Pergamon

Oral Oncol, Eur J Cancer Vol. 32B, No. 6, pp. 388–393, 1996

Printed in Great Britain
0964-1955/96 $15.00 + 0.00

PII: S0964-1955(96)00030-9

Discordance of p53 Status in Matched Primary Tumours and Metastases in Head and Neck Squamous Cell Carcinoma Patients

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To study the use of p53 as a diagnostic tool in head and neck squamous cell carcinoma (HNSCC), we analysed 15 primary tumours (PT) and matched lymph node metastases (LNM) for overexpression and mutations of p53. The primary goal was to study whether differentiation between primary and metastatic disease through their p53 status would be possible. Immunohistochemistry for p53 protein (antibody BP 53-12-1) was performed. Mutations of the p53 gene were detected by exon-specific amplification of DNA (exons 4–9), followed by exon analysis using denaturing gradient gel electrophoresis (DGGE). Mutant exons were sequenced. p53 overexpression was detected in seven (47%) of the PT and in seven (47%) of the LNM. 6 patients (40%) exhibited p53 protein overexpression in both PT and LNM. 2 patients had a different p53 protein expression in each sample. Mutations in the p53 gene were detected in 6 patients (40%) in the PT and in 7 patients (47%) in the LNM. In 2 patients (13%), the same mutation was found in the PT and in the LNM. 9 patients (60%) had a different mutation in each sample.

We conclude that a poor correlation exists between p53 protein overexpression and p53 gene mutation in HNSCC. Also, a poor correlation for both detection techniques exists, when PT and LNM are compared. The p53 status may seem to differ between PT and LNM because of polyclonality in the PT. More sensitive detection techniques could be promising. Copyright © 1996 Elsevier Science Ltd

Key words: p53 alterations, immunohistochemistry, exon analysis, head and neck cancer, metastases

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INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) represents the sixth most common cancer worldwide [1]. In some countries, head and neck cancer is even the fourth cause of death among the male population [2]. Besides the problem of local recurrences after initial curative therapy, these patients have a relatively high risk of developing subsequent primary tumours. A yearly incidence of 3–7% second primary tumours cumulative per annum after the diagnosis of a primary tumour (PT) has been reported [3–6]. This means that approximately 20% of all head and neck cancer patients will develop a second primary tumour after 5 years of follow-up. An explanation for the origin of these second primary tumours has been proposed by Slaughter *et al.* They stated that the entire mucosa of the upper aerodigestive tract has been exposed to the same carcinogens, which caused the PT to arise. This effect has been called "oral field cancerization" [7]. On the other hand, local and distant metastasis, for instance to the lung, can occur. When, during the follow-up period, a tumour in the aerodigestive tract is diagnosed, differentiation between metastatic disease and second primary tumour is very difficult. In the field of head and neck cancer research, one of the most challenging issues is this diagnostic differentiation between subsequent primary and metastatic disease. This differential diagnostic problem occurs when patients, previously treated for a HNSCC, develop a lymph node metas-

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Received 23 Feb. 1996; provisionally accepted 28 Mar. 1996; revised manuscript received 17 Apr. 1996.

tasis (LNM) or a lung lesion. The LNM may be a metastasis from the previously treated HNSCC or from an as yet undiagnosed metachronous HNSCC; the lung lesion could be a distant metastasis from the former HNSCC or a second primary tumour. The outcome of such a diagnostic analysis has profound consequences for the therapeutic decision and therefore for the prognosis of the individual patient. As conventional histology is of no use in respect to this, other markers are needed urgently. Such a marker could be the p53 gene. Mutations and overexpression of the p53 protein have been reported in head and neck cancer [8, 9]. Being mutated in 25–69% of head and neck squamous cell carcinoma [10–14], p53 could prove to be a molecular marker on which these decisions could be made, as the mutations may occur at different locations within the gene.

