, and rs9310738 were 29/49, 16/49, and 4/49, respectively. Frequencies of the CC, CT, and TT genotypes and C allele of the SNP rs844107 were 21/49, 19/49, 9/49, and 61/98, respectively. Frequencies of the GG, GA, and AA genotypes and G allele of the SNP rs1349265 were 27/49, 18/49, 4/49, and 72/98, respectively. Given that the genotype frequencies of the 4 intronic SNPs were similar, linkage disequilibrium of these SNPs was additionally examined. Table 3 shows the estimated frequencies of *THRB* haplotypes; 5 haplotypes were statistically inferred with a frequency >0.1% at the population base. With the major 2 haplotypes, the frequency reached approximately 97%: G-A-G-C (73.4%) and T-C-T-T (23.5%). T-A-G-C (1.0%), G-C-G-C (1.0%), and G-A-T-T (1.0%) were rare, and other haplotypes were not statistically inferred with a frequency >0.1% in the present population.

Severe acute toxicities

Episodes of severe acute leukopenia, stomatitis, and cheilitis occurred in 42.9% (21/49), 14.3% (7/49), and 16.3% (8/49) of cases, respectively. The genotype and allele frequencies of *THRB* with or without severe acute toxicities are presented in Table 4. The 4 intronic SNPs, but not the 2 SNPs in the 3'-UTR, exhibited a significant predictive value for severe acute leukopenia. Subjects with the GG genotype of rs7635707 SNP, the AA genotype of rs6787255, the GG genotype of rs9812034, and the CC genotype of rs9310738 were at a lower risk of developing severe acute leukopenia. None of the SNPs analyzed has a relationship with severe acute stomatitis and cheilitis. Analysis of the relationship between leukopenia and haplotypes revealed a lower rate of leukopenia in subjects with the G-A-G-C haplotype.

Table 2. Genotype and allele frequencies of *THRB* in 49 patients with esophageal squamous cell carcinoma.

dbSNP (NCBI)	Location	SNP allele 1/2	allele 1	allele 2	genotype 1/1	genotype 1/2	genotype 2/2
rs7635707	intron 1	G/T	74	24	29	16	4
rs6787255		A/C	74	24	29	16	4
rs9812034	intron 2	G/T	74	24	29	16	4
rs9310738		C/T	74	24	29	16	4
rs844107	3'-UTR	C/T	61	37	21	19	9
rs1349265		G/A	72	26	27	18	4

THRB, thyroid hormone receptor beta; SNP, single nucleotide polymorphism; dbSNP, the National Center for Biotechnology Information (NCBI) variation database; UTR, untranslated region.

Table 3. Estimated frequencies of *THRB* haplotypes in 49 patients with esophageal squamous cell carcinoma.

	Intronic SNPs across exon 2			Estimated Frequency
rs7635707	rs6787255	rs9812034	rs9310738	N = 49
G	A	G	C	0.7343
T	C	T	T	0.2346
T	A	G	C	0.0102
G	C	G	C	0.0102
G	A	T	T	0.0099

THRB, thyroid hormone receptor beta; SNP, single nucleotide polymorphism.

Table 4. Effects of *THRB* genetic polymorphisms on severe acute toxicities in 49 patients with esophageal squamous cell carcinoma.

dbSNP (NCBI)	Location		Leukopenia			Stomatitis			Cheilitis		
			yes	no		yes	no		yes	no	
			21	28	*P*-value	7	42	*P*-value	8	41	*P*-value
rs7635707	intron 1	GG	7	22	0.004	5	24	NS	4	25	NS
		GT	11	5		2	14		3	13	
		TT	3	1		0	4		1	3	
		G	25	49	0.002	12	62	NS	11	63	NS
		T	17	7		2	22		5	19	
rs6787255		AA	7	22	0.004	5	24	NS	3	26	NS
		AC	11	5		2	14		4	12	
		CC	3	1		0	4		1	3	
		A	25	49	0.002	12	62	NS	10	64	NS
		C	17	7		2	22		6	18	
rs9812034	intron 2	GG	7	22	0.004	4	25	NS	3	26	NS
		GT	11	5		3	13		4	12	
		TT	3	1		0	4		1	3	
		G	25	49	0.002	11	63	NS	10	64	NS
		T	17	7		3	21		6	18	
rs9310738		CC	7	22	0.004	4	25	NS	3	26	NS
		CT	11	5		3	13		4	12	
		TT	3	1		0	4		1	3	
		C	25	49	0.002	11	63	NS	10	64	NS
		T	17	7		3	21		6	18	
rs844107	3'-UTR	CC	10	11	NS	2	19	NS	3	18	NS
		CT	6	13		4	15		4	15	
		TT	5	4		1	8		1	8	
		C	26	35	NS	8	53	NS	10	51	NS
		T	16	21		6	31		6	31	
rs1349265		AA	1	3	NS	1	3	NS	0	4	NS
		AG	9	9		7	11		2	16	
		GG	16	11		13	14		5	22	
		A	11	15	NS	9	17	NS	2	24	NS
		G	41	31		33	39		12	60	

Episodes of leukopenia, stomatitis, and cheilitis during the first 2 courses and the subsequent 2 weeks (until day 70) were recorded as acute toxicities and those of grade 3 or higher, as severe acute toxicities. Statistical significance was assessed with Fisher's exact test. dbSNP, the National Center for Biotechnology Information (NCBI) variation database; NS, not significant.

