

Impact of MDM2 single nucleotide polymorphism on tumor onset in head and neck squamous cell carcinoma

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Impact of *MDM2* Single Nucleotide Polymorphism on Tumor Onset in Head and Neck

Squamous Cell Carcinoma

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Abstract

Objective

This study was designed in order to evaluate the association between the single nucleotide polymorphism (SNP) 309 in the *MDM2* gene with head and neck squamous cell carcinoma (HNSCC). An MDM2 protein downregulates the p53 pathway. Recently, an important SNP was discovered in the *MDM2* promoter region, which could affect the tumorigenesis of HNSCC by attenuation of the p53 pathway.

Material and Methods

Patients with 103 HNSCC were genotyped using direct sequencing and real-time PCR. The relationship between the SNP309 genotypes and the clinicopathological features was statistically analyzed.

Results

The number of patients genotyped to TT, TG, and GG was 29, (28%), 46 (44.7%), and 28 (27.2%), respectively. The average age of tumor onset was 65.6 years in the TT, 62.9 years in the TG, and 56.7 years in the GG. The patients with the GG genotype had a

significantly earlier tumor onset in comparison to those with the TT genotype ($P = 0.032$).

Conclusions

The GG genotype of *MDM2*-SNP309 is associated with an earlier onset of HNSCC in the Japanese population. SNP309 may be a key factor in the tumorigenesis of HNSCC as well as other hereditary or sporadic tumors.

Introduction

Malignant neoplasms are known to occur due to multi-step genetic alterations in tumorigenesis (1). The *murine double minute 2 (MDM2)* gene was originally identified to be an amplified gene on a murine double minute chromosome in the transformed BALB/c 3T3 cell line. A MDM2 protein forms a complex with the p53 protein and promotes the rapid degradation of the p53 protein. As the *p53* gene product plays a central role in cell cycle regulation, such as cell cycle arrest, cellular senescence, and apoptosis (2-3). **These p53-regulated pathways are frequently inactivated in human tumors both by gene alterations, or cellular/viral proteins binding to p53 protein. Genetic alterations of *p53* have been reported in various types of epithelial tumors, including head and neck squamous cell carcinoma (HNSCC). Such mutations of *p53* gene were observed approximately 40-50% of the HNSCC (4).**

The interaction between the p53 and MDM2 proteins inhibits both cell cycle arrest and apoptotic functions (5-7). *In vitro*, the overexpression of MDM2 in the murine cells has been shown to increase the tumorigenic potential and growth rate (7-8). In addition, the overexpression of the *MDM2* gene product and/or gene amplification

has been frequently found in human malignant tumors, especially in sarcomas and soft tissue tumors (9). Therefore, these data suggest the oncogenic role of MDM2 in several tumor models.

Recently, a single nucleotide polymorphism (SNP), i.e., a T to G substitution at the first intron, was discovered in the *MDM2* promoter region, which was named SNP309 (10). Interestingly, in Li-Fraumeni Syndrome patients, who have a somatic mutation in the TP53 gene, those with SNP309 showed a significantly earlier age of onset for malignant tumors in the form of sarcomas and breast cancer. Moreover, the G allele of SNP309 was associated with an earlier tumor onset of sporadic soft tissue sarcoma. These data suggest that the G allele of SNP309 is associated with the acceleration of tumorigenesis by attenuation of the p53 tumor suppressor pathway. The study conducted in Finland reported no association between SNP309 and HNSCC (11). However, the relationship between SNP309 and HNSCC has not yet been elucidated in other ethnic populations.

Estrogen signaling has been shown to regulate MDM2 expression levels.

Previous study suggested that G allele of SNP309 increases the affinity of the promoter

for Sp1, which is a transcriptional activator for multiple hormone receptors, including estrogen receptor (ER) (12). Since the effects of overexpressed MDM2 may be enhanced by ER interactions with Sp1, SNP309 could affect gender differences on tumorigenesis and susceptibility in different types of cancer (10-13). In order to investigate the above issues in Japanese patients with HNSCC, we investigated the relationship between the *MDM2-SNP309* genotype and the clinical factors.

