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p53 Mutation and Multiple Primary Oral Squamous Cell Carcinomas

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1. Introduction

Oral squamous cell carcinoma (OSCC) is a worldwide malignancy and is ranked the sixth most common cancer. At current rates, approximately 45,000 cases in the United States and more than 650,000 cases worldwide will be diagnosed each year (Jemel et al., 2008). One promising strategy for the treatment of OSCC and other cancers, which has developed as a result of breakthroughs in the fields of molecular biology, cancer genetics, and cancer biology, is molecular targeted therapy. Patients with a head-and-neck squamous cell carcinoma (HNSCC) often develop multiple malignant lesions. The oral sites that give rise to the majority of HNSCCs undergo cornification and shed squames during terminal differentiation, a process that is impaired in malignancies. The lymph nodes of the head and neck region form the principle site of primary metastasis, and perineural invasion marks tumours with a poor prognosis. Genetic changes correlate with lymph node metastasis in SCC. It is frequently observed that genetic damage persists beyond the histological border of precancerous lesions and tumours often develop far from the precancerous site (Braakhuis et al., 2003).

The p53 gene is the most frequent target of genetic alterations being, mutated in half of human cancers (Nylander et al., 2000, Iwakuma et al., 2005). Loss of p53 function leads to enhanced accumulation of mutations in other genes and reduced apoptotic responses, processes important to cancer progression and response to treatments. Using laser capture

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microdissection of tumour cells, p53 mutations were found in 100% of SCC (Agar et al., 2004). The p53 gene is mutated in more than 70% of oral SCC (Kashiwazaki et al., 1997, Hassan et al., 2008). In this article, we demonstrate that p53 statuses were diverse in the oral SCCs and leukoplakias, which indicated independent origins of the tumors in the multiple cancers. It is necessary to individual follow-up to each precancerous lesions based on the molecular alteration by the molecular analysis. However, the development of targeted approaches to OSCC requires understanding of the molecular pathogenesis of the disease, as well as further characterization of the specific molecular events involved in multiple cancers. The first part of this chapter will discuss on the molecular mechanisms of multiple carcinoma and the current status of targeted therapies directed toward critical molecular alterations in OSCC and the second part will evaluate a critical analysis of the various technological aspects of p53 analysis and the third part will present a case report with multiple carcinoma and molecular analysis of p53.

2. Molecular pathogenesis of OSCC

OSCC arises as a result of multiple molecular events that develop from the combined influences of an individual's genetic predisposition and exposure to environmental carcinogens (Califano et al., 1996). Chronic exposure to carcinogens such as tobacco, alcohol, oncogenic viruses, and inflammation can damage individual genes as well as larger portions of the genetic material, including chromosomes. Accumulation of such genetic alterations can lead to the development of premalignant lesions and subsequent invasive carcinoma. These genetic alterations include mutations, amplification or translocation of oncogenes that promote cell survival and proliferation, as well as inactivation of tumor suppressor genes involved in the inhibition of cell proliferation. From these alterations of oncogenes and tumor suppressor genes, tumor cells acquire autonomous self-sufficient growth and evade growth-inhibitory signals, resulting in uncontrolled tumor growth. Tumor cells thereby escape programmed cell death and replicate infinitely through the immortalization process by telomere lengthening. As OSCCs grow, invade, and metastasize, new blood vessel formation is critical. OSCCs, like most tumors, are able to create a blood supply by stimulating endothelial cell proliferation and new blood vessel formation. During oral carcinogenesis, there is selective disruption of this process, such that pro-angiogenic factors predominate (Hanahan & Weinberg, 2000). This angiogenesis is an essential part of solid tumor formation. The subsequent progression of OSCC includes tissue invasion and metastasis. Invasion of adjacent normal tissue requires that cellular adhesion molecules, such as integrin and cadherins, are lost, to allow cancer cells to leave their primary site. OSCCs develop through a complex process, as mentioned above. Here, we discuss those processes involving genetic alteration during multistep carcinogenesis, growth regulation, apoptosis, immortalization, angiogenesis, invasion, and metastasis.

2.1 Genetic alterations during development of OSCC

Califano, Sidransky, and colleagues have developed a genetic progression model based on their studies of gene alterations in squamous cell carcinomas of the head and neck (SCCHN) (Sidransky, 1995; Califano et al., 1996). They found that the most common genetic alteration in SCCHN is loss of chromosomal region 9p21, which occurs in 70–80% of dysplastic lesions of the oral mucosa, suggesting that this loss is an early event in oral carcinogenesis (van der Riet et al., 1994; Califano et al., 1996; Mao et al., 1996a). This region of chromosome 9p21, known as

the CDKN2A locus, encodes the tumor suppressors p16 and $p14^{ARF}$, which frequently are inactivated by promoter hypermethylation (Reed et al., 1996). Loss of the chromosome 3p region is another common early genetic alteration in oral carcinogenesis (Garnis et al., 2003; Masayesva et al., 2004). The chromosome 3p region includes FHIT (fragile histidine triad gene) and RSSFIA, tumor suppressor genes inactivated by exonic deletion and hypermethylation (Mao et al., 1996a; Kisielewski et al., 1998; Dong et al., 2003). Loss of heterozygosity (LOH) of chromosome region 17p and mutation of the p53 gene are genetic alterations that occur in the later stage of progression from dysplasia to invasive squamous carcinoma. Alterations of p53, including mutation or deletion, are associated with increased genomic instability in oral dysplasia and may accelerate the rate of genetic alterations in oral carcinogenesis. Amplification of 11q13 and overexpression of cyclin D1 have been described in 40% of cases of oral squamous dysplasia (Rousseau et al., 2001). In general, loss of chromosomal material at 9p, 3p, and 17p is observed in relatively high proportions of dysplastic lesions, indicating that those events are early markers of oral carcinogenesis, whereas losses at 13q and 8p are observed more frequently in carcinomas than in dysplasia and are associated with later stages of carcinogenesis (Califano et al., 1996).

