Detection of Minimal Residual Cancer to Investigate Why Oral Tumors Recur Despite Seemingly Adequate Treatment

Max Partridge,1 Shu-Rui Li, Stelios Pateromichelakis, Rebecca Francis, E. Phillips, Xiao hong Huang, Fis Tesfa-Selase, and John D. Langdon

Maxillofacial Unit/Oncology, King’s College Hospital, London SE5 8RX, United Kingdom

ABSTRACT

Improvements in surgery and radiotherapy techniques have led to only a modest increase in the 5-year survival rate for patients with head and neck cancer. This is because the pattern of clinical disease is changing, such that locoregional recurrence now accounts for fewer treatment failures, but more patients develop a second primary cancer or distant metastatic disease. In this study, we have used the p53 phage plaque assay, immunocytochemistry, and mutational analysis to assess the contribution of minimal residual cancer and genetic aberrations in clinically normal upper aerodigestive tract mucosa to treatment failure. Eighteen consecutive patients with oral tumors, with conventional clear margins, have been followed for a minimum of 36 months. Molecular assessment identified tumor-positive surgical margins for 6 of 11 assessable patients and additional tumor-positive lymph nodes for three cases. Disseminated malignant cells were detected in the hematopoietic cell compartment for six cases, and one patient had molecular evidence of field cancerization. Locoregional recurrence developed in five patients with tumors harboring a p53 gene mutation; four of these were associated with tumor-positive surgical margins, and one was associated with molecular evidence of field cancerization. Radiotherapy to the primary site did not prevent development of local recurrence when the residual tumor harbored a p53 gene mutation. Three of six cases with a tumor-positive bone marrow aspirate developed distant metastases. These findings reveal that molecular and immunocytochemical detection of minimal residual cancer and field cancerization can help identify patients who may develop locoregional or distant recurrence and justify further studies to evaluate the contribution of these remaining malignant cells to treatment failure.

INTRODUCTION

Head and neck cancer represents a major health problem, as evidenced by its high incidence in many parts of the world, the poor survival rates, and the severe functional and cosmetic defects accompanying this disease and its treatment. On a global basis, the number of new cases of head and neck malignancy each year exceeds 550,000, which comprises ~6% of all cancers. Despite very significant improvements in surgery and radiotherapy, comparison with historical controls shows only a modest improvement in overall survival, although there have been significant improvements for tumors at some sites [for examples, see Franceschi et al. (1) and references therein]. There is also evidence that the overall pattern of disease is changing so that today, fewer patients die as a result of locoregional disease but more succumb because of development of distant metastases or a second primary tumor (2–8). These studies suggest that occult tumor cells must remain in the body but are not detected by the current diagnostic procedures. This residual cancer may be present at the surgical margins, in lymph nodes, or be disseminated throughout the body, where it is most readily detected in the hematopoietic cell compartment. Treatment may also fail if a second tumor develops, because the primary lesion develops within a field of cancerization where the mucosa harbors the genetic aberrations required for tumorgenesis.

There is a wealth of evidence identifying tumor-positive surgical margins as an important source of treatment failure, and in view of this, most surgeons aim for a minimum of 1-cm clearance when resecting these cancers. However, the anatomy of the oropharynx means that this is often not achievable, and studies reveal that 12–32% of cases have positive surgical margins (9–12), although the true frequency may be much higher. For example, Brennan et al. (13) have shown, using a sensitive molecular diagnostic based on finding the same p53 gene mutation in the primary tumor and the surgical margins, that malignant cells can be detected for 52% of patients with histopathologically negative margins. The same study reported that 38% of these cases with positive margins after molecular analysis developed local recurrence, whereas no patient with negative margins failed treatment at the local site. This methodology can also be used to provide a sensitive approach to screen lymph nodes, which are negative for tumor by light microscopy, for the presence of micrometastases.

A proportion of recurrent tumors share the same p53 mutation (14–17), and p53 mutations in mucosa adjacent to head and neck tumors (13, 18–21) have been taken as evidence of likelihood of the development of a malignant lesion. There is also data that suggests that second primary tumors develop more frequently when p53 mutations are present in single (18) or multiple biopsies of tumor-distant mucosa (22). This later study

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1 To whom requests for reprints should be addressed, at Maxillofacial Unit/Oncology, King’s College Hospital, Denmark Hill, London SE5 8RX, United Kingdom. Fax: 020-7346-3754.
used a FASAY\(^2\) (23) to detect mutations that abrogate the function of p53, an approach that has the advantage of detecting polyclonal aberrations against a background of wild-type.