Discussion

Administration of chemotherapy without severe toxicities is of great importance to medical oncologists. In the treatment of ESCC, definitive 5-FU/CDDP-based CRTs are promising modality with organ preservation; however, oncologists have noted that some proportion of patients will be exposed to a high risk of severe toxicities without long-term survival. In this regimen, the most common and alarming one is leukopenia, and 8 to 40% of patients show grade 3/4 leukopenia [5-7]. In the current study, we demonstrated that ESCC patients with the GG genotype of the *THRB* rs7635707 SNP, the AA genotype of rs6787255, the GG genotype of rs9812034, and the CC genotype of rs9310738 have a lower probability of severe acute leukopenia than those with the other genotypes.

Germline mutations in *THRB* have been identified in association with resistance to thyroid hormone, which is quite a rare disease [36]. Such patients harbor missense mutations or defects that correspond to amino acid alterations in the ligand-binding domain [36]. Somatic mutations and aberrant expression have been detected in cancers of different organs, including breast, thyroid, kidney, lung, liver, and colon, suggesting a suppressive role in carcinogenesis and metastasis [37, 38]. However, whether non-coding SNPs in *THRB* would is still unclear. The present study is the first to demonstrate that the intronic SNPs in *THRB* could have a clinical impact.

The 4 intronic SNPs analyzed are closely linked together. Given the burden of cost and time, analysis of only one of the 4 intronic SNPs can be a strong predictor of severe acute leukopenia. Analyses of the haplotype frequencies of these SNPs revealed 2 main haplotypes, indicating linkage disequilibrium of the 4 SNPs. However, the analysis did not have enough statistical power to detect the significant association between haplotype or diplotype distribution and severe acute leukopenia. These SNPs were located across exon 2, which is common among the THRB transcript variants. Polymorphism of gene regulatory regions is likely to be one of the major contributors to phenotypic variation between and within human populations [39]. Therefore, this region is considered to be important for the regulation of TRβ mRNA and protein expression, although the data are limited [12, 40]. An intron control region regulates TRβ2 expression in the central nervous system [12], and 3'- and 5'-UTR variants regulate TRβ1 expression in renal cell cancer [41], but this region is not mentioned in these reports. The precise mechanism should be clarified in future studies.

To date, translational control of TRβ is believed to regulate cell proliferation, metabolism, and cellular stress responses [14, 42, 43]. These functions may have direct or indirect influence on the severity of chemotherapy-related toxicities. Although the 4 intronic SNPs analyzed showed a strong association with severe acute myelotoxicity, the effects of these SNPs on the expression, regulation, and biological functions of TRβ are unknown. A large number of *THRB* SNPs have been identified; however, information on TRβ mRNA processing, including transcriptional initiation, and expression and function in each tissue and organ according to SNPs is limited [12, 44], and the molecular mechanisms underlying the effects of these SNPs remain unclear. Based on the limited available data, the mechanism may be explained as follows. First, the proliferation of hematopoietic stem cells may be directly dependent on these SNPs, given the change in TRβ expression in these cells. Second, TRβ may have a variable influence on the phosphatidylinositol 3-kinase (PI3K)-Src homology 2-containing inositol phosphatase-1 (SHIP1) interaction, depending on the SNPs. SHIP1 regulates myeloid cell proliferation, which is controlled by PI3K [45]. PI3K also plays a role in TRβ-regulated cellular survival and proliferation [46, 47]. Finally, aberrant TRβ expression in liver and kidney may influence the pharmacokinetics of 5-FU and CDDP [21-24].

Thyroid function should also be considered when analyzing myeloid function [15-18]. In this study, TH-related parameters were not routinely evaluated because no clinical manifestations were found. We believe that subclinical TH disorders will not eradicate the strong impact of *THRB* SNPs on leukopenia. It is also necessary to consider the influence on thyroid function because irradiation fields often include a part of the thyroid gland. Although it is well known that radiation exposure of the thyroid gland can induce hypothyroidism, its potential influence can be ignored in this study because radiation-induced hypothyroidism would be detected several years after completion of irradiation [48]. Furthermore, possible influence of CDDP-based chemotherapy on thyroid function has been reported in children [49].

Our study has some limitations. First, this is a retrospective study. Second, our pilot study was limited by small sample size and a lack of racial and ethnic diversity. The *THRB* genotype and haplotype frequencies in healthy subjects also remain to be estimated. Further, detailed studies are needed to identify the mechanisms underlying transcriptional regulation and examine the involvement of other possible factors, including related genotypes and the presence

of transcription factors, in these mechanisms. Finally, our exploratory reconnaissance selected only 6 candidate SNPs based on the selection criteria described above, and the criteria for selecting useful SNPs from a large number of SNPs remain unclear. Despite these limitations, the present data contribute significant information on the predictive value of SNPs in leukopenia and may prove to be useful tools for individualizing ESCC treatment strategies.

Conclusion

Our pilot study shows the potential utility of *THRB* polymorphisms in ESCC patients receiving a definitive 5-FU/CDDP-based CRT, suggesting that specific SNPs in *THRB* provide pre-treatment warning of severe acute myelotoxicity. The clinical utility of *THRB* genotyping should be confirmed in prospective studies.

Authors' contributions

TA, SM, MH, and TS conceived, designed, and coordinated the study. IM, TT, TO, and HO evaluated the clinical outcome. MY and KN determined the *THRB* SNPs. AK, MY, KK, and KN performed data management and statistical analysis. IM and TM prepared the manuscript. All authors read and approved the final manuscript.

Acknowledgments

Competing Interests

The authors have no competing interests.

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