2.2 Multiple carcinomas

Individuals with one carcinoma of the head and neck region have an increased risk of developing a second malignancy (Schwartz et al., 1994); the frequency of that event varies from 16% to 36%. When a second malignancy occurs at the same time as the initial lesion, it is called a synchronous carcinoma. Metachronous neoplasm, on the other hand, is additional primary surface epithelial malignancies that develop in a later time period than the original tumor. About 40% of second malignancies of the upper aero digestive tract arise simultaneously and represent a synchronous tumor. The remaining multiple cancers in this population represent metachronous disease and usually develop within 3 years of the initial tumor (Schwartz et al., 1994). Second primary tumors are the chief cause of death in patients with an early -stage diagnosis (Hong et al., 1990). The tendency to develop multiple carcinomas in the upper aero digestive region is known as "field cancerization" (Slaughter et al., 1953). Prolonged and diffuse exposure to local carcinogens, particularly tobacco combined with alcohol, appears to increase the malignant transformation potential of exposed epithelial cells in the upper aero digestive tract and lungs (Franco et al., 1991). The overall risk for developing a second head and neck malignancy is 10 to 30 times higher in populations that use tobacco and alcohol than in the general population (Fijuth et al., 1992).

2.3 Field cancerization

Since the epithelial layer of the upper aero digestive tract is exposed to carcinogenic insult, such as tobacco products and alcohol, the entire area is at increased risk for the development of malignant lesions from the accumulation of genetic alterations of oncogenes and tumor suppressor genes. This led Slaughter and colleagues to develop a theory of "field cancerization", based on their extensive histological examination of dysplastic epithelium adjacent to invasive oral cancers; this dysplasia accounts for the relatively high incidence of second primary tumors in patients treated for OSCC (Slaughter et al., 1953). Many of these second primary tumors are associated with a lower rate of survival than the original tumor (Day et al., 1994; Cianfriglia et al., 1999). In this field cancerization model, multiple oral cancers develop from separate, independent cell clones, and this hypothesis has been

supported by data from chromosome X inactivation studies, microsatellite analysis, and p53 mutational analysis (Bedi et al., 1996; Lydiatt et al., 1998; Tabor et al., 2002). More recent genetic analyses have shown, however, that second or multiple cancers distant from the abnormal fields can be clonally related and derived from expansion of an original clone (Braakhuis et al., 2003).

To reconcile this finding, Braakhuis *et al.* proposed a progression model, in which a stem cell located in the basal cell layer of the epithelium acquires a genetic alteration and subsequently give rise to a clonal unit, consisting of the stem cell with its daughter cells, all of which share the DNA alteration. Next, this patch of cells progresses into an expanding field as a result of additional genetic alterations (Braakhuis et al., 2004). This mucosal field pushes the normal epithelium aside and can expand to a size of several centimeters. These fields are often macroscopically undetectable, but they can also appear as oral lesions such as leukoplakia or erythroplakia. Ultimately, clonal selection leads to the development of carcinoma within this field of preneoplastic cells. The mechanism of this clonal expansion may be intra-epithelial migration of transformed cells or inoculation through saliva.

From these data, it has become clearer that the multifocality of oral carcinogenesis is an important cause of treatment failure in oral cancer. Although primary excision can completely remove an oral carcinoma, the altered field may remain, and the patient can develop a second primary tumor nearby, in the same field, that may be clinically indistinguishable from a local recurrence. Understanding of this field cancerization concept led Hong, Lippman, and other investigators to develop a strategy called 'chemoprevention', in which systemic therapy is administered with the intent of preventing epithelium from the entire upper aero digestive tract from progressing along the multistep pathway of carcinogenesis (Lippman et al., 2005).

2.4 Tumor suppressor genes

Tumor suppressor genes encode proteins that typically transduce negative growth-regulatory signals (Weinberg, 1991). These genes are often involved in cell-cycle regulation, including cell-cycle arrest and apoptosis. Unlike oncogenes, which can be activated by mutation of only one of the two gene copies, tumor suppressor genes are inactivated by any of several mechanisms, including point mutations and/or deletion, in both alleles of the gene, in a "two hit" fashion (Knudson, 1977; Vogelstein & Kinzler, 1993; Yokota & Sugimura, 1993). Once these genes are inactivated, the cell escapes tight cell-cycle control, predisposing it to uncontrolled growth and division, which contributes to the malignant phenotype (Levine, 1997).

2.4.1 p53

p53 is a tumor suppressor gene, located on chromosome 17p13.1, which plays a role in cell-cycle progression, cellular differentiation, DNA repair, and apoptosis. A major function of p53 is to serve as a guardian of the genome. Endogenous or exogenous stresses, such as DNA damage, hypoxia, and oncogene activation, increase p53 levels, leading to cell-cycle arrest that enables DNA repair to occur (Hartwell & Kastan, 1994). The induction of p53 expression can also occur through oncogenic stimulation that leads to $p14^{ARF}$ activation (Vogelstein et al., 2001) or DNA double-strand breaks that activate the ATM/Chk2-dependent pathway (Levine, 1997). p53 is the most commonly mutated gene and is altered

in over 50% of all cancers, including 25–70% of oral cancers (Levine et al., 1991; Kashiwazaki et al., 1997, Baral et al., 1998). Most TP53 alterations are missense mutations, localized in the DNA-binding domain, and abolish the transcriptional activity via p53-responsive elements. The residues such as R175, G245, R248, R249, R273 and R282 in the p53 protein are frequently mutated, and are therefore called "hot spots (Brachmann et al., 1996).

