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)

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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.

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Abbreviations: HNSCC – head and neck squamous cell carcinoma, MASA – mutant allele-specific PCR amplification, SSCP – single-strand conformation polymorphism.

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 by haematoxylin-eosin staining during initial diagnosis 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 CO_2 incubator (Sanyo Electric Co., Ltd., Osaka, Japan) with an atmosphere of 95% air plus 5% CO_2 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 CTC TCA GGC ATA AGG 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 TTG 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 ºC for 3 min was followed by amplification for 35 cycles at 94 ºC for 30 s, annealing for 30 s (the annealing temperatures were 54 ºC for exon 11 and 52 ºC for exon 15), and an extension step at 72 ºC for 1 min. A final extension step at 72 ºC 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% nondenaturing polyacrylamide gel, and electrophoresed in power-Pac 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 ºC. 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 (Wellbrock 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*

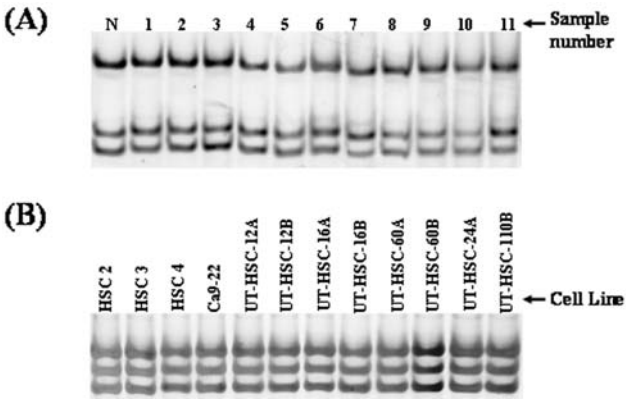

Fig. 1. (A) SSCP gel of exon 15 of *B-RAF*, the same band pattern in both the control sample (N) and the all tumour samples (1-11). (B) SCCP gel of exon 11 of *B-RAF* showing the same band pattern in 12 HNSCC cell lines

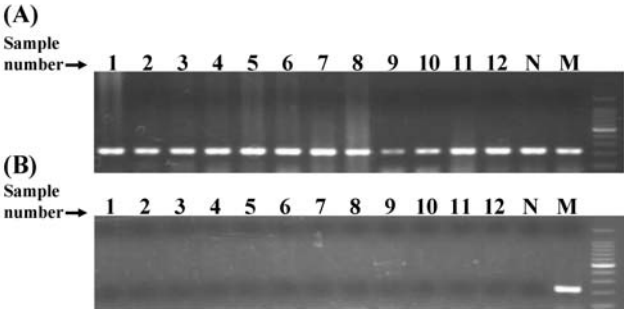

Fig. 2. (A) MASA analysis of *B-RAF*. A fragment of exon 15 of *B-RAF* spanning codon 600 was amplified using primers with the wild-type sequence; this set of primers was able to amplify genomic DNA from HNSCC tumour tissues (1-12), a normal control (N) and a papillary thyroid cancer patient with ^{V600E}*B-RAF* mutation (M). (B) PCR using a mismatch primer that allows amplification of the mutant allele only. A band could be seen only in genomic DNA from a papillary thyroid cancer patient already known to have ^{V600E}*B-RAF* mutation (M). Absence of bands from genomic DNAs of HNSCC patients (1–12) and the normal control (N) indicates evidence of ^{V600E}*B-RAF* mutation

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; Zebisch 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; Ruíz-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 (Jeuken 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 anticancer 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.

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