Assuming that the p53 status in a PT and its LNM will be the same, while the p53 status in a second primary malignancy will be different, a reliable p53 screening test could be of use in discriminating between primary and subsequent PTs [15]. The first step, therefore, is to prove that a p53 mutation, found in a PT, can be detected reliably in its lymph node metastasis. To study this, we used matched PTs and their LNM. Immunohistochemistry was used for the detection of overexpression of the p53 protein. The polymerase chain reaction (PCR) was used for amplification and denaturing gradient gel electrophoresis (DGGE) for exon analysis of the tumour samples.

PATIENTS AND METHODS

Retrospectively, 15 sets of samples were selected, each consisting of a PT and a matched LNM from the same head and neck cancer patient. 14 patients underwent resection of the PT and a neck dissection from which the LNM were obtained during the same surgical procedure. In 1 patient, a neck dissection was performed shortly after excision of the PT. Therefore, LNM were metastatic deposits from the PT. No recurrent tumours were included in this study. 6 patients had an oral cavity carcinoma, 3 patients an oropharyngeal carcinoma, 5 a hypopharyngeal carcinoma and 1 patient a laryngeal carcinoma. All patient material was paraffin-embedded tissue and obtained from the files of the Pathology Department.

Immunohistochemistry

Paraffin blocks were selected in which tumour was present and 4 μm paraffin sections were cut for immunohistochemical detection of p53 protein. The immunohistochemistry was performed on deparaffinized 4 μm sections using a murine monoclonal antibody, BP 53-12-1 (Biogenex, San Ramon, California, U.S.A.), recognising both wild-type and mutant p53 protein.

All sections for immunohistochemistry were treated with a boiling solution of freshly prepared citrate/HCl buffer (10 mM) pH 6.0 for 15 min on an electric hotplate which, in our laboratory, has proved to be as effective as microwave pretreatment in antigen retrieval. After cooling to room temperature, sections were rinsed in phosphate-buffered saline (PBS) (×3) and incubated with anti-p53 protein antibody at a dilution of 1:100 for 1 h. Next, sections were washed in PBS and incubated with biotinylated horse-anti-mouse antibodies (1/500; Vector, Burlingham, California, U.S.A.) for 30 min, followed by washing in PBS and incubation with peroxidase labelled streptavidin (1/400; Boehringer, Mannheim, Germany) for 30 min. The peroxidase activity was developed by 3,3-diaminobenzidine hydrochloride (DAB, Sigma) resulting in a brown reaction product. Sections were counterstained with haematoxylin and mounted. All dilutions were performed using PBS. Controls consisted of omitting the specific antibody or by replacing it by an anti-AA amyloid antibody (Eurodiagnostics, Apeldoorn, The Netherlands; same IgG subclass as the BP53-12-1 antibody); the dilution used for control antiserum was 1:1000 for the anti-AA amyloid antibody. Sections stained for p53 protein were examined for the presence of brown staining nuclei. Lesions were classified as positive when more than 10% of tumour cell nuclei were stained.

Exon analysis

Formalin-fixed, paraffin-embedded tissue was processed essentially as described by Wright and Manos [16]. Neoplastic areas in three 10 μm sections were detected and marked using standard microscopic techniques. The marked neoplastic areas were removed with a clean razorblade and used as starting material, extracted with xylene (twice), ethanol (twice), acetone, dried, incubated overnight at 37°C with 100 μl 50 mM Tris pH 8.5, 1 mM EDTA, 0.5% Tween 20 and 200 μg/ml proteinase K. The samples were heated for 8 min at 96°C to inactivate the proteinase K. Either 1 or 2 μl of these samples were used as template material in the PCR amplifications.

