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Scientific Research

High Incidence of Null-Type Mutations of the *TP*53 Gene in Japanese Patients with Head and Neck Squamous Cell Carcinoma

Yasuhiro Ebihara[1,2], Miwako Iwai[1], Ken Akashi[1,2], Takeshi Ito[1], Go Omura[2], Yuki Saito[2], Masafumi Yoshida[2], Mizuo Ando[2], Takahiro Asakage[2], Tatsuya Yamasoba[2], Yoshinori Murakami[1*]

[1]Division of Molecular Pathology, Institute of Medical Science, The University of Tokyo, Tokyo, Japan
[2]Department of Otolaryngology—Head and Neck Surgery, The University of Tokyo, Tokyo, Japan
Email: [*]ymurakam@ims.u-tokyo.ac.jp

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Abstract

Objective: Molecular targeting therapy has not been generally established in head and neck squamous cell carcinoma (HNSCC) except for cetuximab treatment for targeting epidermal growth factor receptor (*EGFR*). We analyzed alterations of the *TP*53, *KRAS*2, and *EGFR* genes in Japanese HNSCC to identify subpopulations of tumors potentially susceptible or not susceptible to specific therapy based on their genetic alterations. Materials and Methods: A total of 56 Japanese subjects were included in this study. Genomic DNA of exons 5 - 9 of the *TP*53, exons 1 and 2 of the *KRAS*2, exons 19 - 22 of the *EGFR*, and their flanking sequences were amplified by polymerase chain reaction (PCR) followed by direct sequencing. Splicing variants of *EGFR* were examined by reverse transcription (RT)-PCR. Results: Mutations of the *TP*53 and *KRAS* genes were detected in 25 (45%) and 2 (4%) of 56 HNSCC cases, respectively, while neither mutation nor splicing variant of *EGFR* was observed. The *TP*53 mutation did not correlate with clinical stages or primary sites of the tumors. The patterns of nucleotide substitutions specific to HNSCC were not observed. However, the incidence of null-type mutations of the *TP*53, which cannot be detected as abnormal by conventional immunohistochemical (IHC) studies, was significantly higher (10/25; 40%) than that of HNSCC reported in other countries. Conclusion: Frequent *TP*53 mutations, especially null-type mutations, but infrequent or no alterations of the *KRAS* and *EGFR* suggest that the sequencing analysis of the *TP*53 mutation rather than IHC analysis of p53 provides a potentially useful marker to predict the response of HNSCC to chemotherapy or radiotherapy.

Keywords

Head and Neck Squamous Cell Carcinoma, *TP*53 Mutation, *KRAS*, *EGFR*, Molecular Marker

[*]Corresponding author.

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1. Introduction

Head and neck squamous cell carcinomas (HNSCCs) account for 6% of all cancers worldwide, affecting more than 650,000 patients every year. Most of these cases are classified as the advanced stage III/IV, which is characterized by a poor prognosis with a survival rate of only 40% and little improvement with multi-modality therapy [1] [2]. In the past decade, the introduction of molecular targeting therapy had improved the prognosis and resulted in therapeutic gains in patients with HNSCC. Treatment with anti-epidermal growth factor receptor (*EGFR*) monoclonal antibodies, such as cetuximab, is currently used for HNSCC, and a number of additional drugs are being tested for clinical use. To improve the effectiveness of the molecular targeting therapies, it is important to identify biomarkers related to clinical gains. Presence of the *KRAS* gene mutation has been established as a useful indicator for predicting poor response to cetuximab treatment in colorectal cancer [3]. Since *KRAS* acts in the downstream cascade of *EGFR*, its activating mutation would cancel the suppressor effect of the upstream molecule, *EGFR*, with cetuximab treatment. However, neither screening nor extensive analyses of the *KRAS* gene mutation have been performed, at least not in Japanese cases of NHSCC.

One successful example of molecular targeting therapy against solid tumors is that of *EGFR* tyrosine kinase inhibitors, such as gefitinib or erlotinib, for a subset of lung adenocarcinomas, where the presence of the *EGFR* mutation serves as a very strong indicator of a good response to the therapy. In addition, it is reported that a specific splicing variant of *EGFR* (vIII) is overexpressed in a subset of tumors and could provide a predictive marker of poor prognosis in glioblastoma patients [4]. Only a few studies, however, have reported the incidence of the *EGFR* mutation or splicing variants of *EGFR*, at least in Japanese cases of HNSCC.

