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Head and neck cancer and precancer: can we use molecular genetics to make better predictions?

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Over the past two decades, scientific study has identified key genetic errors which play a role in the development of the malignant cell. This knowledge has increased our understanding of the way in which these aberrations alter growth control and the interactions between cells and their surroundings which give rise to invasion and metastasis. Tumour suppressor genes, including the p53, retinoblastoma (Rb) and p16 genes, play a key role in the pathogenesis of head and neck cancers *(1)*, and several other chromosomal areas have been identified which are likely to harbour other sequences associated with development of this tumour type. A variety of oncogene aberrations have also been reported in these tumours *(1)*. However, little has been done to date to utilise this information for the benefit of the cancer patient, and the challenge which we now face is how best to use this new knowledge to make better predictions about tumour behaviour and inform treatment decisions.

At present, the TNM tumour 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 owing to the requirement to take into account tumour site, histology and information about tumour biology in addition to conventional TNM criteria. However, progress in this area can now be made using molecular genetics to profile the spectrum of genetic aberrations in a given cancer and compiling computerised patient-specific mutation databases for a large series of exophytic, invasive and metastatic cancers. Subsequent analysis of these data will indicate whether there are crucial steps which are common to all upper aerodigestive tract cancers, or whether some abnormalities occur at higher frequency in specific clinical subtypes. For example, we may gain insight into why some tumours show exophytic growth whereas others are invasive. Combining results which provide information about the key genetic abnormalities in each tumour with clinico-pathological details will also enable us to take a much broader outlook when evaluating the factors which affect outcome, and distinguish a lesion with a rapid growth rate and metastatic potential from one which is less aggressive and does not harbour the genetic changes associated with metastasis.

In addition to identifying molecular markers which can predict tumour behaviour, research is also required to establish why current treatment protocols fail to cure many patients with head and neck cancer. There have been very significant advances in surgery, reconstructive techniques and radiotherapy, such that the percentage of deaths attributed to locoregional recurrence has fallen from 40% to 27% *(2)*. However, the 5-year survival rate for this group of patients remains at a disappointing 50–60%. This is because more patients now live long enough to be exposed to the risk of developing a second upper aerodigestive tract cancer or distant metastatic disease, which are important and increasing causes of mortality. For example, in the series reported by Fardy and Langdon *(2)*, development of a second primary cancer in the upper aerodigestive tract was the cause of death for 20% of cases and distant metastases were responsible for a further 20% of all deaths. Post-mortem studies show an even higher rate of distant metastases of 40–60%. Thus, if improvements in cure rates are to be achieved, the factors which are responsible for both local and distant failure

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must be examined critically and treatment protocols modified accordingly.

Analysis of the results after treatment for patients with head and neck cancers suggests that local recurrence and distant metastatic disease occur as small deposits of tumour remaining in the body which are not detected by the current diagnostic procedures. This residual cancer may be present at the surgical margins, in the lymph nodes, or there may be dissemination of tumour throughout the body. However, treatment may also fail owing to the existence of genetic abnormalities throughout the upper aerodigestive tract mucosa which predispose patients to the risk of developing a second primary cancer. In addition to the presence of residual cancer, or other mucosa 'at risk', locoregional recurrence may also occur owing to an intrinsic lack of sensitivity of the tumour to radiotherapy or chemotherapy since, at present, there is no objective way of predicting tumour response to these treatment modalities.

In order to achieve better results we need to develop sensitive methods for tumour detection and ways of predicting tumour response to treatment, so that we can identify individuals with a good prognosis so that we do not overtreat them, and those likely to have poor outcome who should be targeted to receive appropriate adjuvant treatment at an early stage. The currently available imaging techniques may all fail to reveal low levels of disseminated disease. However, since genetic changes are essential for development of malignancy, they are ideal targets for tumour detection, and have the potential to provide a powerful new generation of molecular diagnostics for detection of residual disease. Comparison of the results obtained using these new molecular diagnostics with established practice will allow assessment of the contribution of residual cancer at the surgical margins and disseminated tumour to local and distant treatment failure.