In addition to minimal residual cancer and precancerous change within the upper aerodigestive tract, patients may also fail treatment because of the presence of malignant cells in the hematopoietic cell compartment (24–26). At present, the prognostic significance of disseminated tumor cells has not been established conclusively, although tumor cells identified in mononuclear cells obtained from the bone marrow of 32–64% of cases with SCC of the head and neck were associated with poor outcome (24, 25). Subsequent studies have revealed that when density gradients are used to prepare leukocyte subsets, although tumor cells sediment predominantly with the mononuclear fraction, they may also be detected in the granulocyte layer (26). Thus, to improve the sensitivity of tumor cell detection, protocols for detection of disseminated tumor cells for patients with tumors of squamous epithelium must screen all leukocytes, using an approach that incorporates appropriate controls and morphological assessment of immunostained cells, to minimize the risk of false-positive results (26).

To shed light on the vexing question as to why a proportion of patients develop local and distant recurrence despite seemingly adequate treatment, we have used the p53 phage plaque assay (13) to detect occult tumor cells in surgical margins, immunocytochemistry to detect disseminated tumor cells (26), and FASAY (23) to search for molecular evidence of field cancerization by detecting p53 mutations in tumor-distant mucosa. Our preliminary findings confirm that detection of minimal residual cancer can provide additional prognostic information and support the need for long-term prospective studies to assess the contribution of these remaining malignant cells to treatment failure for this group of patients.

MATERIALS AND METHODS

For this prospective study, biopsies of 22 consecutive primary oral SCCs were taken at the time of surgery and stored at \(-70^\circ\)C until required. Four cases reported as having tumor-positive margins by the histopathologist were excluded from the analysis. To facilitate detection of systemic disease, all patients were screened for distant metastases preoperatively by alkaline phosphatase assay, chest X-ray, liver ultrasound, and a bone scan. The biochemical tests, X-ray, and ultrasound examination were repeated every 6 months, and the bone scan was repeated every 2 years.

The indications for postoperative radiotherapy to the local site were close resection margins (all cases with involved resection margins were excluded from the study), a noncohesive tumor margin, or perineural spread. The neck was irradiated when two or more tumor-positive lymph nodes were identified and when extracapsular spread was present. In addition, elective irradiation was used when the risk of microscopic tumor involvement was considered to be at least 20% in clinically negative necks, where no surgery was performed. Details of the radiation dose and timing are not shown for these cases because it was felt that this was outside the purview of this report.

All clinical samples were coded and analyzed blind. Local recurrence was confirmed by histopathologically demonstrable SCC at a site adjacent to the index lesion. All patients have been followed for a minimum period of 36 months. Ethical Committee Approval for the minimal residual cancer program was granted at King’s College Hospital.

Surgical Margins and Lymph Nodes. After excision of the primary tumor, four mucosal and two deep margins, each approximately 1 cm \(\times\) 8 mm \(\times\) 8 mm, were harvested. The mucosal biopsies were taken at both ends of the defect for T\(_1\) and T\(_2\) lesions, but for larger T\(_3\) and T\(_4\) tumors and lesions which involved bone, the shape of the defect meant that the mucosal margins were often taken at random. Two deep margins were taken from the floor of the resection unless the tumor was excised in continuity with the neck dissection and there were no other sites that could be sampled. Each margin was divided into two; the inner portion was processed for conventional light microscopy, and the outer half frozen for molecular analysis. Sections of paraffin-embedded lymph nodes >1 cm in diameter, considered to be tumor-free after light microscopy, were also analyzed.

Biopsies of Tumor-distant Mucosa. Three to five biopsies of clinically normal mucosa were taken from patients undergoing preoperative diagnostic endoscopy or at the time of primary surgery. Each biopsy was divided in two; one half was sent for histology, and the other was used for molecular analysis. All samples were taken at least 5 cm from the tumor, and the distance between biopsies was at least 2 cm. Samples to be analyzed using the modified FASAY (22) were placed directly in 500 \(\mu\)l of RNA lysis buffer [500 mm LiCl, 1% LiDS, 100 mm Tris (pH 8), 10 mm EDTA, and 5 mm DTT], immediately frozen on dry ice, and stored at \(-80^\circ\)C until use. All biopsies were examined by light microscopy for evidence of hyperplasia or dysplasia.