Another potentially useful biomarker of cancer treatment is the *TP*53 mutation because it is the most frequent mutation observed in human cancers and is deeply involved in the cellular responses against chemotherapy and radiotherapy. In fact, it has been reported that the presence of *TP*53 mutations serves as an essential biomarker that correlates well with the efficacy of induction chemotherapy [5]-[7], the local control rate of radiotherapy [8]-[10], and the survival rate of HNSCC patients [11] [12]. Mutation of the *TP*53 is usually tested by immunohistochemical (IHC) analysis because mutant p53 proteins generated by missense mutations show a longer half-life and can be detected by IHC analysis, whereas wild-type p53 proteins cannot be detected by IHC analysis due to their shorter half-lives. On the other hand, null-type mutations of the *TP*53 cannot be detected by IHC analysis, which might be one reason why the usefulness of the p53 mutation in predicting cancer response to chemotherapy or radiotherapy is limited and controversial [13]-[15]. Although a number of studies on the *TP*53 mutation have been reported for HNSCC from the USA and Europe, only a few reports are available for HNSCC in Japanese and Asian populations.

In this study, we analyzed mutations of the *TP*53, *KRAS*, and *EGFR* genes as well as a splicing variant of *EGFR* in 56 Japanese cases. A high incidence of null-type mutations of the *TP*53 gene strongly suggests that sequencing, rather than IHC analysis, is necessary to examine the mutation status of the *TP*53 gene as a possible biomarker for the treatment of Japanese HNSCC.

2. Materials and Methods

2.1. Surgical Materials

A total of 56 Japanese subjects (48 men and 8 women; median age: 65 years old; age range: 24 - 87 years old) provided informed, written consent in accordance with the ethics board of the University of Tokyo (ID: 20-39-1016) and were included in this study. All subjects were diagnosed as having HNSCCs and were operated on at the Department of Otolaryngology—Head and Neck Surgery at the University of Tokyo from April, 2008, to June, 2009. Primary sites were the oral cavity in 21 cases, the oropharynx in 8 cases, the hypopharynx in 12 cases, the larynx in 13 cases, the nasal cavity in 1 case, and the auditory canal in 1 case (**Table 1**). The clinical stages were I, II, III, and IV in 4, 9, 15, and 28 cases, respectively, including 12 recurrent cases (Stage III in 4 cases and Stage IV in 8 cases). The majority of subjects in this cohort (71%; 35 of 49 patients) were heavy smokers (≥25 pack years). Specimens were collected at surgical resection and were quickly frozen in liquid nitrogen and stored at −80˚C or preserved in Allprotect Tissue Reagent (Qiagen, Alameda, CA, USA).

2.2. Sequencing Analysis

Genomic DNA was extracted using the AllPrep Mini Kit (Qiagen). Exons 1 and 2 of the *KRAS*, exons 19 - 22 of

Table 1. Clinical stages and primary sites of HNSCC tumors with mutation of the *TP*53 and *KRAS* genes.

	No of tumors with *TP*53 mutation				No of tumors with the *KRAS* mutation	No. of tumors examined
	Total	Missense	Nonsense	Others		
Clinical stage						
I	3 (75%)	2	1	0	0	4
II	6 (67%)	2	4	0	0	9
III	8 (53%)	4	3	1	1	15
IV	8 (26%)	5	2	1	1	28
Primary sites						
Oral cavity	11 (52%)	5	5	1	0	21
Oropharynx	2 (25%)	1	1	0	2	8
Hypopharynx	7 (58%)	3	3	1	0	12
Larynx	4 (31%)	3	1	0	0	13
Nasal cavity	1 (100%)	1	0	0	0	1
Auditory canal	1 (0%)	0	0	0	0	1

the *EGFR*, exons 5 - 8 of the *TP*53 genes, and their flanking sequences were amplified by polymerase chain reaction (PCR) using KOD FX polymerase (Toyobo, Osaka, Japan). Primers used for PCR are described in **Table S1**. The PCR mixtures contained 10 μl of 2× PCR buffer, 4 μl of 2 mM dNTPs, 0.4 unit of KOD FX, 0.6 μl of forward and reverse primers (each 10 μM), 40 ng of template DNA, and distilled water to make a reaction mixture of 20 μl. PCR cycles were as follows: denaturing at 94˚C for 2 min followed by 35 cycles of denaturing at 98˚C for 5 sec, annealing at 57˚C for 10 sec, and extension at 68˚C for 10 sec, with a final extension at 68˚C for 7 min. PCR products were separated by electrophoresis on agarose gel, and fragments were cut from the gel and purified by Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). Sequencing was conducted by reactions with Applied Biosystems' BigDye 3.1 kit (Applied Biosystems, Foster City, CA, USA) on a 3130xl DNA sequencer. Sequence results were analyzed using Lasergene (Dnastar, Madison, WI, USA), and statistical analysis was done using SPSS software. The significance level was set at $P < 0.05$.