When resecting head and neck cancers, most clinicians aim for a minimum of 1 cm clearance. However, the anatomy of the region means that this is often not achievable and studies reveal that 12–32% of cases have positive surgical margins *(3–5)*, although the true frequency of positive margins may be much higher. For example, Brennan *et al. (6)* have shown that, using a sensitive molecular diagnostic based on finding the same p53 gene mutation in the primary tumour and the surgical margins, malignant cells can be detected for 52% of patients with histopathologically negative margins. The same study reported that 38% of cases with positive margins after molecular analysis developed local recurrence, whereas no patient with negative margins failed locally. This methodology can also be used to provide a sensitive approach to screen lymph nodes, which are negative for tumour by light microscopy, for the presence of metastases.

The prognostic significance of disseminated tumour, determined by the finding of tumour cells in the peripheral blood and bone marrow, has been demonstrated for many solid tumours (Jauch *et al. (7)*). Preliminary studies involving patients with head and neck cancer have revealed that about one-quarter of patients with early stage tumours and one-half of those with advanced lesions have a tumour-positive bone marrow at presentation *(8,9)*. This suggests that although the skeleton is not the most common site of relapse for oral cancer, epithelial cells in the haematopoietic cell compartment reflect the general disseminative capacity of a tumour and identify patients with poor outcome. At present two approaches are used to detect these disseminated epithelial cells. One method uses immunocytochemistry (ICC) to look for expression of epithelial cell-specific targets, the other uses reverse transcription–polymerase chain reaction (RT–PCR) to screen for these gene transcripts. However, the frequency of these disseminated tumour cells in the haematopoietic cell compartment is often low *(8–10)*. To date, most studies have examined only micrometastases in the mononuclear cell fraction (MNC). However, tumour cells can be detected in both the MNC and granulocyte (GC) subfractions when leucocyte subsets are examined for patients with advanced head and neck cancer *(11)*, suggesting that previous studies may also underestimate the true frequency of disseminated tumour for this group of patients. The aim of this investigation was to use molecular techniques to start to build databases summarising the genetic aberrations associated with head and neck tumours, and apply knowledge about the key targets to develop sensitive and specific protocols for detection of residual cancer. The findings have been related to clinical outcome to determine whether this new information can help oncologists make better predictions about tumour behaviour.

Methods

Clinical samples and microsatellite analysis

Fifty-one primary head and neck SCC were snap frozen in liquid nitrogen. Surgical margins were harvested from four deep and two lateral margins of the tumour resection. One half was processed for conventional light microscopy, the other for molecular analysis. Peripheral venous blood (30 ml) and bone marrow aspirates (5–10 ml) were collected from cancer patients and healthy volunteers, and buffy coat cells prepared by mixing the sample with 0.1 vol dextran (mol. wt 50 000, 6% w/v). Ethical Committee approval for this project was granted at King's College Hospital. Clinicopathological features of the tumour examined are shown in Table I. SCC 4, SCC 9, SCC 15, SCC 25, HN5, CAL 27, Detroit 562, HepE2, KB, RPMI2650 (ATCC), UM-SCC-22A (kindly provided by Dr T Carey, Ann Arbor, Michigan) were maintained in DMEM and 10% FCS.