Exons 4–8 and 8 + 9 of the p53 gene were amplified in two successive PCR-runs using the primers listed in Table 1 [17]. The 5′ primers used in the first PCR have a 5′ 15 nucleotide GC-rich extension. The 50-nucleotide GC-rich primer (universal clamp) used as the 5′ primer in the second PCR does anneal to the complement of the 15 nucleotide extension. This results in the second PCR in a product with a 50 nucleotide "GC-clamp" as described by Top [18]. A more inward-located 3′ primer was used in most cases in the second PCR. The first PCR was performed in 50 μl 75 mM Tris/HCl pH 9.0, 20 mM $(NH_4)_2SO_4$, 0.01% Tween 20, 0.25 mM of each dNTP, 1.5 mM $MgCl_2$ with 1 or 2 μl DNA-template and 20 pmol of each primer. The samples were overlaid with 50 μl mineral oil and incubated in a New Brunswick Scientific Thermocycler TC-1 or an Eppendorf Mastercycler 5330 with the following cycle conditions: 3 min at 95°C; 5 cycles 30 s 95°C, 30 s 62°C and 1 min 72°C, 5 cycles 30 s 95°C, 30 s 59°C, 1 min 72°C, 25 cycles 30 s 95°C, 30 s 56°C and 1 min 72°C; and finally, 10 min 72°C for termination of the reaction. One unit of Taq polymerase (Ampli Taq, Perkin-Elmer Cetus) was added after the 3 min 95°C step. An aliquot of 1 μl of the first PCR was used as the template in the second PCR that was performed in the same way as the first PCR.

Samples of 5 μl of the second PCR were run on a 1.5% agarose gel to estimate the yield of the reactions. Amplification of exons 8 + 9 was unsuccessful in three cases, possibly due to DNA fragmentation after fixation and embedding in paraffin.

Denaturing gradient gel electrophoresis was used for exon analysis. The PCR fragments with GC-clamp were analysed on an 8% polyacrylamide gel with a linear gradient of denaturing agents (urea and formamide, 7 M urea/40% forma-

Table 1. p53 primer sequences, positions and gradients used for polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE)

		Sequence	Position	DGGE gradient
Exon 4	5′	*-AGGACCTGGTCCTCTGACTG	11981–12000	30–80%
	3′	TACGGCCAGGCATTGAACGTC	12350–12331	
	3′	TCTCATGGAAGCCAGCCCCT	12332–12313	
Exon 5	5′	*-TTCCTCTTCCTACAGTACTC	13040–13059	60–90%
	3′	CTGGGCAACCAGCCCTGTCGT	13281–13261	
	3′	AGAGCAATCAGTGAGGAATC	13316–13297	
Exon 6	5′	*-GAGACGACAGGGCTGGTT	13258–13273	30–80%
	3′	TCCTCCCAGAGACCCCAGTT	13463–13444	
	3′	GCCACTGACAACCACCCTTA	13488–13469	
Exon 7	5′	*-CCAAGGCGCACTGGC	13957–13971	40–80%
	3′	CAGTCTGCAGGGTGGCAAGTG	14139–14119	
	3′	CAAGCAGAGGCTGGGGCACA	14165–14146	
Exon 8	5′	*-TGATTTCCTTACTGCCTCTTG	14407–14427	50–80 or
	3′	CTGCACCCTTGGTCTCCTCC	14633–14614	60–90%
	3′	AATCTGAGGCATAACTGCAC	14647–14628	
	3′	AGGAAAGAGGCAAGGAAAGGT	14678–14658	
Exon 8 + 9	3′	GCATTTTGAGTGTTAGACTG	14813–14794	30–90%
	Universal clamp:			
	CCGCGCCCCGCGCCCGCCCCGCCGCCCCCCCCCCGCGCCGCCGCCGCCCG			

* The sequence is preceded by the GC-rich sequence CGCCGCCGCCGCCCG. This is the sequence which corresponds to the last 3′ 15 nucleotides of the universal clamp which is used in the second PCR. A number of the primers have been described by DiGuiseppe *et al.* [17].

mide is defined as 100% denaturant) as described by Myers *et al.* [19]. The gradient conditions for the different regions are listed in Table 1.