2.3. Detection of Splicing Variants

Total cellular RNA were extracted using the FastTrack 2.0 Kit (Invitrogen, Carlsbad, CA) and reverse-transcribed by Superscript II (Invitrogen, Carlsbad, CA) as described previously [16]. A 1037-bp cDNA fragment of *EGFR* corresponding to exons 1 - 8 and a possible 236-bpfragment of *EGFR*vIII were amplified by PCR using a pair of primers, *EGFR*vIIIOneStepF and *EGFR*vIIIOneStepR as described previously [17], while a 452-bp fragment of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified as an internal control in the same reaction. Primers used for PCR are described in **Table S1**. The PCR product was subjected to electrophoresis on agarose gel as described previously [16].

3. Results

3.1. Analysis of the *KRAS* and *EGFR* Mutations as Well as Splicing Variants of *EGFR*

Sequencing analysis of exons 1 and 2 and their flanking regions of the *KRAS* gene identified 2 activating mutations (3.6%) out of 56 HNSCC cases (**Figure 1(a)**, **Figure 1(b)**). The G to A transition at codon 14 (valine to isoleucine) is an uncommon but an already reported activating mutation of the *KRAS* gene. Interestingly, both cases are oropharyngeal HNSCCs (2 of 8; 25%); one is in clinical Stage III and the other is in IV. In contrast, none of the other 48 HNSCCs shows any *KRAS* mutation, suggesting that the *KRAS* mutation could not provide a practical marker for predicting response to cetuximab treatment in Japanese HNSCCs except for oropharyngeal squamous cell carcinomas (SCCs).

On the other hand, sequencing analysis of exons 19 - 22 and their flanking regions of the *EGFR* gene did not show any mutations. Moreover, reverse transcription (RT)-PCR analysis covering the sequences corresponding

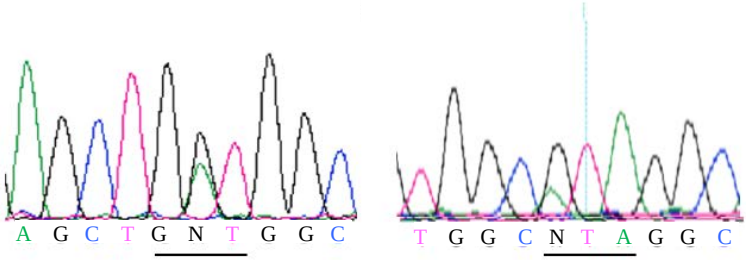
to exons 1 - 8 did not show any extra products, suggesting that the splicing variant III, which is reported to be present in glioblastomas and to be associated with poor prognosis for glioblastoma patients, is not expressed in HNSCC.

3.2. High Incidence of Null-Type Mutations of the *TP*53 Gene in Japanese HNSCCs

Since most of the *TP*53 mutations were found in its hotspot corresponding to exons 5 - 9, we amplified the genomic DNA fragments of exons 5 - 9 as well as their flanking sequences by PCR followed by direct sequencing. Representative results are shown in **Figure 2**, in which a missense mutation in codon 134 from TTT (phenylalanine) to TGT (cysteine) in exon 5 (**Figure 2(a)**), a nonsense mutation in codon 242 from TGC (cysteine) to TGA (stop) in exon 7 (**Figure 2(b)**), and a nonsense mutation in codon 306 from CGA (arginine) to TGA (stop) in exon 8 of the *TP*53 gene (**Figure 2(c)**) are demonstrated. Similar analysis identified the *TP*53 mutations in 25 (44.6%) of 56 primary HNSCC cases. Clinicopathological analyses of HNSCC-carrying mutations demonstrate that the *TP*53 mutation does not significantly correlate with the clinical stages of HNSCC tumors (**Table 1**). It also does not correlate with the primary sites of the tumors, although the incidence in oropharyngeal HNSCC (2 of 8; 25%) is relatively low. Regarding the patterns of nucleotide substitutions, the G:C to A:T substitution is the most frequent change (36%), followed by the G:C to T:A (24%) and A:T to G:C (20%) substitutions (**Table S2**). On the other hand, when focused on the mutation spectrum, missense mutations, nonsense mutations, frameshift mutations, and splicing mutations are found in 13 (52%), 10 (40%), 1 (4%), and 1 (4%), respectively, of 25 cases with *TP*53 mutations (**Table 2**).