FASAY for Identification of a Signature p53 Gene Mutation in the Primary Tumor. The presence or absence of a p53 mutation was determined by screening p53 cDNA by FASAY, according to the method modified by Tada et al. (27), with the yeast reporter strain yIG397 (23). Ten-\(\mu\)m frozen sections were prepared from 50–100 mg of tumor tissue and lysed with LiDS buffer as described above. The sample was homogenized in 500 \(\mu\)l of LiDS lysis/binding buffer by passing it repeatedly through a 22-gauge needle and freeze/thawing twice on dry ice. After centrifugation, mRNA was prepared using Dynabeads (Dynal, Oslo, Norway) according to the manufacturer’s instructions. RNA was eluted in 20 \(\mu\)l of water containing 50 units of human placental RNase inhibitor (Roche, Lewes, United Kingdom). p53 cDNA was synthesized from 5 \(\mu\)l of mRNA at 46°C for 1 h in a final volume of 20 \(\mu\)l containing 100 units of Superscript II reverse transcriptase (Life Technologies, Inc., Paisley, Scotland) and 0.1 \(\mu\)g of RT1 primer (CGG GAG GTA CAA), as described by Flaman et al. (23). The p53 cDNA was amplified in 20 \(\mu\)l of reaction mixture containing 2 \(\mu\)l of the reverse transcriptase reaction product, 1.25 units of pfu DNA polymerase (Stratagene, Amsterdam, Holland), 10 pmol of primers, 10% DMSO, and 50 mm deoxynucleotide triphos-
phates. Primers P3 and P4 were used for the standard FASAY (22). PCRs were run in the Thermal Cycler Model 2400 (Perkin-Elmer, Warrington, United Kingdom) at 96°C for 1 min; then 35 cycles of 95°C for 40 s, 65°C for 70 s, and 78°C for 90 s; followed by 78°C for 2 min. Satisfactory amplification was confirmed by examining the PCR product on a 1% agarose gel. Each crude PCR product was used for yeast transformation. More than 200 colonies were examined for each sample analyzed, and yeast plasmids harboring a p53 mutation and wild-type p53 were included in each run. Plasmid DNA was rescued from yeasts yielding red colonies by alkaline lysis (Wizard Minipreps; Promega, Southampton, United Kingdom) and electroporated into Escherichia coli, strain DH5a. At least six plasmids were rescued from all samples yielding >10% red colonies, and mutations were identified on both strands by automated sequencing. Genomic DNA was prepared for cases found to have intronic p53 gene deletions, and the mutation was determined by sequencing a PCR fragment encompassing the deletion.

Modified FASAY for Identification of Polyclonal Mutations in Tumor-distant Mucosa. To increase the sensitivity of the assay and discriminate clonal mutations from PCR-induced mutations, the modified split form of this assay was used to examine three to five samples of normal mucosa with primers P3, P17, P4, and P16 (22) for each case. cDNA obtained from normal mucosa yields 3–15% red colonies, which represents the background for the FASAY (22, 27). Plasmid DNA was rescued from at least six colonies for all patient samples, yielding >10% red colonies for sequencing.

p53 Phage Plaque Assay for Detection of Residual Disease at the Surgical Margins and Micrometastases in Lymph Nodes. Twenty 5-μm sections were examined for the paraffin-embedded portion of each surgical margin and lymph nodes >1 cm for cases 1–3. The sections were stained with H&E and examined by light microscopy. Only cases pronounced tumor free by the histopathologist were forwarded for the molecular analysis. DNA was prepared from the frozen portion of the surgical margins and paraffin-embedded lymph nodes using DNA Stat 50 (Biogenesis, Poole, United Kingdom) according to the manufacturer’s instructions. Exons harboring the p53 mutation detected in the primary tumor were amplified by PCR; products were cloned into a bacteriophage vector and amplified further in E. coli (13). Each sample was plated in duplicate to prepare filters for hybridization with mutant and wild-type-specific oligonucleotide probes. At least 2000 clones were transferred to nylon membranes for each margin analyzed. Filters were hybridized with probes end-labeled with [γ-32P]ATP. After hybridization, the membranes were washed to identify plaques after exposing the membranes to X-ray film. The assay included positive and negative controls for each set of margins and lymph nodes examined. The positive control was the amplified p53 gene product derived from the patient’s primary carcinoma; the negative control cloned p53 products derived from patients with a different p53 mutation in the primary tumor. All positive assays resulting in 1–10 plaques/plate were repeated from the genomic DNA step. Because the ploidy status of the tumors examined was unknown, we assumed that each cancer cell contained one mutant p53 allele and estimated the percentage of clonal (mutated) tumor cells in each sample by counting the number of plaques labeled with the mutant-specific oligonucleotide probe and dividing this number by the total number counted.