Immunohistochemical positivity or various mutation analyses of the DNA-binding domain of p53 are known to be useful markers for predicting prognosis of patients with oral SCC (Hassan et al., 2008, Yamazaki et al., 2003, De Vicente et al., Marx et al., 2007). Mutation most often occurs at a 'hot spot' region from codon 238 to codon 248 (Somers et al., 1992; Hainaut et al., 1998; Kropveld et al., 1999) and causes defects in the binding of specific DNA sequences and the transactivation of genes whose expression is up-regulated by the wild-type protein (Vogelstein et al., 2001). Some human tumor-associated p53 mutants possess unique properties not found in the wild-type protein (Sigal & Rotter, 2000). Based on functional interactions with the remaining wild-type (WT) p53 allele, the p53 mutations are classified into two types, recessive and dominant-negative (DN) mutations. The most frequent p53 mutations are dominant negative (DN). Such "gain of function" activities include the ability to transform cells, increase tumorigenicity, and modulate the sensitivity of cancer cells to drugs (Sigal & Rotter, 2000; Song & Xu, 2007). We have found that oral SCC patients with DNp53 mutations have a significantly worse outcome than patients with recessive mutations, in terms of recurrence free survival [Hassan et al., 2008]. This has also been noted in other cancers (Marutani et al., 1999; Sakuragi et al., 2005). The over-representation of DN mutants, accounting for about 27% of all p53 mutations (6414 of all 23,544 in the IARC database R11), suggests an advantage for tumour development of the cancer cell harbouring a DN mutation. But why does this occur? The mechanism responsible for the poor clinical outcome in DNp53 mutants is unknown at present. One explanation is that the DNp53 allele suppresses the remaining wild-type p53 allele, promoting tumour progression. Another explanation is that DNp53 mutant proteins often possess a gain-of-function (GOF) related to malignancy, other than transdominance over WT p53 function. Indeed, some DN p53 mutants are known to bind to other transcription factors and to transactivate or repress specific target genes, such as MYC (Frazier et al., 1998), MDR-1 [Sampath et al., 2001], CD95 (Fas/APO-1) (Zalcenstein et al., 2003) and EGR1 (Weisz et al., 2004). It is important to understand malignant properties acquired by GOF activity in each mutation to enable better use of genetic information for diagnosis and therapy. Interestingly, we recently showed that different p53 mutants have a different quality in gain-of-function (GOF) activities even for different mutations occurring at the same codon (Yoshikawa et al., 2010).

Even in the absence of p53 mutations, p53 function can be inactivated by other mechanisms, such as infection with an "oncogenic" human papillomavirus type, such as HPV16 or HPV18. In HPV-positive SCCHN, p53 interacts with the E6 protein, which leads to increased ubiquitin-dependent proteolysis of p53 (Min et al., 1994; Nagpal et al., 2002). Another mechanism of p53 inactivation is elevation of expression of the MDM2 protein, which binds to p53 and promotes ubiquitination of the C-terminus of p53 and subsequent degradation (Oliner et al., 1993). $p14^{ARF}$ interacts with MDM2, preventing association of p53 and MDM2 and thereby stabilizing p53 (Pomerantz et al., 1998). Therefore, degradation of p53 may be inappropriately stimulated by over expression of MDM2 or by deletion or epigenetic silencing of $p14^{ARF}$.

p53 mutations commonly arise as a result of alcohol and/or tobacco exposure, and their presence is associated with the early recurrence and development of second primary tumors (Shin et al., 1996). Wild-type p53 gene therapy has been attempted in preclinical studies and in clinical trials in heavily treated patients. These studies demonstrated the feasibility of delivering the wild-type p53 gene to human tumors and yielded some clinical response and induction of apoptosis in the tumors (Clayman et al., 1998, 1999). However, difficulty in obtaining uniform delivery of the gene throughout the tumor has limited the utility of this therapeutic strategy. Other reports have demonstrated associations between p53 mutation and unfavorable responses to chemotherapy or radiation therapy (Temam et al., 2000; Warnakulasuriya et al., 2000).

Inactivation of the p53 gene is essentially due to small mutations (missense and nonsense mutations or insertions/deletions of several nucleotides), which lead to either expression of a mutant protein (90% of cases) or absence of protein (10% of cases). No inactivation of p53 gene expression by hypermethylation of transcription promoters has been demonstrated at the present time, which supports the hypothesis of a function for p53 mutants. We can analyse p53 gene alteration in cancer in different way.

3. Analyse p53 gene alterations in cancers

3.1 Molecular analysis

Direct sequencing of the p53 gene after PCR amplification remains the "Gold Standard" of molecular analysis. For the p53 gene, this approach is facilitated by the fact that the 10 coding exons are smaller than 350 bp and can therefore be easily amplified individually. Mutations involving partial or total gene deletions are relatively rare. Unfortunately, although considerable progress has been made in the field of DNA sequencing in terms of throughput, its sensitivity still remains limited. The major problem of molecular analysis of tumour specimens is the presence of normal cells (lymphocytes, stromal cells) that contaminate the tumour samples. According to the type of tumour or the type of sample, the rate of contamination can range from several percent (surgical tumour sample) to 50% (biopsies) or even more than 95% (urine, stools or bronchial lavage). It is generally accepted that direct sequencing requires at least 20% of mutant alleles, but this can vary considerably according to the quality of the sample. This qualitative aspect is generally underestimated. The quantity and quality of DNA obtained varies considerably according to the origin of the sample (frozen tumour, formalin- or paraffin-embedded tissues). This variability can lead to the generation of PCR artifacts, which can be falsely interpreted as mutations. In the case of heavily contaminated samples, microdissection can be performed in order to enrich the tumour cell content, but this complicates the manipulations and cannot be performed routinely at the present time. The application of molecular technologies to routine analysis in hospital is a very important aspect. Many extremely sensitive molecular analysis methodologies have been developed, but their clinical application is generally limited because of the complex installation, their low throughput, the use of radioactivity or the need for highly qualified personnel.