Tumour and leucocyte DNA were prepared for microsatellite assay as previously described *(12)*. Three polymorphic markers for polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) analysis and 13 microsatellite markers, close to, or within, the sequences which are frequently altered when head and

Table I. Clinicopathological features and allelotyping results of tumours examined

Case	TNM	Stage	Outcome	Survival (months)	D3S192	THRB	D3S686	D3S32	D3S1241	D3S1296	D3S1562	D3S30	D8S261	D8S298	Ank 1	D9S162	INF_α	D9S171	D9S43	D9S177	Rb	p53	DCC	FAL score
1			DoC	15	MSI	R	NI	NI	AI	AI	NI	AI	MSI	MSI	R	MSI	AI	R	AI	MSI	AI	MSI	AI	0.7
2			DoD	48	MSI	R	R	AI	MSI	MSI	ND	NI	NI	MSI	MSI	MSI	MSI	MSI	MSI	MSI	MSI	MSI	MSI	0.3
3			A	48	NI	ND	NI	R	R	ND	ND	R	ND	R	ND	R	ND	R	ND	ND	R	R	NI	0
4			DoD	24	R	ND	R	R	AI	NI	AI	NI	MSI	R	NI	R	R	R	R	R	R	R	R	0.14
5	T1 N0	1	A	120	NI	R	R	R	NI	AI	ND	R	NI	R	R	R	R	NI	AI	NI	R	NI	NI	0.18
6	T1 N0	1	DoD	63	R	R	R	NI	R	R	ND	NI	AI	AI	NI	AI	AI	R	AI	R	R	AI	MSI	0.42
7	T1 N0	1	A	60	NI	R	NI	NI	R	NI	NI	AI	NI	NI	R	R	AI	R	R	R	R	R	AI	0.25
8	T1 N0	1	DoD	57	AI	R	R	R	R	R	AI	R	NI	NI	NI	AI	R	NI	R	NI	R	AI	R	0.28
9	T1 N0	1	DoD	24	R	R	AI	R	NI	R	AI	NI	R	R	NI	R	R	AI	AI	R	R	R	AI	0.31
10	T1 N0	1	A	36	R	R	R	R	R	ND	ND	NI	NI	AI	ND	NI	AI	R	AI	R	R	NI	MSI	0.27
11	T1 N0	1	A	36	R	ND	R	R	R	R	NI	R	NI	AI	R	R	R	R	R	R	NI	AI	ND	0.14
12	T2 N0	2	DoD	48	R	NI	NI	AI	NI	R	MSI	NI	R	NI	NI	NI	R	AI	R	AI	R	NI	AI	0.4
13	T2 N0	2	A	60	R	R	AI	NI	R	ND	ND	R	R	R	ND	R	ND	NI	ND	ND	AI	ND	R	0.2
14	T2 N0	2	DoD	18	NI	ND	AI	NI	ND	ND	ND	R	ND	NI	ND	NI	ND	AI	ND	ND	AI	AI	NI	0.8
15	T2 N0	2	DoD	23	AI	R	R	R	R	NI	NI	AI	ND	ND	MSI	R	ND	NI	R	NI	R	AI	NI	0.3
16	T2 N0	2	A	140	R	R	R	NI	NI	R	ND	NI	R	NI	R	NI	R	R	R	AI	AI	NI	R	0.17
17	T2 N0	2	DoD	52	R	ND	NI	NI	R	MSI	ND	AI	R	R	AI	AI	AI	AI	R	NI	MSI	R	MSI	0.45
18	T2 N0	2	A	48	R	R	NI	NI	R	R	ND	R	AI	R	R	NI	NI	R	R	R	AI	R	NI	0.15
19	T2 N0	2	A	42	R	R	NI	AI	R	R	R	R	AI	NI	MSI	R	NI	AI	R	R	R	R	R	0.2
20	T2 N0	2	DoD	24	R	R	R	NI	NI	AI	AI	AI	AI	NI	R	R	NI	R	R	R	AI	NI	R	0.38
