Original Article

Lack of B-RAF Mutations in Head and Neck Squamous Cell Carcinoma

(B-RAF / mutation / head and neck squamous cell carcinoma / single-strand conformation polymorphism)

M. AL SHEIKH ALI¹, M. GUNDUZ¹,4, E. GUNDUZ³, R. TAMAMURA¹, L. BEDER⁴, S. TOMINAGA², T. ONODA², N. YAMANAKA⁴, R. GRENMAN⁵,6, K. SHIMIZU³, N. NAGAI¹, H. NAGATSUKA¹

Departments of ¹Oral Pathology and Medicine, ²Otolaryngology, Head and Neck Surgery, ³Molecular Genetics, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, Okayama, Japan
⁴Department of Otolaryngology, Head and Neck Surgery, Wakayama Medical University, Wakayama, Japan
Departments of ⁵Otorhinolaryngology, Head and Neck Surgery, and ⁶Medical Biochemistry and Molecular Biology, University of Turku and University Central Hospital, Turku, Finland

Abstract: B-RAF is one of the most commonly mutated oncogenes in human cancer. However, the mutation status of B-RAF has not been established completely in HNSCC. We have analysed the mutation status of the kinase domain of the B-RAF gene (exons 11 and 15) in 91 Japanese HNSCC patients as well as 12 HNSCC cell lines. DNA was extracted and amplified by PCR. Mutations were then analysed by SSCP mutation detection method. Since V600E B-RAF constitutes 90 % of the mutations identified in B-RAF in human cancers, we also used MASA analysis to specifically detect this mutation in exon 15 of B-RAF. Using both methods, no mutation was found in both exon 11 and 15 in all patients and cell lines. Mutations are absent or rare in the kinase domain of B-RAF in Japanese HNSCC. However, more studies are still needed to determine its usefulness as a target for molecular therapy in these patients.

Introduction

Head and neck cancer is the sixth most frequently occurring cancer worldwide, with an incidence of 500,000 cases annually (Shah and Lydiatt, 1995). More than 90 % of diagnosed head and neck cancers are of the squamous cell histology. Despite improvements in surgical techniques and treatment regimens including combination of radiotherapy with chemotherapy, three-year overall survival in patients with locally advanced head and neck squamous cell carcinomas (HNSCC) is only around 30 % (Adelstein et al., 2003). Nowadays, developing new strategies for treating progressive HNSCC is an important field of research. This can be achieved by identifying new molecular targets which can be used as the Achilles heel in cancer therapy.

The EGFR-RAS-RAF-MEK-ERK pathway is an important pathway for cell proliferation, migration and survival. Aberrant activation of this pathway has been implicated in cancer development and progression. Overexpression of EGFR was also found in the majority of head and neck tumours (Al Sheikh Ali et al., 2008). In this pathway B-RAF, a serine threonine-kinase, plays an important role in the signal transduction downstream of the membrane-bound RAS protein, which is activated by EGFR. Three isoforms of the RAF protein have been identified in humans as named A, B, and C (Marais and Marshal, 1996). Among these, the B and C isoforms showed mutation in cancer, with mutations in the B-RAF being the majority (Davies et al., 2002; Emuss et al., 2005; Zebisch et al., 2006).

Mutations of the B-RAF have been identified in different types of cancers, particularly in malignant melanoma (27–70 %), papillary thyroid cancer (36–53 %), colorectal cancer (5–22 %) and ovarian cancer (around 30 %) (Garnett and Marais, 2004). All of the mutations of B-RAF have been found in exons 11 and 15, which
encode the kinase domain of this protein. A single nucleotide substitution V600E (also referred to as V599E according to the numbering system used) in the kinase domain accounts for about 90% of the mutations found in B-RAF. These mutations lead to constitutive activation of B-RAF, regardless of activation by RAS, which in turn leads to constitutive activation of MEK and ERK, leading to proliferation and survival of the cancer cells. Since the mutation status of B-RAF in HNSCC has not been established completely, we have performed mutation analysis of the B-RAF gene in 91 Japanese HNSCC patients as well as 12 HNSCC cancer cell lines using the SSCP mutation detection method. Another method, MASA-PCR (mutant allele specific amplification-polymerase chain reaction), which specifically detects the V600E substitution, was also used in the same study cohort.