Electrophoresis was performed overnight at 5 V/cm at 60°C. Gels were stained with ethidium bromide and photographed. Fragments that showed an altered pattern at the DGGE analysis were sequenced.

The PCR fragments were cloned in the pGEM-T vector using the vectorsystem as suggested by the supplier (Promega). Plasmid DNA from mixtures of 20 positive clones were sequenced using the appropriate amplification primers as sequence primers, a T7 DNA polymerase sequencing kit (Pharmacia) α^{35}S-dATP and a BioRad Sequi-Gen sequencing (BioRad, Hercules, California, U.S.A.). In three cases, the cloning of the PCR fragments was unsuccessful.

RESULTS

Immunohistochemistry

The immunohistochemical analysis showed p53 protein overexpression in 7 (47%) of the PTs and in 7 (47%) of the LNM. Figure 1 shows immunohistochemical staining as performed in our study. 6 patients (40%) exhibited p53 protein overexpression in both PT and LNM. Matching results for immunohistochemistry between PT and LNM were found in 13 patients (87%). In 1 patient (7%), the LNM was positive, while the PT showed no staining. The opposite was observed in 1 patient.

Exon analysis

Exon analysis of the matched samples showed p53 mutations in 10 patients (67%) in either PT, LNM or both (Fig. 2). Mutations in the p53 gene were detected in 6 patients (40%) in the PT and in 7 patients (47%) in the LNM. In 2 patients (13%), the same mutation was found in the PT and in the LNM. In 1 case, there were two different mutations in the PT, which were not found in the LNM, The LNM, however, contained a third mutation (Table 2; patient 5) [20], which is a silent mutation that does not result in a different amino acid. In 3 patients (20%), a mutation was detected in the PT, while the LNM did not contain a mutation. The opposite was observed in 4 cases (27%). In 4 patients, mutations were found in two exons. Sequencing of the mutant exons showed that the mutations result, in 3 cases, in a stopcodon (Fig. 3) and, in 2 cases, in a stopcodon downstream from the mutated site as a result of a frame shift. In 4 cases, a point mutation resulted in a different amino acid in the p53 protein. The mutation at position 13432 (patient 8) results in an altered exon/intron splice donor site, which probably results in a defective splicing. The results of the immunohistochemistry, exon analysis and the sequence analysis are listed in Table 2.

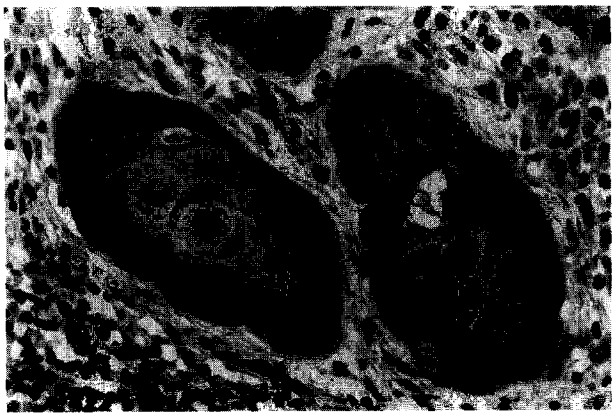

Fig. 1. p53 immunostaining with antibody BP 53-12-1 in head and neck squamous cell carcinoma.

Fig. 2. The DGGE analysis of amplified p53 (exon 8) from patient number 2. Lane A represents the metastasis; lane B represents the primary tumour.