Materials and Methods

Patients and Controls

This study included 103 patients with HNSCC and 120 cancer-free controls.

Table 1 shows detailed information about the characteristics of the HNSCC patients (84 males, 19 females). The average age of patients at diagnosis was 62.0 years (range 18-92 years, $SD \pm 13.4$). The most common tumor site was the larynx (n=40), followed by the hypopharynx (n=14), oral cavity (n=13), nasopharynx (n=12), and oropharynx (n=12). Seventy-six patients (73.8%) were smoker (male: 70/84, female: 6/19) and 55 patients (54.1%) were habitual alcohol drinker (male: 55/84, female: 0/19). The female patients' group was consisted of mostly postmenopausal women (89.5%, 17 of the 19 patients), and no one had taken exogenous estrogens. Table 2 shows information about cancer-free controls. The cancer-free control cohort consisted of 59 males and 61 females. The average age of the patients was 33.7 years (range 16-78 years, $SD \pm 13.5$). All of the subjects were genetically unrelated Japanese; they were from Kanazawa, Japan and the surrounding regions. All of the patients had histopathologically confirmed

HNSCC and were diagnosed and recruited from December 2003 to March 2005 at the Department of Otolaryngology of Kanazawa University Hospital. Written informed consent was obtained from each subject at recruitment. This study was approved by the institutional review board of Kanazawa University.

Genetic Analysis

A 3 mL blood sample was collected from the patients and the controls. The genomic DNA was extracted using a QIAamp[®] DNA Mini Kit (QIAGEN, Hilden, Germany). The *MDM2* SNP309 was genotyped by genomic sequencing and real-time PCR (RT-PCR). In order to confirm the result of RT-PCR, we performed direct genomic sequencing. Genotyping was performed without knowledge of patient or control status of the study subjects. The sequencing PCR primers that were used in the reactions were as follows: 5'-GTTCAGGGTAAAGGTCACG-3' and 5'-GTCTGAACTTGACCAGCTC-3'. PCR was performed in 100 μ l volumes containing 100 ng genomic DNA, 10 \times PCR buffer, 2.5 mmol each dNTP mixture, 0.1 μ mol of both primers, and 2.5 U of Takara Ex *Taq* DNA polymerase (TAKARA BIO,

Otsu, Japan).

PCR was carried out with an initial melting step of 5 min at 94°C, followed by 40 cycles of 30 sec at 94°C, 30 sec at 62°C, and 30 sec at 72°C, and a final elongation step of 7 min at 72°C. The PCR products were purified using a QIAGEN quick PCR Purification Kit (QIAGEN), and then sequenced using an ABI PRISM 310 sequencing system (Applied Biosystems, Foster City, CA).

RT-PCR was performed utilizing a Light-Cycler (Roche, Mannheim, Germany) with hybridization probes in combination with the Light-Cycler DNA master hybridization probes kit (Roche). The PCR primers were the same as those used in sequencing. **SNP analysis on the Light-Cycler instrument using melting curve analysis represents amplification and detection with specific probes.** The hybridization probes were as follows: 5'-LC Red-CGCGCCGCAGCGGCC-3' and 5'-CAGGCACCTGCGATCATCCGGACCTC-Fluorescein. The hybridization probes were synthesized by Nihon Gene Research Laboratories (Sendai, Japan). The PCR reaction mixture consisted of 2 mM MgCl₂, 4 pmol of each hybridization probe, 10 pmol of each PCR primer, 2 µl of Light-Cycler DNA master hybridization mix (Roche),

and 10 ng of DNA in a final volume of 20 μ l. After 10 min of denaturation at 95°C, the PCR cycles were performed with 10 sec denaturation at 95°C, 10 sec annealing at 59°C, and 8 sec extension at 72°C for 45 cycles. The melting curves were achieved following a start temperature of 40°C and an end temperature of 95°C with a temperature increase of 0.05°C /sec. *MDM2* SNP309 genotypes were differentiated based on melting temperatures of normal and mutant alleles in the homozygous and heterozygous configuration.