Up until now, molecular analyses have been performed on exons 5-8 of the p53 gene, as the majority of mutations are located in these regions. It is generally established that 90% of mutational events are missense mutations leading to the synthesis of an abnormal protein

that is not degraded and which accumulates in the nucleus of tumour cells. The remaining 10% of mutational events are nonsense mutations or small deletions that do not lead to accumulation of p53. This type of mutation excludes the possibility of using molecular methodologies such as PTT (Protein truncature test) based on expression of truncated proteins. More recently, molecular studies have been extended to the other exons, as exons 4, 9 and 10 have been found to contain a considerable number of mutations (about 15%) (Soussi & Béroud, 2001). Analysis of molecular events also shows a high proportion of nonsense mutations in these exons. Analysis of the latest version of the p53 gene mutation database shows that about 20% to 25% of mutations do not lead to the synthesis of a p53 protein. These mutations also present a marked variability as a function of the type of cancer: they are more frequent in lung cancers and breast cancers than in colon cancers (Soussi & Béroud, 2001).

About 280 of the 393 codons of the p53 gene can be affected by a mutation. Furthermore, as each codon comprises 3 bases, which can each be altered generating a different amino acid, there are a very large number of theoretical combinations. 1,300 different variants have been identified in the p53 mutation database, which comprises more than 15,000 mutations derived from as many tumours (Béroud et al., 2000).

Many prescreening methodologies have been used to increase the sensitivity of detection of mutations and to concentrate the sequencing exclusively on the mutant exon. Unfortunately, many of these methods, possibly with the exception of DHPLC (denaturing high-performance liquid chromatography), remain confined to specialized laboratories and the sensitivity of detection of some of them is incompatible with the needs of clinical diagnosis. However, they present the advantage of being able to detect mutations in samples heavily contaminated by normal DNA.

3.2 Immunohistochemical analysis

Immunohistochemical studies concerning p53, as for other markers, suffer from a lack of standardization, leading to very heterogeneous results. The sources of heterogeneity are multiple: i) the various antibodies used; ii) methodological aspects (amplification, epitope unmasking); iii) the initial material (paraffin block, frozen tumour) and storage conditions; iv) the positive cut-off value, which can vary from 1% to 20% according to the authors; and v) individual variability of interpretation of the results (McShane et al., 2000; Schmitz-Drager et al., 2000).

Nonsense or frame shift mutations do not lead to accumulation of p53 protein. This is certainly due to instability of truncated proteins, which are generally not detectable despite the use of monoclonal antibodies which recognize an epitope situated in the amino-terminal domain of p53. It is beyond the scope of this chapter to present an exhaustive review of the literature concerning immunohistochemical analysis of p53 and its clinical applications (Hall & Lane, 1994; Save et al., 1998).

3.3 Serological analysis of p53 gene alterations

Since 1992, a new series of studies has shown that p53-Abs can be found in the serum of patients with various types of cancer, whereas the prevalence of these antibodies in the

normal population remains very low. To date, the majority of published studies suggest that most patients with p53 antibodies have a p53 mutation leading to p53 accumulation. It is also clear that not all patients with a p53 alteration develop p53 antibodies. Comparison of the frequency of p53 alterations in the literature indicates that 30% to 40% of patients with an alteration of the p53 gene develop p53 antibodies (Lubin et al., 1995a).

The majority of the literature clearly demonstrates the specificity of this serological analysis, as such antibodies are very rare in the normal population. The specificity of this assay can be estimated to be 95%. This high specificity is supported by the fact that p53 specifically accumulates in the nucleus of tumour cells after gene mutation. One of the disadvantages of this assay is its lack of sensitivity, as only 20% to 40% of patients with p53 mutations develop p53-Abs. This lack of sensitivity totally precludes the use of the assay to evaluate p53 alterations in human tumour. Nevertheless, if we estimate that there are 8 million patients with various types of cancer throughout the world, and 50% of them have a mutation in their p53 gene, then we can deduce that about 1 million of these patients would have p53- Abs.

3.4 FASAY functional assay

FASAY (Functional Assay in Yeast) is used for the detection of mutations in tumour samples (Flaman et al., 1995; Ishioka et al., 1993). Here cDNA obtained from tumour RNA. PCR amplification of this cDNA, using primers corresponding to codons 52to 364 (68% of exons 4 to 10), followed by introduction of the PCR product into an indicator yeast, where it recombines with an expression vector, can be used to define the transactivating activity of the protein expressed. Red yeast colonies express mutant p53, while white colonies express wild-type p53 (Fronza et al., 2000). The amplified region corresponds to 95 % of the mutations identified to date, which makes FASAY a very good approach for exhaustive analysis of p53 gene mutations. All alterations leading to absence of RNA expression will obviously not be detected, but this is a relatively rare situation for p53. The only criticism that can be formulated in relation to this methodology is that it provides no information about the type of mutation, so that sequencing must always be performed subsequently. As sequencing is performed on DNA extracted from red colonies (mutant p53), problems of sensitivity are eliminated. This methodology can also be used to demonstrate splicing alterations. Waridel et al. have modified the FASAY technique to increase its sensitivity and robustness (Waridel et al., 1997).