21	T2 N1	2	A	110	NI	R	R	NI	NI	R	R	R	NI	NI	NI	R	NI	R	R	R	R	R	NI	0
22	T2 N1	2	A	48	R	R	R	NI	NI	R	MSI	NI	R	NI	R	R	R	NI	R	NI	R	R	AI	0.08
23	T2 N0	2	A	52	R	R	R	R	AI	NI	AI	R	R	NI	R	R	R	R	R	R	AI	NI	R	0.18
24	T2 N0	2	A	48	MSI	ND	R	AI	ND	AI	ND	AI	ND	ND	ND	ND	NI	R	MSI	R	ND	R	R	0.37
25	T2 N0	2	DoD	3	R	AI	AI	R	NI	NI	AI	R	NI	R	NI	R	R	AI	R	R	AI	R	R	0.33
26	T2 N0	2	DoD	12	NI	ND	R	R	R	AI	MSI	R	ND	ND	R	NI	AI	R	NI	AI	R	R	NI	0.27
27	T2 N0	2	A	136	NI	ND	R	NI	AI	ND	ND	R	NI	NI	MSI	AI	NI	R	AI	R	R	R	NI	0.33
28	T2 N1	3	DoD	4	AI	ND	AI	NI	NI	NI	ND	NI	MSI	AI	R	R	R	NI	R	NI	NI	MSI	R	0.37
29	T3 N0	3	A	72	R	R	ND	R	NI	R	NI	NI	NI	R	NI	AI	AI	AI	R	R	R	R	MSI	0.25
30	T2 N1	3	A	50	NI	R	NI	R	R	R	R	NI	R	R	R	R	R	R	R	R	R	AI	R	0.06
31	T2 N1	3	DoD	18	NI	AI	R	R	R	R	R	NI	R	R	NI	NI	AI	R	AI	NI	R	NI	R	0.23
32	T2 N1	3	DoD	36	AI	AI	R	AI	AI	NI	NI	R	NI	R	R	NI	NI	NI	R	R	NI	AI	R	0.41
33	T3 N0	3	DoD	1	R	R	R	R	R	NI	R	NI	R	NI	R	R	NI	R	R	R	R	R	NI	0
34	T2 N1	3	DoC	42	R	R	NI	N	R	AI	R	AI	R	ND	NI	ND	MSI	R	NI	AI	R	R	AI	0.33
35	T2 N1	3	A	40	AI	ND	AI	N	NI	R	R	R	NI	R	R	NI	R	R	R	NI	R	R	R	0.15
36	T3 N0	3	A	166	R	R	R	R	NI	R	NI	R	R	R	NI	ND	R	R	R	MSI	AI	R	MSI	0.07
37	T3 N0	3	DoD	80	MSI	ND	R	N	NI	R	NI	NI	AI	AI	AI	NI	R	R	R	AI	AI	AI	R	0.5
38	T4 N2	4	DoD	8	MSI	AI	R	N	AI	AI	AI	AI	R	ND	AI	NI	AI	R	AI	R	R	AI	AI	0.66
39	T4 N2	4	DoD	36	NI	AI	AI	AI	AI	R	MSI	NI	NI	AI	NI	AI	AI	NI	R	AI	NI	NI	AI	0.8
40	T4 N2	4	DoD	12	NI	AI	NI	R	R	NI	AI	R	NI	NI	NI	AI	AI	R	R	NI	NI	AI	R	0.45
41	T4 N2	4	DoD	4	R	AI	AI	AI	R	AI	AI	R	R	NI	NI	MSI	MSI	AI	AI	AI	AI	AI	AI	0.73
42	T3 N0	4	A	36	AI	R	R	AI	R	R	R	NI	R	R	R	ND	R	NI	MSI	R	R	NI	NI	0.15
43	T4 N2	4	DoD	24	NI	AI	AI	AI	AI	AI	R	AI	MSI	ND	AI	MSI	MSI	AI	AI	MSI	AI	R	AI	0.84
44	T3 N2	4	DoD	18	R	AI	R	N	NI	R	NI	R	NI	R	MSI	R	R	AI	NI	AI	R	NI	NI	0.27
45	T3 N1	4	NA	50	AI	AI	NI	N	R	R	ND	AI	NI	AI	AI	NI	NI	R	NI	R	R	R	R	0.41
46	T3 N2	4	A	28	AI	R	R	R	NI	NI	R	AI	ND	ND	AI	AI	R	NI	NI	AI	MSI	NI	NI	0.5
47	T4 N1	4	NA	75	AI	R	R	R	R	R	ND	R	NI	R	MSI	NI	NI	AI	R	ND	NI	R	R	0.16
48	T4 N0	4	DoD	24	AI	R	AI	AI	R	R	ND	NI	MSI	MSI	R	NI	R	AI	ND	ND	AI	AI	MSI	0.54