4. Discussion

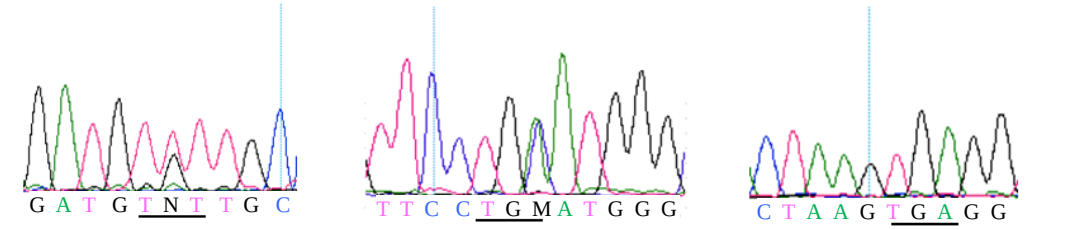
In the treatment of both solid tumors and lymphoma/leukemias, the importance of molecular targeting therapies



codon 12: GGT (Gly) → GAT (Asn) (a)

codon 14: GTA (Val) → ATA (Ile) (b)

Figure 1. Mutation of the *KRAS* gene in HNSCC. (a) The glycine codon (GGT) at position 12 is mutated to the asparagine codon (GAT) in an oropharyngeal SCC with T2N1M0. (b) The valine codon (GTA) at position 14 is mutated to the isoleucine codon (ATA) in an oropharyngeal SCC with T3N2bM0.



codon 134: TTT (Phe) → TGT (Cys) (a)

codon 242: TGC (Cys) → TGA (Stop) (b)

codon 306: CGA (Arg) → TGA (Stop) (c)

Figure 2. Representative results of *TP*53 gene mutation in HNSCCs. (a) The phenylalanine codon (TTT) is mutated to the cysteine codon (TGT) in a laryngeal SCC with T3N0M0. (b) The cysteine codon (TGC) at position 242 is mutated to the stop codon (TGA) in an oral cavity SCC with T2N0M0. (c) The arginine codon (CGA) at position 306 is mutated to the stop codon (TGA) in a hypopharyngeal SCC with T3N0M0.

Table 2. Mutation spectra of the *TP*53 gene in HNSCC.

	Percentage of tumors with specific spectrum of mutation							No. of cases with *TP*53 mutation	*TP*53 mutation incidence
	Missense	Nonsense	Frameshift	Splicing	Silent	Others	Null type*		
The present study	52%	40%	4%	4%	0%	0%	48%	25	45%
Erber (Germany, Oncogene 1998)	88%	5%			5%	5%	5%	41	47%
Alsner (Denmark, RadioTxOncol 2001)	53%	14%	9%	12%	12%	0%	34%	58	39%
Eriksen (Denmark, RadioTxOncol 2005)	54%	25%		3%	17%	0%	28%	125	60%
Peronne (Italy, JCO 2010)	83%	8%	8%	0%	0%	0%	17%	24	45%
IARC database (HNSCC)	60%	13%	14%	5%	3%	5%	32%	614	42%
IARC database (All primary sites)	74%	8%	9%	2%	4%	4%	18%	26597	NR

*Null type mutation includes nonsense mutation, frameshift mutation, and splicing mutation.

in association with specific genetic markers is growing. However, it is not well established in HNSCC, except for cetuximab treatment. In the present study, we examined mutations of the *KRAS*, *EGFR*, and *TP*53 genes as possible genetic markers for molecular targeting therapy of HNSCC. Specific mutations in exons 19 - 22 of the *EGFR* gene are potent genetic markers for selecting a subset of lung adenocarcinomas that will respond well to treatment with *EGFR* tyrosine kinase inhibitors (TKIs), such as gefitinib and erlotinib. However, we failed to detect any *EGFR* mutations in 56 HNSCC cases, supporting the current findings that *EGFR* TKI treatment is not effective against HNSCC.