Bone Marrow and Central Venous Blood. Central venous blood (30 ml) and bone marrow aspirates (5–10 ml) were collected preoperatively, and buffy coat cells were prepared, mixing the sample with 0.1 volume of dextran (average $M_r$ 500,000; 6% w/v) to sediment the erythrocytes. Bone marrow aspirates were diluted 1:1 with PBS prior to preparation of leukocyte-rich plasma, and the leukocytes were washed in 10× volume PBS with 2% FCS to reduce the viscosity of the sample (26).

Detection of Disseminated Tumor Cells. Leukocytes ($6 \times 10^7$) were enriched for tumor for each patient using M450 Dynabeads (Dynal, Oslo, Norway) coated with anti-CD45 (recognizing the common leukocyte antigen; Ref. 26). After negative immunomagnetic selection, the remaining cells (approximately $2 \times 10^7$) were sedimented onto slides coated with Cell-Tak tissue adhesive (Becton Dickinson, Oxford, United Kingdom) for immunocytochemistry with the pancytokeratin antibody AE1/AE3, diluted 1:50 (Dako, High Wycombe, United Kingdom), using an alkaline phosphatase/monoclonal mouse anti-alkaline phosphatase technique (26). Briefly, slides were fixed in acetone for 10 min. After washing in Tris-buffered saline (TBS), tissues were incubated for 10 min in fetal bovine serum (Life Technologies, Inc., Paisley, Scotland) diluted 1:5 in TBS to block nonspecific binding before incubation with 100 μl of AE1/AE3 in a humid chamber. After washing in TBS, slides were incubated for 30 min with 100 ml of rabbit antimouse immunoglobulin antibody (Dako; 1:25) diluted in 20% human serum and washed again, and 100 μl of alkaline phosphatase/mouse anti-alkaline phosphatase (Dako; 1:30) were added for 30 min. After additional washing, the reaction product was developed with New Fuchsin substrate solution (Dako) containing levamisole (1 mM) to block endogenous alkaline phosphatase activity for 10 min. Slides were washed with water and counterstained with hematoxylin. Isotype controls were stained with mouse anti-FITC (Sigma, Poole, United Kingdom), of the same subclass (IgG1), using the same concentration of immunoglobulin. Positive controls were SCC cell lines. Immuno reactive cells were scored as tumor using the criteria defined previously (26) if they lacked recognizable hematopoietic characteristics and were typically large, often with an irregular shape and a high nuclear/cytoplasmic ratio when compared with the surrounding leukocytes. Cases found to have a single immuno reactive cell, which could not be categorically identified as tumor, were scored as tumor negative.

RESULTS

Clinical details and results after screening for minimal residual cancer and molecular evidence of field cancerization are shown in Table 1. Thirteen of 18 (72%) tumors examined were found to harbor a p53 gene mutation. Four to six surgical margins from 12 patients, assessed as tumor negative after conventional light microscopic examination, were probed with mutant-specific and wild-type oligonucleotides (for an example, see Fig. 1). The amplified p53 region from two or more surgical margins hybridized to the tumor-specific probe for six cases
The number of nodal metastases showed extracapsular tumor spread. There was no relationship between the number of nodal metastases and the frequency of disseminated tumor cells. Tumor cells were also detected in the central venous blood preoperatively, was found for 6 of 18 cases (nos. 3, 4, 5, 7, 16, and 18; for examples, see Fig. 3). Two of these cases developed multiple lung lesions (cases 3 and 5) and a third case developed a solitary lung nodule (case 4), which were ultimately the causes of death for these patients. The presence of SCC was confirmed by bronchoscopy for cases 3 and 4. All cases with evidence of widespread tumor dissemination had at least one tumor-positive lymph node after conventional light microscopic examination, and two of these nodal metastases showed extracapsular tumor spread. There was no relationship between the number of nodal metastases and the frequency of disseminated tumor cells. Tumor cells were also detected in the central venous blood preoperatively for two cases (3, 7), both with extracapsular nodal deposits of tumor detected by conventional light microscopy.