Statistical Analysis

The genotype frequency was analyzed using the χ^2 test. The correlation between *MDM2*-SNP309 and age at tumor onset was studied with one-way ANOVA, and *post hoc* analyses of the differences among the means were performed using the Tukey-Kramer multiple comparison test. The tumor onset age separated patients into male and female were compared with Fisher's protected least significant difference (PLSD) test. *P* values of < 0.05 were considered to be significant. All of the statistical analysis was performed with the Dr. SPSS II software program for Windows (SPSS,

Chicago, IL) and Statview software for Macintosh (Abacus concepts, Inc., Berkeley, CA).

Results

Analysis of *MDM2*-SNP309

The genotype and distribution of the *MDM2* SNP309 in the controls and patients are shown in Table 3. In the controls, 37 individuals (30.8%) were wild type (TT), 50 (41.7%) were heterozygous (TG), and 33 (27.5%) were homozygous (GG) for SNP309. In the HNSCC patients, 29 individuals (28.2%) were TT, 46 (44.7%) were TG, and 28 (27.2%) were GG for SNP309. In the male patients, 24 individuals were TT genotype, 38 were TG, and 22 were GG for SNP309. In the female, 6 individuals were TT, 8 were TG, and 5 were GG for SNP309. The observed genotype frequency of the patients was in agreement with the Hardy-Weinberg equilibrium in the controls. There was no significant difference between the patient groups and the controls regarding the distribution of genotypes.

Tumor Onset of HNSCC

The average age of tumor onset in the HNSCC patients was 65.6 years in the TT group, 62.9 years in the TG group, and 56.7 years in the GG group, respectively. Figure 1 shows the distributions of the age at diagnosis for each genotype. The median age at diagnosis was 70 years in the TT genotype, 64 years in the TG, and 58 years in the GG (Table 4). A significant difference was observed in the average age of tumor onset between the TT and GG groups ($P = 0.032$), but no significant differences were observed between the TT and TG groups ($P = 0.67$), or between the TG and GG groups ($P = 0.12$), respectively. These results indicate that patients with the GG genotype develop HNSCC earlier than those with the TT genotype.

Since several reports suggest this SNP restricted with female, we also investigated whether this SNP associated with gender. Figure 2 shows the tumor onset age separated the patients into female and male for each genotype. The average diagnosed age of the male HNSCC patients was 65.2 years in the TT group, 63.2 years in the TG, and 57.0 years in the GG. These data are similar to the result from all of the HNSCC patients, and significant difference was observed in the average age between the TT and GG groups of male ($P = 0.47$). In the female patients, the average diagnosed

age was 67.4 years in the TT group, 61.6 years in the TG group, and 55.5 years in the GG group. These results are similar to those of all HNSCC patients. In spite of 11.9 years earlier tumor onset was observed, there was no statistically significant association between the different genotypes and tumor onset age in the female patients ($P = 0.086$).

Association of *MDM2*-SNP309 with Clinical Status of HNSCC

We evaluated the effects of *MDM2* SNP309 on the clinical status of HNSCC at the time of diagnosis. However, there was no statistically significant association between SNP309 and primary tumor stage (T classification), lymph node stage (N classification), and the overall stage (data not shown). The average Brinkman Index (BI) of patients was 919.1 (SE \pm 142.7) in the TT group, 733.5 (SE \pm 91.5) in the TG group, and 653.7 (SE \pm 130.2) in the GG group. The BI showed a decreasing tendency according to an increase the number of G alleles. However, there was no statistically significant association between *MDM2* SNP309 and the smoking status. Similarly, there was no association between *MDM2* SNP309 and alcohol consumption (data not shown). In addition, no significant association was observed between SNP309 and status of the

female patients: smoking, exogenous estrogen intake, and menopausal state (data not shown).

Discussion

MDM2 is known to be an oncogene, and its product is one of the essential negative regulators of p53 (14). Various human tumors show an overexpression of the MDM2 protein with or without the *MDM2* gene amplification. Moreover, an overexpression of the MDM2 protein has been reported to correlate with a poor prognosis. In such tumors, an overexpression of MDM2 probably plays an important role in tumorigenesis, thus causing the suppression of tumor suppressor p53, or other mechanisms.