**Material and Methods**

**Patients and Samples**

Archival tumour samples were randomly collected from 91 patients diagnosed and treated for HNSCC, at Okayama University Hospital, between 1994–2003. According to the site of the primary tumour, 50 samples were from the oral cavity, 20 were laryngeal, 11 were hypopharyngeal and 10 were oropharyngeal tumours. Informed consent was obtained from each patient. All tissues were frozen in liquid nitrogen immediately after surgery and stored at −80°C until the extraction of DNA. Histopathological examinations were also performed at the Department of Pathology, and all tumours were confirmed as squamous cell carcinoma. Also, it has been confirmed that the tumour cell ratio in the samples is greater than 70%. Genomic DNAs were isolated from frozen tissues by SDS/proteinase K treatment, phenol-chloroform extraction, and ethanol precipitation as described previously (Gunduz et al., 2005; 2008). The ethical committee of the institution approved the study.

**Cell Lines**

Twelve HNSCC cell lines were also analysed for mutations of B-RAF. The four human oral cancer cell lines, HSC-2, HSC-3, HSC-4, Ca9-22 were obtained from the Cell Resource Center for the Biomedical Research Institute of Development, Aging and Cancer, Tohoku University. The remaining eight cell lines, UT-SCC-12A, UT-SCC-12B, UT-SCC-16A, UT-SCC-16B, UT-SCC-60A, UT-SCC-60B, UT-SCC-24A and UT-SCC-110B, were provided by Dr. Reidar Grenman, University of Turku, Finland. The first six cell lines were paired and derived from the primary and metastatic tumours of the same patients. All cell lines were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) (Gibco Invitrogen Co. Ltd., Tokyo, Japan) supplemented with 10% foetal bovine serum (FBS) (Gibco Invitrogen), 100 units/ml penicillin, 100 µg/ml streptomycin (Invitrogen Ltd., Tokyo, Japan), and 2.5 mg/l amphotericin B (Gibco Invitrogen) in a CO₂ incubator (Sanyo Electric Co., Ltd., Osaka, Japan) with an atmosphere of 95% air plus 5% CO₂ at 37°C. DNA was extracted for mutation analysis using the same method mentioned above.

**SSCP Mutation Analysis of B-RAF**

PCR was used to amplify exon 11 and 15 of B-RAF using two pairs of intron-flanking primers. For exon 11 the sequence of the forward primer was: 5’-TCC TCA GGC ATA AAG TAA-3’, the reverse primer: 5’- CGA ACA GTG AAT ATT TCC TTT GAT-3’. For exon 15 the forward primer was: 5’-TCA TAA TGC TGT CTC TGA TAG GA-3’ and the reverse primer: 5’-GGC CAA AAA TTT AAT CAG TGG A-3’. The PCR reaction was carried out in 20 µl of reaction mixture with 20 pmol of each primer, 200 ng of genomic DNA, 1x PCR buffer, 200 µM of each deoxynucleotide triphosphate, and 0.5 unit of Taq DNA polymerase (Takara, Kyoto, Japan). Initial denaturation at 94°C for 3 min was followed by amplification for 35 cycles at 94°C for 30 s, annealing for 30 s (the annealing temperatures were 54°C for exon 11 and 52°C for exon 15), and an extension step at 72°C for 1 min. A final extension step at 72°C for 7 min was also added. To check the efficiency of amplification, all PCR products were run on 1.5% agarose gel (BIO-RAD, Hercules, CA).

One µl of each PCR product was then mixed with 8 µl of denaturing buffer (40 mM NaOH, 0.8 mM EDTA), heated for 5 min at 96°C, and immediately chilled on ice. The samples were then loaded onto 8% non-denaturating polyacrylamide gel, and electrophoresed in powerPac 3000® system (BIO-RAD), with constant temperature of 13°C. Silver staining of the gel was performed as described previously (Gunduz et al., 2005) and band patterns were examined. The experiment was repeated twice to confirm reproducibility of the results obtained in the first round.