On the other hand, the *KRAS* gene mutation is known to serve as a marker for a subpopulation of colorectal cancer that is resistant to molecular targeting therapy by anti-*EGFR* antibodies, such as cetuximab, because the activated *KRAS* mutant acts in the downstream cascade of *EGFR*. Although several studies have demonstrated that the clinical gain with cetuximab treatment alone is limited for HNSCC patients, we failed to detect a high incidence of the *KRAS* mutation in Japanese HNSCC. It is noteworthy, however, that a relatively high incidence (2 of 8; 25%) of the *KRAS* mutation was detected in oropharyngeal HNSCC in this study, because previous studies reported that the *KRAS* gene was mutated in none of 71, none of 12 or only 4 of 123 (totally, 4 of 204; 2%) oropharyngeal HNSCCs [17]-[19]. Further studies focusing on the oropharyngeal HNSCCs would be required to assess the response to cetuximab according the presence or absence of the *KRAS* mutation as well as the human papilloma virus (HPV) infection.

The *TP*53 mutation is not a genetic marker for molecular targeting therapy. However, it is well known that it provides a good predictive marker for resistance to chemotherapy and radiotherapy and for poor prognoses in various cancers, including HNSCCs [5]-[12]. In this study, we found the *TP*53 mutation in 25 (45%) of 56 Japanese HNSCCs. This incidence is comparable to those reported previously [7] [9]-[11] (**Table 2**) and shows no correlation to the clinical stages of the tumors (**Table 1**). Regarding the sites of the tumors, it is noteworthy that the incidence of the *TP*53 mutation is relatively low (25%) in oropharyngeal HNSCC. Since infection with the human papillomavirus (HPV) is specifically observed in this type of tumor, the p53 function of a portion of the oropharyngeal HNSCCs is inactivated by protein interaction with the HPV E6 protein instead of by the gene mutation. The patterns of the nucleotide substitutions in our study are essentially the same as those of previous studies on HNSCC or those summarized by the International Agency for Research on Cancer (IARC) in the *TP*53 database [20] [21]. The relatively high incidence of G:C to T:A transversions in HNSCC appears to be caused by tobacco carcinogens, including benzopyrene.

On the other hand, when we consider the mutation spectrum, the incidence of null-type mutations, especially that of nonsense mutations, is clearly higher than that of previous reports or the *TP*53 database of the IARC for HNSCC [7] [9]-[11] [22]. The relatively high incidence of null-type mutations of the *TP*53 in HNSCC (48% in our study and 32% of HNSCCs in the IARC database) in comparison with that of all tumor types in the IARC database (18%) suggests that loss of the p53 function by null-mutation would be beneficial in HNSCC. It is

noteworthy that among 56 HNSCCs examined, 12 were derived from recurrent tumors, in which only 2 (17%) tumors showed the *TP*53 mutation with nonsense mutations, suggesting that the therapeutic intervention might not play a role in the high incidence of null-type *TP*53 mutations in HNSCC. On the other hand, a markedly high incidence of nonsense mutations (40%) in this study as compared with that in the IARC *TP*53 database for HNSCC (13%) suggests that Japanese HNSCC might develop through some unique etiological background as compared with HNSCC cases from Europe or the USA. Further studies would be required to determine the etiologic factors as well as the significance of the *TP*53 mutation in Japanese HNSCCs.

At present, mutant p53 is screened by IHC staining of tumors in many hospitals and laboratories because aberrant p53 proteins with missense mutations show a longer half-life than do wild-type p53 proteins, and, therefore, only mutant p53 with missense mutations, but not wild-type p53, can be detected by IHC analysis. It should be noted, however, that p53 proteins with null-type mutations cannot be detected by IHC analysis due to the nonsense-mediated RNA decay and the resultant decreased amount of the protein with truncation. Therefore, our findings demonstrate that the sequencing analysis of the *TP*53 gene, rather than IHC analysis, is essential for assessing the mutational state of the *TP*53 in Japanese HNSCCs for the genetic marker to predict the response to chemotherapy, local control of radiotherapy, and the overall survival rate. A systematic approach using next-generation sequencing of the surgically resected primary tumors would be beneficial for determining the mutational state of the relevant genes and for establishing a gene-based medicine for HNSCC. The limitation of this study, however, is that we have not systematically examined the clinical features of the HNSCC cases with or without the *TP*53 mutations. Further study would be necessary to assess the significance of the *TP*53 mutation in predicting the clinical outcome of 56 cases of HNSCCs.