A previous study demonstrated that *MDM2* SNP309 with a G allele has a strong potential toward tumorigenesis (10). The presence of the G allele could increase the affinity of the stimulatory protein 1 (Sp1) to this region of the *MDM2* promoter, resulting in higher levels of MDM2 RNA and protein and the subsequent attenuation of the p53 pathway. Therefore, the naturally occurring variations in the *MDM2* gene may influence an individual's susceptibility to cancer by affecting the p53 signal transduction. On the other hand, the clinical role of SNP309 remains controversial

(10-13, 15-36). Regarding sporadic cancers, many previous studies showed that SNP309 was associated with an earlier onset of cancer, or an increased risk of cancer (11-13, 15-32). In contrast, several investigators reported no association between SNP309 and cancer (33-36). Few reports have examined the relationship between SNP309 and the risk of developing squamous cell carcinoma, but Hong et al reported an association between the *MDM2* SNP309 GG genotype and a risk of developing poorly differentiated and advanced esophageal squamous cell carcinoma in comparison to the GT or TT genotypes (21). In addition, Zhou et al. reported SNP309 associated with increased risk of nasopharyngeal carcinoma (NPC) occurrence and advanced neck lymph node metastasis of NPC (32). Similarly, our results demonstrated that the GG genotype, not existence of the G allele, in SNP309 is significantly associated with an earlier tumor onset in HNSCC patients in comparison to the TT genotype cohort. In contrast, a report showed no significant association between the SNP309 genotype and tumor onset in HNSCC patients in the Finnish population, suggesting that a difference in ethnicity affects the tumorigenic potential of the GG genotype in SNP309 (11). Previous studies demonstrated that the G-allele of SNP309 accelerates tumorigenesis of

diffuse large B-cell lymphoma, soft tissue sarcoma, breast cancer, and colorectal cancer in women (10-13). Our results showed an 11.9-years earlier tumor onset in women with the GG genotype and an 8.2-years earlier onset in men with the GG genotype compared with TT. There was significant difference was observed in men ($P = 0.047$). However, there was no statically significance in women ($P = 0.086$), in spite of 11.9 years earlier tumor onset was observed. This is probably because the number of women is too small ($n=19$). This is come from the majority of HNSCC patients is men, because HNSCC is strongly related with habits of cigarette smoking and alcohol drinking, and the prevalence of women is low in HNSCC. Previous study demonstrated that increasing estrogen levels and ER signaling in women with the GG genotype could increase their risk to develop cancers. However, in our study, most of women were postmenopausal state and we could not observe gender-specific difference with tumor development. Therefore, we consider the estrogen levels and ER signaling is not strongly affect in HNSCC development. Moreover, both male and female showed the trend toward earlier tumor onset with the GG genotype compared with TT in our study. Thus, SNP309 may contribute to acceleration of tumor formation independent from influence of estrogen

hormone or ER signaling upon HNSCC. In addition, thus suggesting that the gender-associated effect of SNP309 on tumorigenesis varies according to the anatomic site in which the cancer develops. More studies will be necessary involving cancers of various anatomic sites with a larger sample size, especially women involved in, in order to elucidate this issue.

In addition, the HPV infection status is known to correlate with some of HNSCC, especially tonsillar carcinoma (37-38). A larger study may reveal the relationship between HPV infection and mdm2.

This study suggests that the SNP genotype provides interesting information, including predisposition to HNSCC, which is represented by early onset age. However, a larger scale prospective study examining the relationship between SNP 309 and p53 status is called for.

In conclusion, the GG genotype of *MDM2* SNP309 is associated with an earlier onset of HNSCC in the Japanese population according to our results. These results suggest that *MDM2* SNP309 may affect the tumorigenic potential of the head and neck mucosa in the Japanese population, which eventually results in the earlier

onset of HNSCC as well as other hereditary and sporadic tumors.

Figure Legends

Figure 1 shows the distributions of the age of HNSCC patients at diagnosis for each genotype.

Figure 2 shows mean tumor onset age separated the patients into male and female for each genotype. Vertical bars indicate 95% CI.