**DISCUSSION**

At present, the Union International Contre Cancer TNM tumor classification system is an essential component of the measures used for predicting outcome for patients with head and neck cancers, although it does not always provide accurate prognostic information. This failure is attributable to the limited sensitivity of the current methods for detecting spread of tumor cells and the requirement to take into account information about tumor site, histology, thickness, and tumor biology in addition to conventional TNM criteria when making important predictions about outcome and planning treatment (28). However, progress in this area can now be made by applying a new generation of ultrasensitive diagnostics to detect small numbers of malignant

### Table 1  Clinicopathological features of tumors examined and p53 gene status

<table>
<thead>
<tr>
<th>Case</th>
<th>Tumor site</th>
<th>Age</th>
<th>TNM</th>
<th>Post-op radiation</th>
<th>Outcome</th>
<th>Development of recurrence</th>
<th>Primary tumor p53 mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Lingual tongue</td>
<td>40</td>
<td>pT4N0M0</td>
<td>DOC 25 m</td>
<td>Local</td>
<td>Codon 248 CGG-CAG</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Alveolus</td>
<td>80</td>
<td>pT3N0M0</td>
<td>DOD 18 m</td>
<td>Local</td>
<td>Codon 175 CGC-CAC</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Retromolar</td>
<td>51</td>
<td>pT4N0M0</td>
<td>DOD 21 m</td>
<td>Nodal + lung lesion</td>
<td>Codon 248 CGG-CAG</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Floor of mouth</td>
<td>67</td>
<td>pT4N0M0</td>
<td>DOD 22 m</td>
<td>Local + lung mets</td>
<td>Codon 280 AGA-GGA</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Floor of mouth</td>
<td>52</td>
<td>pT4N0M0</td>
<td>DOD 32 m</td>
<td>Local</td>
<td>Codon 175 CGC-ACG</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Retromolar</td>
<td>62</td>
<td>pT4N0M0</td>
<td>DOD 33 m</td>
<td>No</td>
<td>Codon 152 CGC-ACG</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Floor of mouth</td>
<td>54</td>
<td>pT4N0M0</td>
<td>Alive 36 m</td>
<td>No</td>
<td>Codon 245 GGC-AGC</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Retromolar</td>
<td>74</td>
<td>pT4N0M0</td>
<td>Alive 42 m</td>
<td>No</td>
<td>Wild-type</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Alveolus</td>
<td>75</td>
<td>pT4N0M0</td>
<td>Alive 40 m</td>
<td>No</td>
<td>Codon 282 CGG-TGG</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Lateral tongue</td>
<td>57</td>
<td>cT4N0M0</td>
<td>DOC 8 m</td>
<td>No</td>
<td>Codon 242 TGC-TAC</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Alveolus</td>
<td>65</td>
<td>cT4N0M0</td>
<td>Alive 40 m</td>
<td>No</td>
<td>Codon 152 CGG-CCCG</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Floor of mouth</td>
<td>54</td>
<td>pT4N0M0</td>
<td>Alive 36 m</td>
<td>No</td>
<td>Wild-type</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Retromolar</td>
<td>63</td>
<td>cT4N0M0</td>
<td>Alive 40 m</td>
<td>No</td>
<td>Deletion 26 bp intron 4</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>Buccal mucosa</td>
<td>56</td>
<td>cT4N0M0</td>
<td>DOD 26 m</td>
<td>Local</td>
<td>Wild-type</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>Lateral tongue</td>
<td>85</td>
<td>pT4N0M0</td>
<td>DOD 8 m</td>
<td>Local</td>
<td>Codon 278 CCT-TCT</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>Retromolar</td>
<td>83</td>
<td>pT4N0M0</td>
<td>Alive 7 m</td>
<td>No</td>
<td>Wild-type</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>Alveolus</td>
<td>58</td>
<td>cT4N0M0</td>
<td>Alive 12 m</td>
<td>No</td>
<td>Wild-type</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>Lateral tongue/floor of mouth</td>
<td>51</td>
<td>pT4N0M0</td>
<td>Documentation</td>
<td>Wild-type</td>
<td>Wild-type</td>
<td>Wild-type</td>
</tr>
</tbody>
</table>

* DOD, died of disease; DOC, died of other causes; m, months; mets, metastases.