In addition, the DN potential of the detected *p53* mutant was tested using a yeast-based transdominance assay as described previously (Marutani et al., 1999). Briefly, yIG397 was transformed with both a plasmid with wild-type *p53* and a plasmid with the mutant *p53* that had been sequence-verified. For each transformation, 50 µl of yeast suspension were mixed with 100 ng of pTSHP53 (Trp 1 marker), 100 ng of mutant *p53*-containing pSS16 (Leu 2 marker), 50 µg of sonicated single-stranded salmon sperm DNA and 300 µl of LiOAc containing 40% polyethylene glycol 4000. The mixture was incubated at 30 °C for 30 min and heat-shocked at 42 °C for 15 min. Yeast was then plated on SD medium minus leucine and tryptophan, but with a limited amount of adenine (5µg/ml). The plates were then incubated for 48 hr in a 30 °C-humidified atmosphere. Double-transformant clones (Leu^+, Trp^+) giving rise to white (Ade^+) or pink/red (Ade^-) colonies were interpreted as expressing recessive and DN mutant, respectively.

Recently, we used this approach to detect p53 gene mutations in biopsies containing only 5% of tumour cells (Fouquet et al., 2004). These mutations could not be detected by direct sequencing. In addition to this high level of sensitivity, the FASAY technique also presents the advantage of being simple and robust. FASAY avoids selecting active variants (see below for problems related to this biological activity). It also has the advantage of being the only method able to rigorously demonstrate codon 72 polymorphism linked to the mutant allele (see beginning of the chapter for the significance of this polymorphism) (Tada et al., 2001). The cloning and sequencing of cDNA in the indicator yeast provide a non-fragmented molecule corresponding to the initial RNA expressed by the p53 gene. New indicator yeasts allowing more accurate evaluation of p53 activity have been developed. It remains to be seen whether the use of these yeasts can help to increase the sensitivity of this test.

4. Case report

This study reported on a patient with multiple primary carcinomas, consisting of five separate carcinomas and three leukoplakias of the head and neck region. To determine whether the individual head and neck carcinomas were of multiple origins but genetically related, or whether they were metastases, analysis of p53 mutation by yeast functional assay and subsequent sequencing analysis were performed. The yeast p53 functional assay tests the ability of p53 to activate transcription in vivo in yeast (Flaman et al., 1995). It was demonstrated that the p53 status is diverse in oral SCCs and leukoplakias, suggesting that the tumours in a multiple carcinoma may have independent origins.

A 67-year-old man was referred to the Outpatient Clinic of Hokkaido University Dental Hospital in September 2003. He had an ulcerative lesion at the right (lesion 1) and left (lesion 2) lower gingiva and left side of the tongue (lesion 3) with a continuous dull pain in the most part of the mandibular area. He also had white patches (leukoplakia) in the upper left (lesion 4) and right (lesion 5) gingiva and left side (lesion 6) of the buccal mucosa (Figure 1).

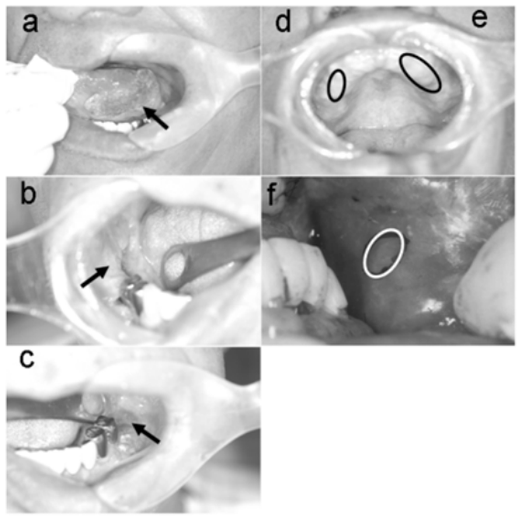


Fig. 1. Preoperative state of the tumors. Arrows indicate cancerous ulcerations; on left side of the tongue (a), right side of the lower gingiva (b) and left side of the lower gingiva (c). Circles indicate leukoplakic lesions in the right (d) and left upper gingiva (e) and left side of buccal mucosa (f).

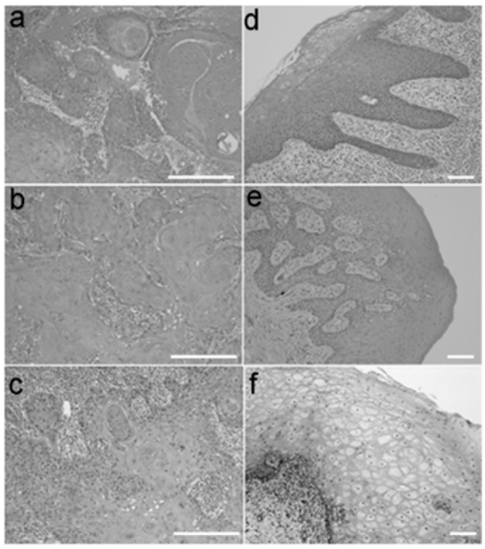


Fig. 2. Histopathology of the biopsy specimens corresponding to the lesions in Figure 1. Well differentiated squamous cell cancers (a, b, c), and leukoplakias (d, e, f) showing hyperkeratosis with low grade dysplasia but no evidence of malignancy (H&E stain, bar indicates 100 micrometer)

He had a long history of cigarette smoking (20 cigarettes a day) and alcohol intaking (beer 500 ml + sake 300 ml a day) for about 48 years. Family history was unremarkable except that his mother had died of gall bladder carcinoma. Physical examination disclosed hard swelling of sub-mandibular lymph nodes at both sides of the neck: one at the right sub-mandibular, two in the right jugulo-digastric area, two in the left submandibular and one in the left jugulo-digastric area. Magnetic resonance imaging (MRI) revealed ill-defined, hypointense masses in the lower right and left gingival regions and in the left side of the tongue. Both lesions in the gingival regions showed invasion of the medial surface of the mandible and extension into the bone marrow (Figure 3).