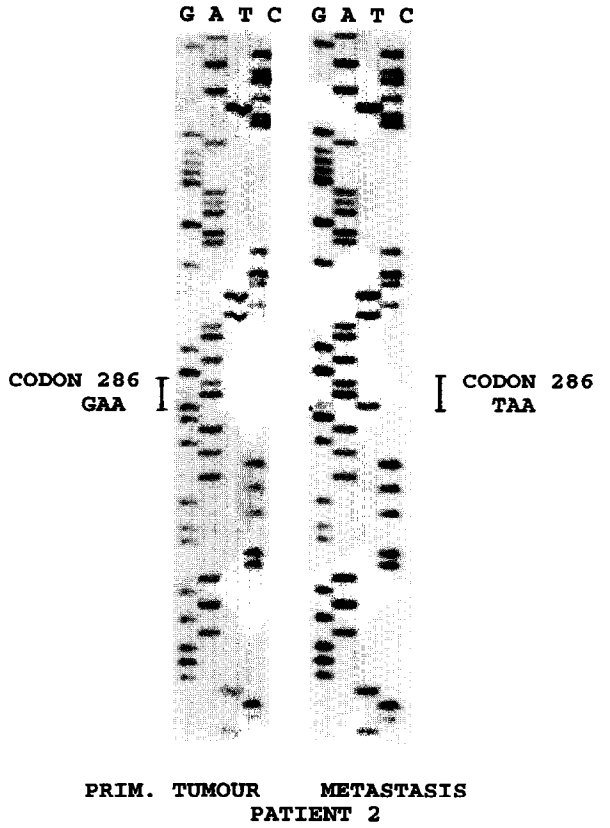

Fig. 3. Autoradiograph of part of the sequence of p53 exon 8 from patient number 2. The example shows the GAA → TAA mutation at codon 286 in the metastasis, which results in a stop sequence.

DISCUSSION

Comparing the immunohistochemical analysis of the primary tumours and their metastases, we found a concordance of p53 protein expression in 87%. This, and our finding of 40% p53 mutations in primary head and neck tumours, is consistent with other observations [10–14]. Comparing immunohistochemistry with DGGE and sequencing showed an overall concordance of 50%. The discordance between immunohistochemistry and DGGE was 53% for the PTs (8 patients) and 47% for the LNM (7 patients). A mutation without overexpression of the p53 protein in a single sample may be the result of the formation of a stopsequence, as indeed could be found in 3 cases. Overexpression of the p53 protein without mutation could be the result of a mutation or a disturbance elsewhere in the G1 cell cycle checkpoint, which could result in elevated protein without a detected mutation in the p53 gene [21–23]. Therefore, the absence of a correlation between p53 overexpression and the presence of a mutation can be explained.

The exon analysis itself showed a poor correlation of p53 status between PT and LNM. In our study, we found the same mutation in a PT and its LNM in only 2 cases (13%). In 1 patient, the LNM contained a mutation different from the PT. Surprisingly, in 7 cases, a mutation was found only in one of the two matched samples, either in the PT or in the LNM. In 3 patients (20%), a mutation was found in the PT, while the LNM did not contain a mutation. The opposite was observed in 4 cases (27%). The overall concordance, which includes the patients without a mutation (5 cases) in both PT and LNM, was 47%. Some authors, who studied small cell lung carcinoma and head and neck cancer patients, found a 100% concordance between mutations in primary tumours and their metastases [10, 14, 24, 25]. Others, who studied prostate cancer, breast carcinoma, lung carcinoma and gastrointestinal adenocarcinomas [26, 27], found similar discordances as found in our study. When we compare tumours from different sites of origin, we must always bear in mind that a wide variety in hot-spot sites exists, which differs for each tumour type and location. We conclude that the p53 mutational status is not concordant in PTs and matched LNM. It has been observed that HNSCC may harbour different p53 mutations in one and the same tumour [28]. As a p53 mutation gives rise to genetic instability, additional mutations could produce subclones with growth advantages over other tumour cells with regard to the environmental pressure in the tumour environment [29]. Also, these subclones could have an increased metastatic potential. The metastasis could originate from a small cell clone, which could be smaller than one cell in a sample of 10^5 or 10^6 cells in the primary tumour cell population. A cell fraction this small will not be detected using standard PCR methods [30]. In the case where the LNM only harboured one p53 mutation, while the PT harboured two mutations, the PT probably consisted of multiple subclones of which one gave rise to a metastatic cell [31]. Although it is generally accepted that p53 mutations are an early event in HNSCC, some data point to the opposite. Additional p53 mutations can occur in metastatic tissue as