(nos. 1, 2, 3, 4, 5, and 16), demonstrating residual tumor cells harboring p53 mutations at the operative site. The estimated percentage of occult tumor cells in the surgical margins ranged from 0.05 to 10%. All patients received standard adjuvant treatment, including postoperative radiotherapy. During the period of study, six patients (nos. 1, 2, 3, 4, 5, and 16) with tumor-positive surgical margins by molecular analysis had biopsy-proven recurrences of carcinoma at the site of the primary tumor in the neck. In addition, one case (12), which could not be assessed by the phage plaque assay because of the nature of the tumor or in the neck. In addition, one case (12), which could not be assessed by the phage plaque assay because of the nature of the p53 mutation (insertion codon 152 CGG-CCCG), and a case with wild-type p53 (14) developed a recurrence at the local site.

Samples of mucosa, assessed as histologically normal, gave 5–43% red colonies (Table 2). For each sample giving a positive reaction, at least six plasmids were rescued from red colonies and sequenced. Nonclonal mutations were detected for 17 cases, but this is the expected result when the mutant content approaches the background. One case (no. 6, primary tumor in the buccal sulcus and recurrence in the fauces) was found to have 16–43% red colonies when multiple biopsies of tumor were examined. Two of the three biopsies examined harbored clonal mutations at codon 205 (TAT-GAT); nonclonal mutations were found for the other sample tested. The mutation detected in the primary tumor was at codon 175 (CGC-ACG), whereas the local recurrence in the fauces harbored codon 205 (TAT-GAT) and lower levels of 175 (CGC-ACG). Local recurrence also developed in one case (14) for which the margins could not be approached by the mutagenic assay. Cases 1, 2, and 4 were all found to have occult deposits in two of six (cases 1 and 2) and two of four nodes (case 4), which comprised 0.05–0.4% of all cells examined. Case 4 developed a nodal recurrence at the apex of the posterior triangle after a selective neck dissection. If the molecular information had been taken into account when the staging was performed, cases 1 and 2 would have been upstaged from N0 to N1.

Evidence of systemic disease, manifested by the finding of tumor cells in the bone marrow preoperatively, was found for 6 of 18 cases (nos. 3, 4, 5, 7, 16, and 18; for examples, see Fig. 3). Two of these cases developed multiple lung lesions (cases 3 and 5) and a third case developed a solitary lung nodule (case 4), which were ultimately the causes of death for these patients. The presence of SCC was confirmed by bronchoscopy for cases 3 and 4. All cases with evidence of widespread tumor dissemination had at least one tumor-positive lymph node after conventional light microscopic examination, and two of these nodal metastases showed extracapsular tumor spread. There was no relationship between the number of nodal metastases and the frequency of disseminated tumor cells. Tumor cells were also detected in the central venous blood preoperatively for two cases (3, 7), both with extracapsular nodal deposits of tumor detected by conventional light microscopy.
cells that have spread away from the primary site, following the cases to assess the prognostic significance of minimal residual cancer and precancer.

The present study used the \( p53 \) phage plaque assay in a prospective study to detect residual cancer at surgical margins considered to be tumor free after conventional light microscopy. We found a \( p53 \) mutation rate that is considerably higher (13 of 18; 72%) than the findings reported for a previous series [40–60%; reviewed by Partridge and Warnakulasuriya (29)]. The high mutation detection rate could reflect the small number of cases examined or a true difference in sensitivity; because the FASAY tests a larger region of the \( p53 \) cDNA than is normally examined (amino acids 53–364), the technique can detect mutations in the presence of large amounts of normal tissue, and the simple red/white read-out means that mutations are not overlooked easily.

Our findings confirm the pilot studies conducted by Brennan et al. (13), showing that molecular detection of malignant cells in apparently tumor-free surgical margins can identify patients likely to fail treatment at the local site. Application of the modified FASAY identified one patient with clinically and histologically normal mucosa harboring a \( p53 \) mutation, confirming that genetic aberrations can precede histological changes. The analysis also provides information about the likely clonal relationship of the index and second tumor, because a different \( p53 \) mutation (codon 205, TAT-GAT) was detected in several biopsies of tumor-distant mucosa and was present, together with very low levels of the mutation detected in the index tumor (codon 175, CGC-ACG), in the second tumor. These data strongly suggest that the recurrence arose as a result of a polyclonal process of field cancerization in the oral cavity and oropharynx. However, screening tumor-distant mucosa for \( p53 \) gene mutations only revealed molecular evidence of field cancerization for a single case. A further disadvantage of the FASAY is that this approach cannot differentiate between non-clonal PCR-induced mutations and true mutations in a very small number of cells. Thus, in view of the large number of yeast plasmids that must be rescued, purified, and sequenced to detect clonal aberrations, our data suggest that it is not be feasible to use this assay to screen patients for molecular evidence of field cancerization in future studies.