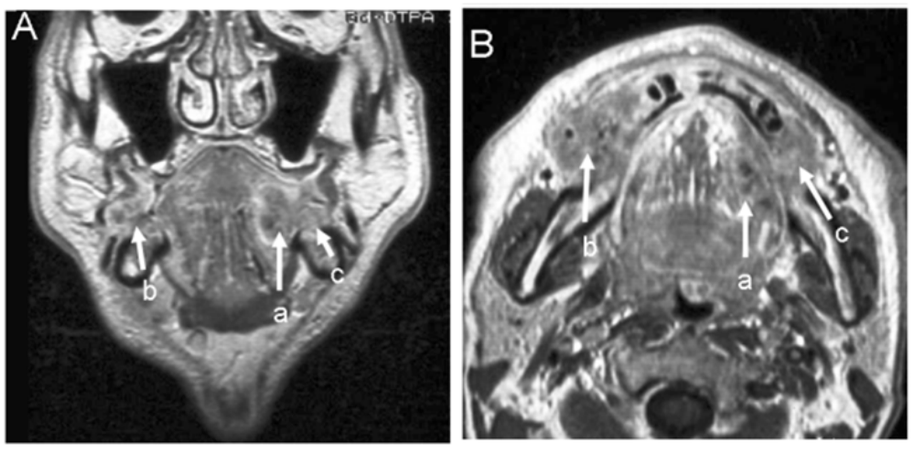


Fig. 3. T1- weighted coronal (**A**) and axial (**B**) magnetic resonance images showing ill-defined, hypo-intense masses surrounded by a Gd-enhanced outer margin in left side of the tongue (a), right molar region (b), and the left molar region (c).

After being admitted to the Department of Oral Maxillofacial Surgery, the patient received 40 Gy preoperative irradiation in 20 sessions over 4 weeks along with chemotherapy consisting of CDDP (6 mg/m2, four times a week) and TXT (15 mg/m2, once a week).

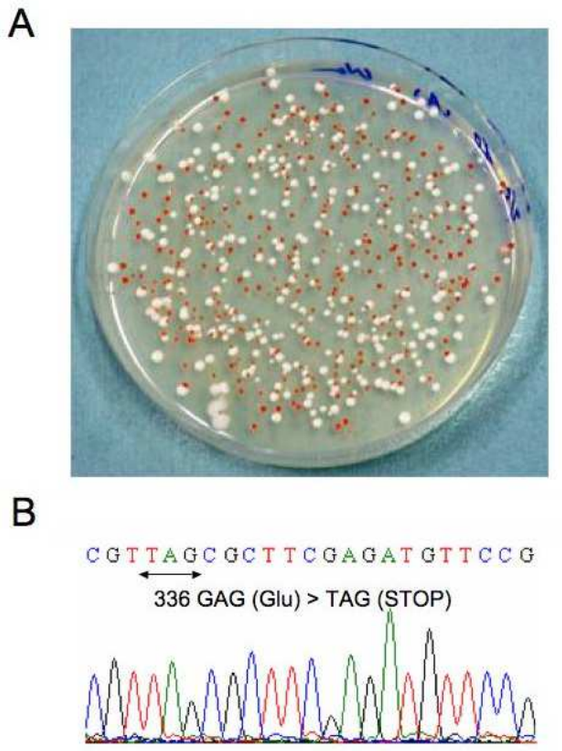


Fig. 4. **A**, A representative result of the yeast p53 functional assay. Colonies containing mutant- type p53 are red due to accumulation of ADE2 substrate, while colonies containing wild type p53 are white. Tumor specimen (left side of the tongue) gave 62.5% red colonies, indicating 62.5% p53 mRNA in the tumor specimens were mutant.
B, Sequence chromogram of the p53 cDNA recovered from a red colony. Wild-type sequence was CGT GAG CGC TTC GAG ATG TTC, in which the underlined glutamic acid changed into a stop codon at the codon 336 (GAG > TAG).

In December 2003, removal of the tumours and radical neck dissection were carried out under tracheostomy and general anaesthesia. Resection of the left half of the tongue and segmental resection of the most part of mandible body were performed. The mandible was reconstructed with titanium plates and the defects of the oral mucosa were repaired with rectus abdominis myocutaneous free flap. After the surgery, the patient was observed without adjuvant therapy. Lesions 1, 2 and 3 were pathologically diagnosed as squamous cell carcinomas. The final TNM staging was pT4N2cM0. The patient was discharged in May 2004. Four months after discharge, he was admitted to a local hospital for terminal care, where he died of sudden severe bleeding due to a locally recurrent invasive tumour. The histopathological study of the surgical specimens confirmed that lesions 1 and 3 were well - differentiated SCC, while lesion 2 was a moderately differentiated SCC. Leukoplakias (lesions 4, 5 and 6) showed hyperkeratosis with a low-grade dysplasia but no evidence of malignancy (Figure 3, Table I).