5. Conclusion

To assess the genetic markers for molecular targeting and gene-based therapy, mutations of the *EGFR*, *KRAS*, and *TP*53 genes were examined in 56 Japanese HNSCCs. No mutation of the *EGFR* gene supports the fact that gefitinib is not effective against HNSCC. A low incidence of the *KRAS* mutation (3.6%) demonstrates that the *KRAS* mutation is not an effective marker for predicting response to cetuximab treatment, which shows a limited clinical gain in HNSCC. On the other hand, a high incidence of the *TP*53 mutation (45%) suggests that screening for the *TP*53 mutation could provide a potentially useful marker for predicting responsiveness to chemotherapy, the rate of local control in radiotherapy, or the overall survival with HNSCC, although further studies would be required to assess the significance of the *TP*53 mutation in predicting the clinical features of HNSCC in individual cases. On the basis of a high incidence of null-type mutations in Japanese HNSCCs in the present study, we propose that sequencing analysis, instead of IHC analysis, is essential for establishing the *TP*53 mutation as a genetic marker for the optimal treatment of HNSCC.

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References

[1] Vermorken, J.B., Remenar, E., van Herpen, C., Gorlia, T., Mesia, R., Degardin, M., Stewart, J.S., Jelic, S., Betka, J., Preiss, J.H., van den Weyngaert, D., Awada, A., Cupissol, D., Kienzer, H.R., Rey, A., Desaunois, I., Bernier, J. and Lefebvre, J.L. (2007) Cisplatin, Fluorouracil, and Docetaxel in Unresectable Head and Neck Cancer. *The New England Journal of Medicine*, **357**, 1695-1704. http://dx.doi.org/10.1056/NEJMoa071028

[2] Leemans, C.R., Braakhuis, B.J. and Brakenhoff, R.H. (2011) The Molecular Biology of Head and Neck Cancer. *Nature Review Cancer*, **11**, 9-22. http://dx.doi.org/10.1038/nrc2982

[3] Karapetis, C.S., Khambata-Ford, S., Jonker, D.J., O'Callaghan, C.J., Tu, D., Tebbutt, N.C., Simes, R.J., Chalchal, H., Shapiro, J.D., Robitaille, S., Price, T.J., Shepherd, L., Au, H.J., Langer, C., Moore, M.J. and Zalcberg, J.R. (2008)

K-ras Mutations and Benefit from Cetuximab in Advanced Colorectal Cancer. *The New England Journal of Medicine*, **359**, 1757-1765. http://dx.doi.org/10.1056/NEJMoa0804385

[4] Montano, N., Cenci, T., Martini, M., D'Alessandris, Q.G., Pelacchi, F., Ricci-Vitiani, L., Maira, G., De Maria, R., Larocca, L.M. and Pallini, R. (2011) Expression of *EGFR*vIII in Glioblastoma: Prognostic Significance Revisited. *Neoplasia*, **13**, 1113-1121.

[5] Temam, S., Flahault, A., Périé, S., Monceaux, G., Coulet, F., Callard, P., Bernaudin, J.F., St Guily, J.L. and Fouret, P. (2000) p53 Gene Status as a Predictor of Tumor Response to Induction Chemotherapy of Patients with Locoregionally Advanced Squamous Cell Carcinomas of the Head and Neck. *Journal of Clinical Oncology*, **18**, 385-394.

[6] Cabelguenne, A., Blons, H., de Waziers, I., Carnot, F., Houllier, A.M., Soussi, T., Brasnu, D., Beaune, P., Laccourreye, O. and Laurent-Puig, P. (2000) p53 Alterations Predict Tumor Response to Neoadjuvant Chemotherapy in Head and Neck Squamous Cell Carcinoma: A Prospective Series. *Journal of Clinical Oncology*, **18**, 1465-1473.