Table 2. p53 overexpression and mutations in 15 primary head and neck tumours (p) and matched neck node metastases (m). Detection by immunohistochemistry (IHC), denaturing gradient gel electrophoresis (DGGE) and sequencing of the mutant bands. Numbering of the base pair positions according to the p53 sequence submitted by P.M. Chumakov (Genebank accession X54156). TNM stage according to pTNM classification* [20]

Tumour no.	TNM*	IHC	Exon	Position	Codon	Sequence change	Result
1.p	T2N2bM0	—	—				
m		—	—				
2.p	T1N2cM0	—	—				
m		—	8	14525	286	GAA → TAA	Stop
3.p	T2N2aM0	—	—				
m		+	—				
4.p	T2N2bM0	+	—				
m		+	—				
5.p	T4N2bM0	+	7	14060	245	GGC → AGC	Gly → Ser
			8 + 9	14702	314	Deletion C	Downstream stop
m		+	8 + 9	14572	301	CCA → CCG	Pro → Pro
6.p	T4N2bM0	+	6	13398	213	CGA → CTA	Arg → Leu
m		+	—				
7.p	T4N2cM0	—	6	13399	213	CGA → CGG	Arg → Arg
			7	14098		Deletion 8 bases 14098–14105	Downstream stop
m		—	—				
8.p	T2N1M0	—	6	13432	224	GAG → GAA	Exon/intron splice donor
m		—	5	13160	161	GCC → ACC	Ala → Thr
			6	13432	224	GAG → GAA	Exon/intron splice donor
9.p	T4N2cM0	+	—				
m		+	4	12260	112	GGC → GGG	Gly → Gly
			5	13107	143	GTG → GCG	Val → Ala
10.p	T2N2bM0	—	—				
m		—	5	13168	163	TAC → TAA	Stop
11.p	T4N2bM0	—	—				
m		—	—				
12.p	T3N2aM0	—	8 + 9				Sequence not determined
m		—	—				
13.p	T4N2bM0	+	—				
m		+	—				
14.p	T4N2cM0	+	8				Sequence not determined
m		+	8				Sequence not determined
15.p	T1N1M0	+	—				
m		—	5	13168	163	TAC → TAA	Stop

this tissue is even more genetically unstable than its PT [32]. The absence of a mutation in the LNM that is present in the PT indicates that the metastasis is derived from a cell clone without a p53 mutation. Another possible explanation for the discrepancy in p53 mutations between PT and LNM could be that in the case of a substantial time interval between removal of PT and LNM, additional mutations have occurred. In our study, this explanation does not apply as tissue from PT and LNM were obtained at the same time and in 1 patient only with a time interval of a few weeks. Moreover, detection of some p53-specific clones and failure to detect others may be due to sampling factors, e.g. only one clone being present in the analysed tissue sections. Therefore, we conclude that with our current technique, demonstration of concordance in p53 mutational status between several tumour deposits proves their origination from one single tumour, but that discordancy does not for reasons outlined above. To study the clonal origin of tumours with regard to their p53 mutational status, it is obvious that new and more selective methods need to be found. Detection of specific mutations in metastases by using mutation-specific oligomer probes seems to be a very promising technique for investigating the monoclonal origin of metastatic spread. With this technique, it is possible to search for a specific mutation sequence in the PT, which has been detected previously in the metastasis using standard methods. Koch *et al.* used this technique and found the mutated sequence of one PT in metastases in both sides of the neck in a patient suffering from two synchronous primary cancers [33].

We conclude that immunohistochemistry and DGGE are not reliable techniques in differentiating between second primary and metastasis, as small clones with different p53 mutations in one individual lesion could be missed. Other methods, like direct sequence analysis, combined with PCR with mutant-specifc oligomer probes, as used by Koch *et al.* [33] seem more promising and are presently in development in our department.

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