**DNA Sequencing**

As a confirmation of the results obtained in SSCP mutation analysis method, 20 randomly selected bands in the same and different locations of the SSCP gel were sequenced. The bands were cut from the SSCP gel and re-amplified by PCR under the same as previous conditions. The PCR products were then run on 1.5% agarose gel (BIO-RAD) and the DNA was purified from the agarose gel using GENECLEAN® kit (Qbiogene, Carlsbad, CA). One µl of purified DNA was then re-amplified with BigDye® terminator sequencing kit (Applied Biosystems, Foster City, CA), ethanol precipitated and directly sequenced in an automated sequence machine ABI prism 3100® (AppliedBiosystems).

**MASA-PCR**

MASA-PCR was conducted as previously described (Hasegawa et al., 1995). A fragment derived from exon 15 which encodes codon 600 was amplified by PCR two times using different primers each time. One of the
primers, 5'-TAG GTG ATT TTG GTC TAG CTA CAG T-3', flanking the sequence of exon 15, was used to amplify the wild-type as well as the mutant allele; this primer was used as a positive control. The other primer 5'-GGT GAT TTT GGT CTA GCT ACA AA-3' with substitution of two bases at the 5' end, allows amplification of the mutant allele only in patients who have mutations. For both primers, the reverse primer was 5’-GGC CAA AAA TTT AAT CAG TGG A-3’. Genomic DNA from a papillary thyroid carcinoma patient already known to have V600E B-RAF mutation was used as a positive control. PCR was carried out under the same previous conditions except that the annealing temperature was 52 °C. When the mismatch primer was used, the PCR was repeated under the same conditions but with a higher cycle number (40 cycles) for all the samples.

Results and Discussion

The EGFR-RAS-RAF-MEK-ERK signalling pathway has gained a lot of interest recently. Aberrant signalling through this pathway due to genetic alterations and mutations can drive the cells into an uncontrolled growth state, leading to cancer. Ligand binding to EGFR leads to activation of the membrane-bound RAS, which in turn recruits the cytosolic RAF to the plasma membrane, leading to its activation. Activation of RAF by RAS occurs by phosphorylation of critical amino acids in the kinase domain of RAF. On the other hand, constitutive activation of B-RAF by mutations in the kinase domain has also been reported. We have performed mutation analysis in the hot spot region of the kinase domain of B-RAF encoded by exons 11 and 15. Both exons have been successfully amplified by PCR with expected sizes of amplification 182 bp for exon 11 and 243 bp for exon 15. In the mutation analysis using SSCP gel electrophoresis, no aberrant band was observed, and all the samples revealed the same band pattern in both exon 11 and 15, indicating absence of mutations in these exons in the samples and cell lines analysed (Fig. 1). To confirm these results, direct sequencing was performed in 20 randomly selected bands, and all sequences revealed the same sequence as the wild-type allele.

Since V600E B-RAF constitutes about 90% of the mutations identified in B-RAF so far, we have also used MASA/PCR mutation analysis to specifically analyse this mutation in the patient samples and cell lines. The specificity of this method in detecting mutations of B-RAF has been tested previously (Xu et al., 2003). Using MASA-PCR, the mismatched primer failed to amplify exon 15, whereas all the samples were successfully amplified using the primer with the wild-type sequence, indicating absence of V600E B-RAF in all the samples and cell lines that we have used (Fig. 2). This result is compatible with the result obtained by the SSCP mutation detection method.

There are several reports indicating that B-RAF can act as a human oncogene. RAF kinase inhibitor (RKIP) was identified as a metastatic suppressor in prostate cancer (Fu et al., 2003). Also, it has been found that mutated B-RAF genes constitute a powerful drive for proliferation of cancer cells. In melanocytes, mutated B-RAF leads to transformation and these transformed cells induce tumour formation if implanted in nude mice (Wellingbrock et al., 2004).