* $P < 0.05$

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Squamous Cell Carcinoma

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Table 1. Characteristics of HNSCC Patients

Characteristics	Number of Patients (%)
Total patients	103 (100)
Sex	
Male	84 (81.6)
Female	19 (18.4)
Age	
60 ≤	48 (46.6)
60 >	55 (53.4)
Tumor site	
Larynx	40 (38.8)
Hypopharynx	14 (13.6)
Oral cavity	13 (12.6)
Nasopharynx	12 (11.7)
Oropharynx	12 (11.7)
Paranasal sinuses	8 (7.8)
Ear canal	3 (2.9)
Primary unknown	1 (1.0)
T classification	
1+2	59 (57.3)
3+4	43 (41.7)
N classification	
N0	57 (55.3)
N1-3	46 (44.7)
Overall stage	
I + II	45 (43.7)
III+IV	58 (56.3)

Table 2. Characteristics of Controls

Characteristics	Number of Controls (%)
Total Controls	120 (100)
Sex	
Male	59 (49.2)
Female	61 (50.8)

Table 3. The Number of *MDM2* SNP309 Genotype of the Controls, Patients, and Male or Female Patients

SNP309	No. Controls (%)	No. Patients (%)	Male Patients (%)	Female Patients (%)
TT	37 (30.8)	29 (28.2)	24 (28.6)	5 (26.3)
TG	50 (41.7)	46 (44.7)	38 (45.2)	8 (42.1)
GG	33 (27.5)	28 (27.2)	22 (26.2)	6 (31.6)
Total No.	120	103	84	19

Table 4. *MDM2* SNP309 status with the Average and Median Tumor Onset Age of Patients

SNP309	Tumor Onset Age (mean \pm SD)	Median age
TT	65.6 \pm 14.3*	70
TG	62.9 \pm 11.4	64
GG	56.7 \pm 14.3*	58

* $P < 0.05$

Figure 1

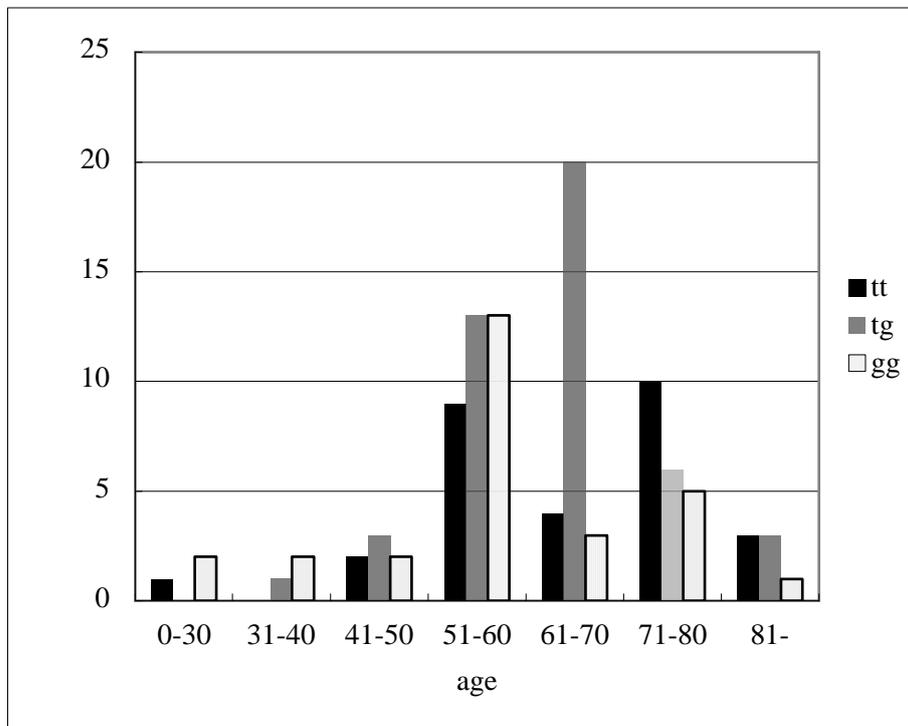


Figure 2