Key: A = alive. NA = data not available. DoD = died of disease. DoC = died of other causes.
AI-allelic imbalance. R = retention of heterozygosity. MSI = microsatellite instability. NI = non-informative.
ND = not done. FAL = fractional allelic loss.

neck tumours are examined, were selected for analysis (Table II). Cases were scored by visual inspection of band patterns and considered to show allelic imbalance (AI) if the ratio of the two alleles in the tumour was 50% less than that detected for the normal sample. Novel microsatellite alleles were identified by the presence of bands which were absent in the normal sample. A fraction allelic loss (FAL) score *(13)* was calculated for each patient by dividing the number of loci showing AI by the number of informative loci. Survival curves were derived using the Kaplan–Meier product-limit method and compared by the log rank test.

Table IIa. Microsatellite markers used for analysis of allelic imbalance

Locus	*% Informative cases showing allelic imbalance*
D3S192 (3p24.1-26)	32
THRB (3p24. 1-2)	28
D3S686 (3p21.33)	34
D3S32 (3p21.32)	28
D3S1241 (3p21.1)	27
D3S1296 (3p13)	29
D3S1562 (3p13)	47
D3S30 (3p13)	35
D8S261 (8p23.1)	25
D8S298 (8p22)	29
Ank-1 (8p12)	26
D9S162 (9p21)	31
INFα (9p21)	37
D9S171 (9p21)	32
D9S43 (9p13)	38
D9S177 (9q22.3)	37
Rb (13q14.2)	34
TP53 (17p13.1)	36
DCC (18q21.1)	35

Chromosomal location of microsatellite markers is shown in parentheses

Table IIb. Relationship between outcome and allelic imbalance at chromosomal loci tested

Loci examined	*Chromosomal region*	*HR for survival*	*P*
D3S192, THRB	3p24–26	4.21	0.0002
D3S32, D3S1241, D3S1562	3p21	1.99	0.07
D3S1296, D3S1562, D3S30	3p13	2.52	0.02
D8S261, D8S298, Ank-1	8p12-23	1.25	NS
D9S162, INFα, D9S171	9p21	2.65	0.01
D9S43	9p13	1.56	NS
D9S177	9q22.3	1.78	NS
Rb	13q14.2	1.16	NS
p53	17p13.1	1.85	NS
DCC	18q21.1	2.39	0.05

HR = Hazard ratio

Molecular assessment of tumour at the surgical margins and within lymph nodes

Frozen 10 μm sections were lysed using DNA Stat 60 (Biogenesis, UK) and RNA and DNA prepared according to the manufacturer's instructions. The presence or absence of a mutation affecting the p53 gene was determined for each tumour by screening p53 cDNA with a Functional Analysis of Separated Alleles in Yeast (FASAY *(14)*), followed by sequencing of plasmid DNA rescued from yeast-yielding red colonies. DNA extracted from the tumour and surgical margins was used to amplify exons 5–9 of the p53 gene by PCR and the products cloned into a bacteriophage vector for further amplification in *E Coli*. Colonies were transferred to nylon membranes and hybridised with oligonucleotide probes recognising the mutant p53 gene identified for each tumour, and wild type p53 as described by Brennan *et al. (6)*.