[7] Perrone, F., Bossi, P., Cortelazzi, B., Locati, L., Quattrone, P., Pierotti, M., Pilotti, S. and Licitra, L. (2010) *TP*53 Mutations and Pathologic Complete Response to Neoadjuvant Cisplatin and Fluorouracil Chemotherapy in Resected Oral Cavity Squamous Cell Carcinoma. *Journal of Clinical Oncology*, **28**, 761-766. http://dx.doi.org/10.1200/JCO.2009.22.4170

[8] Koch, W.M., Brennan, J.A., Zahurak, M., Goodman, S.N., Westra, W.H., Schwab, D., Yoo, G.H., Lee, D.J., Forastiere, A.A. and Sidransky, D. (1996) p53 Mutation and Locoregional Treatment Failure in Head and Neck Squamous Cell Carcinoma. *Journal of the National Cancer Institute*, **88**, 1580-1586. http://dx.doi.org/10.1093/jnci/88.21.1580

[9] Alsner, J., Sørensen, S.B. and Overgaard, J. (2001) TP53 Mutation Is Related to Poor Prognosis after Radiotherapy, but Not Surgery, in Squamous Cell Carcinoma of the Head and Neck. *Radiotherapy and Oncology*, **59**, 179-185. http://dx.doi.org/10.1016/S0167-8140(01)00301-2

[10] Eriksen, J.G., Alsner, J., Steiniche, T. and Overgaard, J. (2005) The Possible Role of TP53 Mutation Status in the Treatment of Squamous Cell Carcinomas of the Head and Neck (HNSCC) with Radiotherapy with Different Overall Treatment Times. *Radiotherapy and Oncology*, **76**, 135-142. http://dx.doi.org/10.1016/j.radonc.2005.05.004

[11] Erber, R., Conradt, C., Homann, N., Enders, C., Finckh, M., Dietz, A., Weidauer, H. and Bosch, F.X. (1998) TP53 DNA Contact Mutations Are Selectively Associated with Allelic Loss and Have a Strong Clinical Impact in Head and Neck Cancer. *Oncogene*, **16**, 1671-1679. http://dx.doi.org/10.1038/sj.onc.1201690

[12] Poeta, M.L., Manola, J., Goldwasser, M.A., Forastiere, A., Benoit, N., Califano, J.A., Ridge, J.A., Goodwin, J., Kenady, D., Saunders, J., Westra, W., Sidransky, D. and Koch, W.M. (2007) TP53 Mutations and Survival in Squamous-Cell Carcinoma of the Head and Neck. *The New England Journal of Medicine*, **357**, 2552-2561. http://dx.doi.org/10.1056/NEJMoa073770

[13] Bodner, S.M., Minna, J.D., Jensen, S.M., D'Amico, D., Carbone, D., Mitsudomi, T., Fedorko, J., Buchhagen, D.L., Nau, M.M. and Gazdar, A.F. (1992) Expression of Mutant p53 Proteins in Lung Cancer Correlates with the Class of p53 Gene Mutation. *Oncogene*, **7**, 743-749.

[14] Hashimoto, T., Tokuchi, Y., Hayashi, M., Kobayashi, Y., Nishida, K., Hayashi, S., Ishikawa, Y., Tsuchiya, S., Nakagawa, K., Hayashi, J. and Tsuchiya, E. (1999) p53 Null Mutations Undetected by Immunohistochemical Staining Predict a Poor Outcome with Early-Stage Non-Small Cell Lung Carcinomas. *Cancer Research*, **59**, 5572-5577.

[15] Mroz, E.A. and Rocco, J.W. (2010) Functional *p*53 Status as a Biomarker for Chemotherapy Response in Oral-Cavity Cancer. *Journal of Clinical Oncology*, **28**, 715-717. http://dx.doi.org/10.1200/JCO.2009.26.3475

[16] Kikuchi, S., Iwai, M., Sakurai-Yageta, M., Tsuboi, Y., Ito, T., Masuda, T., Tsuda, H., Kanai, Y., Onizuka, M., Sato, Y. and Murakami, Y. (2012) Expression of a Splicing Variant of the CADM1 Specific to Small Cell Lung Cancer. *Cancer Science*, **103**, 1051-1057. http://dx.doi.org/10.1111/j.1349-7006.2012.02277.x

[17] Fujii, S., Uryu, H., Akashi, K., Suzuki, K., Yamazak, M., Tahara, M., Hayashi, R. and Ochiai, A. (2013) Clinical Significance of *KRAS* Gene Mutation and Epidermal Growth Factor Receptor Expression in Japanese Patients with Squamous Cell Carcinoma of the Larynx, Oropharynx and Hypopharynx. *International Journal of Clinical Oncology*, **18**, 454-463. http://dx.doi.org/10.1007/s10147-012-0402-z