Mutations of the B-RAF gene have been detected in different types of cancers (Garnett and Marais, 2004). These mutations lead to substitution of critical amino acid residues in the kinase domain of B-RAF, mimicking the phosphorylation state (the active state), which leads to constitutive activation of B-RAF regardless of EGFR or RAS activation. Most of the mutations of the RAF gene have been detected in the B isoform. This isoform can be activated by a single amino acid substitution in the kinase domain. On the other hand, C-RAF...
and A-RAF require two mutations for oncogenic activation, and since this can be a rare event, mutations are rare in C-RAF and A-RAF (Emuss et al., 2005; Zeibisch et al., 2006).

Referring to the literature, mutations in the kinase domain of B-RAF have been analysed in two studies in HNSCC. Both studies were in western patients. Davies et al. analysed 19 primary HNSCC using capillary-based modified heteroduplex mutation detection method in exons 11 and 15. No mutations were detected in any of the analysed samples (Davies et al., 2002). However, this was a wide study for detection of B-RAF mutations in a wide panel of cancer types and cell lines, and the sample size used for HNSCC was comparatively low for a mutation analysis. Weber et al. sequenced exons 11 and 15 in 89 patients with HNSCC and found three mutations in B-RAF (3%); two of these were substitutions in exon 15 V600E, leading to conversion of valine 600 to glutamic acid, the other was a substitution mutation in exon 11 and resulted in substitution of glycine into alanine in codon 469 (Weber et al., 2003). Considering these two studies together with our study, B-RAF mutations were analysed in 199 HNSCC patients so far and mutations were found only in three of them (1.5%), indicating that mutations are rare events in head and neck cancers.

Recently, molecular targeted therapy of EGFR and other related signalling molecules such as K-RAS and B-RAF has gained a lot of interest in cancer research. A considerable success has been achieved in inhibiting EGFR in HNSCC by using tyrosine kinase inhibitors TKIs (gefitinib, erlotinib) and monoclonal antibodies (cetuximab) (Cohen et al., 2003; Bauman et al., 2007). However, the majority of these patients still don’t show a response to these drugs (Kirby et al., 2006). This might be due to a constitutive active signal in the downstream signalling molecules, like RAS and RAF. However, we didn’t find any mutation in the B-RAF gene in this study, which implies that another mechanism might be responsible for this activation. Turning to RAS, most of the studies revealed that mutations of RAS genes were either very few or lacking in HNSCC (Yarbrough et al., 1994; Kiaris et al., 1995; Ruiz-Godoy et al., 2006). We have also shown that mutations of the K-RAS gene were lacking in HNSCC (Al Sheikh Ali et al., 2008). It’s worth noting that B-RAF mutations occur in the same cancers in which RAS is mutated, such as malignant melanoma, colorectal cancer, and border-line ovarian cancers (Davies et al., 2002).

In one study it was found that RAS-RAF pathway activation in gliomas is achieved much more frequently by gene copy number gains of RAS, RAF or the upstream growth factor receptor and its ligands, rather than by activating RAS/RAF mutations (Jeukens et al., 2007). It is worth noting that similar genetic aberrations, such as gene amplification of EGF and its receptor EGFR, occur in HNSCC (Chung et al., 2006). It would be interesting to study whether activation of the EGFR-RAS-RAF pathway is achieved by gene amplification of RAF and RAS as proposed for glioblastoma. Although mutations were lacking in B-RAF, targeting B-RAF might still be of clinical value by indirectly blocking the oncogenic signal initiated by EGFR or RAS genes upward in the pathway. Moreover, oncogenic activation of B-RAF might occur by mechanisms other than mutation. Recently, sorafenib, an oral inhibitor of the serine-threonine kinases C-RAF and B-RAF, showed a modest anti-cancer activity in HNSCC patients in a phase clinical trial (Elser et al., 2007). In this trial, one patient has shown a partial response and 10 patients have shown disease stabilization, out of 26 patients evaluated. These data are encouraging more studies of B-RAF genetic alterations in HNSCC.

In conclusion, B-RAF mutations are rare events in HNSCC. However, other studies are still needed to determine the role of genetic and epigenetic alterations in the activation of this oncogene in HNSCC.

References