Immunomagnetic separation to enrich clinical samples for tumour, immunocytochemistry, immunohistology and keratin 19 RT-PCR

In model experiments to compare the efficiency of positive and negative immunomagnetic selection, varying numbers of SCC 25 cells (10–1000) were positively selected from 2×10^7 leucocytes, using M280 Dynabeads coated with the mouse monoclonal antibody Ber-EP4 (Dynabeads epithelial cell detection kit, DYNAL, Oslo, Norway). Rosetted cells were isolated and washed using a magnet and attached to microscope slides for ICC using A45-B/B3 (fab) (EPIMET® epithelial cell detection kit, Micromet, Munich, Germany) (Fig. 1). Negative immunomagnetic selection was assessed in similar spiking experiments with M450 Dynabeads coated with anti-CD45 (recognising the common leucocyte antogen). Non-rosetted cells were sedimented on to microscope slides and stained with a pancytokeratin antibody AE1/AE3, diluted 1:50 (Dako, High Wycombe, UK), using an APAAP technique *(15)*. Negative controls were leucocytes obtained from healthy volunteers stained with mouse anti-FITC (Sigma, Poole, UK), of the same subclass (IgG_1), using the same

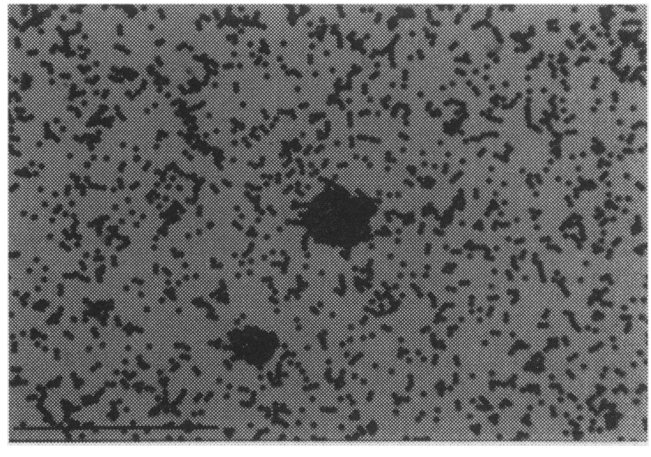

Figure 1. Tumour cell detection using positive immunomagnetic separation and immunocytochemistry with the EPIMET® epithelial cell detection system.

concentration of immunoglobulin. Positive controls were head and neck SCC cell lines. Leucocytes (6×10^7), obtained from peripheral blood and bone marrow aspirates, were enriched for tumour for each patient using anti-CD45 immunomagnetic beads and epithelial cells identified by ICC with AE1/AE3 as described above. Leucocytes, 2×10^6, were also examined for each case using the standard ICC technique without negative immunomagnetic selection. SCC cell lines and 5 μ frozen sections prepared from 20 oral SCC were examined with an antibody recognising the Ber-EP4 epitope (diluted 1:20, Dynal, Oslo, Norway) and the antibody reaction developed with APAAP.

RNA was prepared from bone marrow and peripheral blood using RNAStat 50 (Biogenesis, UK). mRNA was isolated from 10 μg total RNA using the Dynabeads mRNA DIRECT kit (Dynal, Oslo, Norway) and reverse-transcribed to produce cDNA. cDNA (5 μl) was used for each PCR reaction. The suitability of RNA for RT–PCR was confirmed by amplification of β_2 microglobulin cDNA (primer sequences CTCGCGCTACTCTCTC TTTCT, TGTCGGATTGATGAAACCCAG). Primer sequences used for PCR amplification of keratin 19 were designed to maximise mismatches between the keratin 19 gene and the two processed pseudogenes (K1 outer forward GTGGAGGTGGATTCCGCTCC, K2 outer reverse TGGCAATCTCCTGCTCCAGC, K3 inner forward ATGGCCGAGCAGAACCGGAA, K4 inner reverse CCATGAGCCGCTGGTACTCC, Dynal, Oslo, Norway *(16)*. Positive and negative controls were included in each round of PCR. RT–PCR products were analysed by agarose gel electrophoresis and visualised after staining with ethidium bromide. To minimise the risk of contamination, and hence false-positive results, the different steps of the RT and PCR processes were carried out in different laboratories.