In the present study, radiotherapy was given according to standard protocols, and the additional information obtained from the molecular assay was not taken into account when the decision regarding the need for adjuvant therapy was made. Four cases who received postoperative radiotherapy developed local recurrence, despite aggressive adjuvant therapy. This lack of response may reflect an aggressive tumor phenotype or a failure of \( p53 \)-dependent apoptosis, because the product of the \( p53 \) gene modulates a significant proportion of the cytotoxic effects of radiotherapy (30). At this stage, analysis of further cases is required to clarify whether \( p53 \) status influences the response to radiotherapy. Nevertheless, the possibility that this group of patients may show a poor response to this treatment modality emphasizes the need for adequate surgery (31) or effective repeat surgery when positive surgical margins are found (11, 32–35).

It is well recognized that conventional light microscopy can miss the early stages of metastatic disease in lymph nodes (13, 36), and the finding of occult micrometastases in the present study confirms the value of the molecular approach. Clinically, this information is very important because two patients would have been designated as having a more advanced stage of carcinoma if the results of molecular analysis had been taken into account. The current TNM tumor classification system (37) is based on size rather than the number of tumor-positive lymph nodes. Patients found to have more than two tumor-positive nodes are treated routinely with postoperative radiotherapy. Thus, if this information had been taken into account when planning adjuvant treatment for cases 1 and 4, found to have additional tumor-positive nodes with the molecular assay, postoperative radiotherapy to both the primary site and the neck might have prevented development of recurrence for these cases.

The present strategies for supraomohyoid (levels I, II, and III) neck dissection and postoperative radiotherapy are largely based on the patterns of cervical lymph node metastases described by Shah et al. (38). However, recent evidence suggests that this pivotal study may underestimate the true frequency of micrometastases, because molecular analysis identified more tumor-positive lymph nodes and can stage patients more precisely. Today, the majority of patients have at least a functional neck dissection carried out as a staging procedure or to provide

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access to vessels for microanastomosis. Thus, the prognostic significance of these nodal micrometastases will never be known because they are removed routinely. In view of this, although the present study serves to highlight the fact that nodal micrometastases may be present, we did not compare the frequency of nodal micrometastases detected by light microscopy and molecular methods for many patients, because this would be labor intensive and of questionable value in the absence of prognostic data. Nevertheless, we need to bear in mind that the change in treatment protocol from radical to selective forms of neck dissection may leave micrometastases in the body that may not necessarily give rise to recurrences in the neck but which may provide a niche for the evolution of tumor cells with a phenotype conducive to development of distant metastases, which are ultimately responsible for the patient’s demise.

The finding of disseminated tumor cells in the bone marrow and central venous blood for cases 3, 4, and 5 developing lung lesions shows that a proportion of the disseminated tumor cells are true micrometastases, capable of forming a focus of tumor at a new site. Whether these disseminated tumor cells permeate the hematopoietic cell compartment indirectly via the lymphatics or as a consequence of direct perivascular extension is unclear at present. In this preliminary series, all patients found to have disseminated tumor cells in the bone marrow had lymph