Lesion ID	Location Size	HPV infection	Histological type	Red colony %	p53 status	Mutated Codon#
a	Tongue 3.9 x1.7cm	Negative	SCC?	62.5	Mutant	336 GAG (E) > TAG (STOP)
b	Rt* lower gingival 3.0 x3.2 cm	Negative	SCC	64.6	Mutant	285 GAG (E) > AAG (K)
c	Lt+ lower gingival 2.0 x2.2 cm	Negative	SCC	4.3	Wild type	
d	Rt upper gingival 2.0 x2.0 cm		Low grade dysplasia	53.4	Mutant	273 CGT (R) > CAT (H)
e	Lt upper gingival 1.0 x1.0 cm		Low grade dysplasia	9.5	Wild type	
f	Rt buccal mucosa 2.0 x2.5 cm		Low grade dysplasia	16.4	Wild type	

*Rt, right side; + Lt, left side; ? SCC, squamous cell carcinoma; # Mutated Codon, codon number, wild type codon (amino acid) > mutant codon (substituted amino acid)

Table 1. p53 mutational status and HPV status.

Specimens of the tumors and leukoplakic lesions were subjected to yeast functional assay. The yeast assay which screens human p53 function in yeast is now described (6). The reporter yeast strain (yIG397) contains an integrated plasmid with the ADE2 open reading frame under the control of a human p53-responsive promoter. When the strain is transformed with a plasmid encoding mutant p53, the yeast strain becomes defective in adenine synthesis due to a mutation in the endogenous ADE2 gene. Therefore, colonies expressing mutant p53 are red, whereas colonies expressing wild-type (WT) p53 become white. In this system, when more than 20% of colonies are red the sample is considered positive for a p53 gene mutation (Kashiwazaki et al., 1997) and plasmids recovered from at least five red colonies are sequenced for verifying the presence of clonal mutation(s). p53 mutation was observed in two of the three SCCs and one of the three leukoplakias. In the SCCs, one missense mutation (lesion 1) at codon 285 (GAG>AAG, Glu>Lys) and one nonsense mutation (lesion 3) at codon 336 were observed. In the leukoplakias, one missense mutation (lesion 5) at codon 273 (CGT>CAT, Arg>His) was observed (Table I). All mutations were identified as clonal because the sequences from the five red colonies were identical. These results showed that two of the three mutations were G to A transition, being consistent with the record in the IARC TP53 database (http; //www.p53.iarc.fr), showing that the G: C to A: T transition is the most prevalent in oral SCCs in smokers (detailed in Discussion).

In addition, to detect human papillomavirus (HPV) infection of SCC samples, genomic DNA extracted from the tumour specimens was tested by multiplex PCR (8). No HPV infection was detected in any form of HPV subtypes (genotypes 6, 11, 16, 18, 30, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 66) (Table I).

SCC is thought to progress through a series of well defined histopathological stages that run parallel to specific genetic changes. Patients with primary malignancy (especially in the head and neck (HN) area) are at high risk for developing additional primary malignancies.

The oral cavity presents a field, which, if exposed to carcinogens may allow multiple precancerous and tumorous lesions to develop synchronously and metachronously. Generally multiple primary tumors are encountered in 3-5% of malignant tumors. They are most often met with secondary malignant tumors; triple tumors occur in 0.5%, quadruple tumors in 0.3% of malignant tumors (Moertel et al., 1958).

The criteria for diagnosing multiple primary tumors are (a) each of the tumors must present a definite picture of malignancy, (b) each must be distinct, (c) the probability that one may be a metastatic lesion from the other one must be excluded and (d) tumors are multicentric when they are formed at the same site and have the same histological type (Moertel et al., 1977). The histological verified cancers (SCC) in the present case were proven distinct by the MRI findings (Figure 3) and macroscopic findings during surgery. It was impossible to exclude the possibility that the tumors might be metastatic or dissemination from one tumor by image diagnosis or pathological findings. The different p53 mutations were found in the tumors (Table 1), which, strongly suggested that the tumors were of different clonal origins. Therefore, the tumors in the present case were defined multiple and multicentric, fulfilling the above criteria (a) to (d).

It is widely accepted that alterations in multiple oncogenes and tumor suppressor genes are the genetic basis for human carcinogenesis (Weinberg et al., 1989). The p53 gene is the most frequent target of genetic alterations, being mutated in half of human cancers. The p53 mutation usually shows clonality in a cancer and therefore suggests that the mutation has occurred in the very beginning stage of carcinogenesis, which is likewise in oral SCC (kashiwazaki et al., 1997). It means that distinct clonal p53 mutation works as a molecular tag that denies the metastatic dissemination of a single-tumor-origin cells (Tjebbes et al., 1999). This was seen in the present case, as the yeast functional assay and sequence analysis showed a distinct but the same (clonal) mutational status in each SCC: 285K in Lesion 1, wild-type p53 in Lesion 2, 336X in Lesion 3. In addition, other genetic alterations such as PTEN or PIK3CA can activate p53-dependent growth suppression in human cells (kim et al., 2007). So, it has been considered that the other gene alterations are also involved in wild type p53 (lesion 2).

The sequences of appearance of multiple tumors is defined as simultaneous (all malignant tumors are observed at the same time), as synchronous (the second tumor appears within 6 months after the first), or metachronous (the multiple tumor is diagnosed more than 6 months after the recognition of the previous one (Németh et al., 1999). In our case, according to the patient statement, three SCCs and leukoplakias of the oral cavity developed within six months. Different p53 mutation and histology proved their distinctness. So that our patient fulfilled the criterion of synchronous multiple primary malignant neoplasm.

Most of the multiple oral carcinomas are associated with leukoplakia, (Moertel et al., 1977) however, in pre-malignant lesion p53 alteration has not been described frequently (kashiwazaki et al., 1997). Perhaps tobacco uses as in the present case have a high incidence of p53 mutations.[12] We can explain by that way the p53 gene alteration has occurred before the carcinogenetic change. In the present case, we also found in each leukoplakias: wild-type p53 in lesion 4, 273H in lesion 5, and wild type p53 in lesion 6 (Table 1).