Results

Commonly occurring genetic aberrations and their prognostic significance for head and neck SCC

Applying microsatellite analysis to examine a series of 48 primary head and neck SCC revealed that these tumours show a high frequency of AI at the short arm (p) of chromosome 3 (for example, 47% AI at D3S1562), at 9p (37% AI at the INFα locus) and within the p53 (36%) and DCC (34%) tumour suppressor genes (Table I and Table IIa, Fig. 2*a*). However, a variety of other chromosomal loci were also found to show similar aberrations at a lower frequency. AI at all loci tested was found for patients with early stage 1 and stage 2 tumours and for those with advanced stage 3 and stage 4 lesions. A variety of different patterns of genetic aberration were detected for tumours classified as being of the same TNM stage, such that no specific pattern of events was associated with early or late stage cancers.

Separation of the cases into three approximately equally sized groups on the basis of the FAL score revealed a significant relationship between FAL and overall survival (Fig. 2*b*), with relative death rates in low, intermediate and high FAL score groups of 1, 5.4 and 8.4, respectively ($P<0.0002$). Multivariate analysis, using Cox's regression, revealed that this relationship is independent of TNM stage (P value on univariate analysis 0.0002, 0.0006, allowing for stage). Univariate analysis was also performed to assess whether AI at sequences within the p53, Rb and DCC tumour suppressor genes, or at one or more loci within the candidate suppressor gene areas for head and neck cancer, was associated with a poor prognosis. (The results for several adjacent loci, which identify the candidate tumour suppressor gene areas, were combined to reduce multiple significance testing (Table IIb)). AI within the three chromosomal regions investigated at 3p (3p24–26, 3p21 and 3p13) at 9p21 and within the THRB and DCC tumour suppressor gene loci, were all associated with reduced survival ($P< 0.01$). The loci or chromosomal regions which were significant on univariate analysis were also examined to see whether they were of independent prognostic significance, and AI at 3p24–26, 3p21 and at 3p13 were found to predict prognosis independently of each other. The hazard ratios for survival were 3.39, 2.6 and 2.56 ($P=0.002$, 0.03 and 0.03, respectively). This implies that any patient with AI at all of these three key chromosomal regions has an approximately 23 times greater increase in their mortality rate, relative to a case showing retention of heterozygosity (ROH) at these key chromosomal regions.

Development of sensitive protocols for disseminated tumour cell detection

In model experiments, when an antibody recognising the Ber-EP4 epitope, coupled to immunomagnetic beads, was used to enrich leucocytes for malignant cells, 23–40% of the tumour cell input was recovered (Table III). In contrast, negative immunomagnetic selection, using an antibody which removes the majority of CD45-positive haematopoietic cells, resulted in 48–70% tumour cell recovery. Subsequently, negative immunomagnetic enrichment was used to screen bone marrow aspirates and peripheral blood obtained from patients with advanced head and neck SCC for the presence of disseminated epithelial cells.

Of SCC 25 cells, 80% were scored as strongly positive for expression of the Ber-EP4 epitope when screened by ICC (*11*). However, examination of 20 primary head and neck SCC revealed heterogeneous expression of this epitope, with expression confined principally to the outer basal type cells of clumps of tumour. Head and neck SCC cell lines also showed varying percentages of positive cells SCC 9 (20%), SCC 15 (15%), SCC 25 (80%), HN5 (20%), CAL 27 (15%).