[18] Bissada, E., Abboud, O., Abou, C.Z., Guertin, L., Weng, X., Nguyen-Tan, P.F., Tabet, J.C., Thibaudeau, E., Lambert, L., Audet, M.L., Fortin, B. and Soulieres, D. (2013) Prevalence of K-RAS Codons 12 and 13 Mutations in Locally Advanced Head and Neck Squamous Cell Carcinoma and Impact on Clinical Outcomes. *International Journal of Otolaryngology*, **2013**, Article ID: 848021. http://dx.doi.org/10.1155/2013/848021

[19] De Carvalho, T.G., De Carvalho, A.C., Maia, D.C., Ogawa, J.K., Carvalho, A.L. and Vettore, A.L. (2013) Search for Mutations in Signaling Pathways in Head and Neck Squamous Cell Carcinoma. *Oncology Reports*, **30**, 334-340.

[20] Wikstrand, C.J., Hale, L.P., Batra, S.K., Hill, M.L., Humphrey, P.A., Kurpad, S.N., McLendon, R.E., Moscatello, D., Pegram, C.N., Reist, C.J., Traweek, S.T., Wong, A.J., Zalutsky, M.R. and Bigner, D.D. (1995) Monoclonal Antibodies against *EGFR*vIII Are Tumor Specific and React with Breast and Lung Carcinomas and Malignant Gliomas. *Cancer Research*, **55**, 3140-3148.

[21] Olshan, A.F., Weissler, M.C., Pei, H. and Conway, K. (1997) p53 Mutations in Head and Neck Cancer: New Data and Evaluation of Mutational Spectra. *Cancer Epidemiology Biomarkers and Prevention*, **6**, 499-504.

[22] (2009) International Agency for Research on Cancer TP53 Database R14 Release. http://www-p53.iarc.fr/

Supplement

Table S1. Sequences of the primers used for this study.

Name of the primers	Sequences
*TP*53-ex5-pF2	TTCAACTCTGTCTCCTTCCT
*TP*53-ex5-pR	CAGCCCTGTCGTCTCTCCAG
*TP*53-ex6-pF2	GCCTCTGATTCCTCACTGAT
*TP*53-ex6-pR	TTAACCCCTCCTCCCAGAGA
*TP*53-ex7-pF3	CTTGCCACAGGTCTCCCCAA
*TP*53-ex7-pR	TCTACTCCCAACCACCCTTG
*TP*53-ex8&9-pF	ACCCATCCACCTCTCATCAC
*TP*53-ex8&9-pR	TGAAAGCTGGTCTGGTCCTT
EGFR-ex18-pF	CCGGAGTTTTCAATCCAGTT
EGFR-ex18-pR	GTCAATGGCCCCTTTCATAA
EGFR-ex19-pF	TGGATGAAATGATCCACACG
EGFR-ex19-pR	AGTGCTGGGTAGATGCCAGT
EGFR-ex20-pF	TCCGACTCCTCCTTTATCCA
EGFR-ex20-pR	GATGGGACAGGCACTGATTT
EGFR-ex21-pF	AAGTTCAAGCCCAGGTCTCA
EGFR-ex21-pR	TCATTCACTGTCCCAGCAAG
KRAS-ex1-pF	AAAGGTACTGGTGGAGTATTTG
KRAS-ex1-pR	TCTGTATCAAAGAATGGTCCTG
KRAS-ex2-pF	TCTTTGGAGCAGGAACAATG
KRAS-ex2-pR	TGCATGGCATTAGCAAAGAC
*EGFR*vIIIoneStepF	GGGGAATTCGCGATGCGACCCTCCGGG
*EGFR*vIIIoneStepR	GGGAAGCTTTCCGTTACACACTTTGCG
GAPDH_FWD	ACCACAGTCCATGCCATCAC
GAPDH_REV	TCCACCACCCTGTTGCTGTA

Table S2. Patterns of nucleotide substitution of the *TP*53 gene in HNSCC.

	No. of tumors with nucleotide substitutions									No. of cases examined
	A:T > C:G	A:T > G:C	A:T > T:A	G:C > A:T at CpG	G:C > A:T at non CpG	G:C > C:G	G:C > T:A	ins/del	Others	
The present study	8%	20%	4%	20%	16%	4%	24%	4%	0%	25
Olshan (CanEpiBioPre 1997)	4%	13%	8%	14%	18%	9%	17%	12%	5%	286
IARC database (HNSCC)	3%	9%	6%	18%	17%	6%	24%	16%	1%	614
IARC database (All primary sites)	4%	11%	5%	25%	20%	7%	14%	11%	2%	26,597