<table>
<thead>
<tr>
<th>Case</th>
<th>Surgical margins</th>
<th>Molecular analysis a</th>
<th>Lymph nodes</th>
<th>Disseminated tumour b</th>
<th>FASAY</th>
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<tr>
<td></td>
<td>p53 phage-plaque</td>
<td>Microscopy</td>
<td>Lymph nodes</td>
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<td>Bone marrow</td>
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<tr>
<td>1</td>
<td>M1+ (1.2%)</td>
<td>M4−</td>
<td>0/7</td>
<td>LN1−</td>
<td>LN4−</td>
</tr>
<tr>
<td></td>
<td>M2+ (10%)</td>
<td>D1−</td>
<td>0/20</td>
<td>LN1−</td>
<td>LN4+ (0.05%)</td>
</tr>
<tr>
<td></td>
<td>M3−</td>
<td>D2−</td>
<td>0/35 EC spread</td>
<td>M4− (0.05%)</td>
<td>D1+ (5%)</td>
</tr>
<tr>
<td>2</td>
<td>M1+ (2%)</td>
<td>M4−</td>
<td>1/16</td>
<td>LN1−</td>
<td>LN4+ (0.3%)</td>
</tr>
<tr>
<td></td>
<td>M2−</td>
<td>D1+ (0.4%)</td>
<td>M4+ (0.1%)</td>
<td>D1−</td>
<td>LN3+ (0.4%)</td>
</tr>
<tr>
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<td>M3+ (2.5%)</td>
<td>D2−</td>
<td>9/43</td>
<td>ND</td>
<td>D1−</td>
</tr>
<tr>
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<td>3/12 EC spread</td>
<td>M4+ (0.2%)</td>
<td>D2− (0.2%)</td>
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<td>M2−</td>
<td>D1−</td>
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<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>M3−</td>
<td>D2−</td>
<td>0/6</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>4</td>
<td>M1−</td>
<td>M4−</td>
<td>0/6</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>5</td>
<td>M1+ (0.7%)</td>
<td>M4+ (0.2%)</td>
<td>1/9 EC spread</td>
<td>M4−</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>M2+</td>
<td>D1−</td>
<td>0/9</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>M3−</td>
<td>D2−</td>
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<td>ND</td>
</tr>
<tr>
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<td>M4−</td>
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<tr>
<td></td>
<td>M2−</td>
<td>D1−</td>
<td>0/6</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>M3−</td>
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<td>0/6</td>
<td>ND</td>
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<tr>
<td>7</td>
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<td>M4−</td>
<td>1/9 EC spread</td>
<td>M4+ (0.8%)</td>
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<tr>
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<td>1/9 EC spread</td>
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<td>ND</td>
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<tr>
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<td>M3+ (1.4%)</td>
<td>D2+ (0.6%)</td>
<td>0/6</td>
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<td>ND</td>
</tr>
<tr>
<td>8</td>
<td>M1−</td>
<td>M4−</td>
<td>1/9 EC spread</td>
<td>M4−</td>
<td>ND</td>
</tr>
<tr>
<td></td>
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<td>D1−</td>
<td>0/3</td>
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<td>M3−</td>
<td>D2−</td>
<td>0/1</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

a Figures in brackets indicate the highest percentage of mutant clones detected for each margin or lymph node, considered to be tumour-free after conventional light microscopy. ND, not done. EC, extracapsular.

b Number of immunostained cells with tumour cell morphology per 6 × 10^7 leucocytes examined.

c Each value refers to a separate biopsy.

d Mutation confirmed. Codon 205 TAT-GAT.
e Technical problems.
node metastases, but there are also reports of tumor dissemination in tumor-free necks (24, 25). We also observed a higher frequency of tumor cells in the hematopoietic cell compartment when extracapsular spread, a recognized risk factor for development of distant metastases, was present. There are reports in the literature suggesting that the presence of three or more tumor-positive lymph nodes correlates with development of distant metastases (5), suggesting that tumor cells with a migratory phenotype are more likely to be disseminated throughout the body and detected in both the hematopoietic and lymphatic systems. The presence of disseminated tumor cells may be a more reliable marker for risk of development of distant metastases than lymph node status alone, and this relationship will be investigated in a definitive study. However, it will also be important to establish whether the number of disseminated cells falls postoperatively. The study of other cancer types suggests that some tumor cells that remain after surgery are recognized and destroyed by the immune system within 3 months, whereas in other cases, disseminated cells persist after this time and represent a true minimal residual disease situation (39).

These preliminary findings support the need for substantive prospective studies to compare the sensitivity of light microscopy and new molecular approaches to detect residual disease and establish the prognostic significance of detection of minimal residual cancer. If these confirm that the presence of disseminated tumor cells and molecular analysis of surgical margins provides additional prognostic information for all or subgroups of patients, the results obtained with this new generation of diagnostics can be combined with conventional prognostic factors to provide a basis for identifying patients likely to benefit from early systemic adjuvant treatment.

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REFERENCES


Detection of Minimal Residual Cancer to Investigate Why Oral Tumors Recur Despite Seemingly Adequate Treatment

Max Partridge, Shu-Rui Li, Stelios Pateromichelakis, et al.


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