The concept of field cancerization is to explain that have strong tendency those who are exposed repeatedly to carcinogenic factors such as tobacco and alcohol, to develop multiple primary tumors, being consistent with the present case. Mutations of p53 gene occur during

early stages in the development of HN SCCs because they are already present in premalignant lesions (Lazarus et al., 1995). In our study, we found hot spot p53 mutation in codon 273 in one leukoplakia. It has been shown that the malignant potential of leukoplakia is as high as 23-38% (Silverman et al., 1984); hence mutations of p53 gene may be indicative of the potential of these lesions to develop into SCC. G to A transition is the predominant mutations observed in oral SCCs caused from tobacco (tobacco specific N-nitrosomines) (IARC database new version12, $p < 0.002$) (Shin et al., 1994; Petitjean et al., 2007). The most prevalent type of p53 mutation is G: C to A: T transitions found in our study were double times within three mutations. The rest one mutation G: C to T: A transition was also found in our study, which is also frequently mutated in tobacco smokers, although it is not significant in IARC data ($p < 0.11$). The overrepresentation of DN mutants, accounting for about 27% of all p53 mutations (6414 of all 23,544 in the IARC database R11) and suggests an advantage of the cancer cell harbouring a DN mutation in tumour development. In this study we found R273H mutant, which is the most common hotspot DN mutations to the IARC TP53 database (release 11, containing a total of 1093 oral SCC). Studies using mouse models of Li-Fraumeni syndromes have reported gain of functions in R175H and R273H mutants (Lang et al., 2004), which were identified as DN mutants in our study (Hassan et al., 2008). R248W and R273 H mutants interfere with recruiting MRE11-RAD50- NBS1 (MRN) complex to the site of DNA damage, leading to inactivation of ATM (Song et al., 2007). It further enhances genomic instability, which is caused by the loss of p53 function. The gain of-function property of p53 mutants is considered to lend further malignant phenotypes to the tumour cells, such as enhancement of tumourigenicity, metastatic potential and therapy resistance and also new function in conferring the increased cell growth and inhibition of apoptosis (Dittmer et al., 1993; kim et al., 2004; Wong et al., 2007).

Some tumors including oral cancer, inherit gene mutation (Patrikidou et al., 2002) but there are no published reports of germ line p53 mutation. To single out the specific cause of multiple primary malignant tumors is difficult. It is possible that exposure to carcinogens capable of causing multiple genetic abnormalities could develop cancers independently each other throughout the entire anatomic region.

5. Future directions

A longer follow-up in a larger number of patients would further confirm and strengthen the usefulness of DN p53 mutation as a predictor of early recurrence in oral cancer. It should warrant further investigations regarding specific types of DN p53 mutations in relation to the prognosis and responses to therapy in patients with oral SCC. In addition, identification of the dominant-negative property of p53 mutation may be useful for tailoring the treatment of oral cancer. Readers can refer to the database for all the known DN p53 mutation at-http://www.igm.hokudai.ac.jp/crg/DNbase/DNp53.html.

6. Conclusions

Multiple cancers can occur after successfully treating tumors. Genetic analysis of one patient who had simultaneously three SCCs and three dysplasia lesions provided important information on molecular mechanisms of oral cancer development and for its therapeutic strategy. In this particular case, p53 mutation was observed in two of three SCCs and one of the three dysplasias. and these three mutations were at different sites in the p53 gene. These

findings indicate that p53 mutations occurs even at a precancerous lesion and that precancerous and cancerous lesions have different genetic backgrounds for their development. Depending on the molecular findings, we should make a multidisciplinary plan for multiple cancer patients, which will give a valuable insight in future cancer prognosis. We should take extra care with awareness of a patient with risk factors of carcinogenesis. It is further necessary to monitor the effects of single p53 mutation-transduction on a global gene expression by using a cDNA microarray or a tilling array combined with chromatin-immunoprecipitation in order to discover the molecules responsible. Such case provides useful information for predicting the risk for multiple cancers. A more detailed understanding of the p53-related mechanisms that lead to cancer will contribute to the development of more effective, tailored intervention strategies. In particular, detailed information of the p53 status, including transdominancy and GOF activity is expected to be useful for diagnosis and therapeutic strategy fitting each individual patient with multiple carcinomas. Elucidating its role and targets in DNp53 function in cancer cells will open up a new avenue for treatment design in the coming years.

7. References

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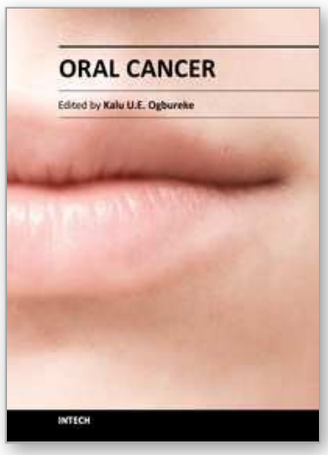
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Oral Cancer

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Oral cancer is a significant public health challenge globally. Although the oral cavity is easily accessible, early diagnosis remains slow compared to the enhanced detection of cancers of the breast, colon, prostate, and melanoma. As a result, the mortality rate from oral cancer for the past four decades has remained high at over 50% in spite of advances in treatment modalities. This contrasts with considerable decrease in mortality rates for cancers of the breast, colon, prostate, and melanoma during the same period. This book attempts to provide a reference-friendly update on the etiologic/risk factors, current clinical diagnostic tools, management philosophies, molecular biomarkers, and progression indicators of oral cancer.

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