Using a single round of PCR, keratin 19 transcripts were readily detected in 10/11 (91%) SCC cell lines and 6/10 venous blood samples obtained from patients with advanced head and neck SCC (Fig. 3). However, transcripts were also found for 2/12 (17%) of bone marrow aspirates and 4/22 (18%) of peripheral bloods obtained from healthy volunteers. To exclude the possibility of contamination of blood and bone marrow

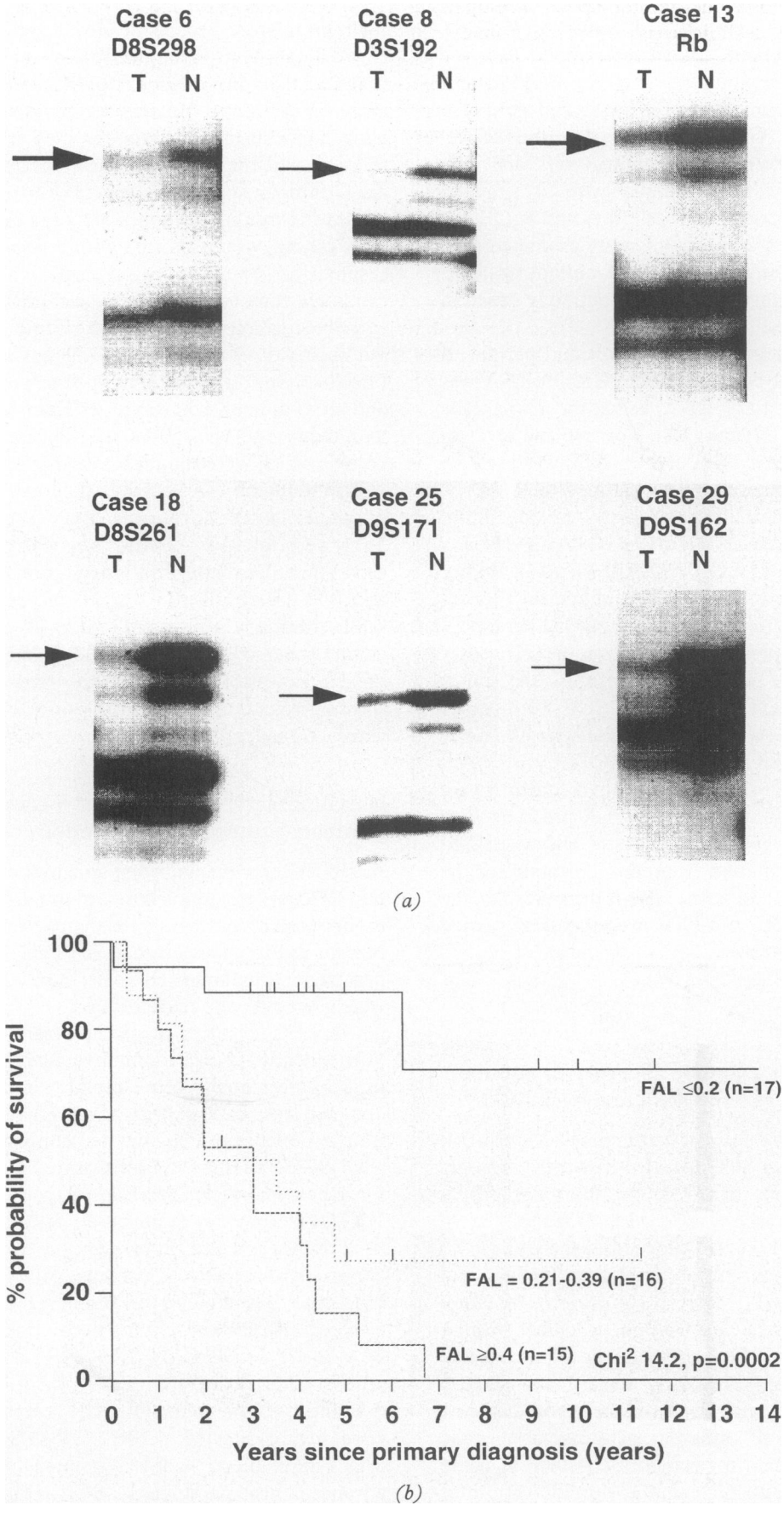

Figure 2. (a) Allelotyping results of representative SCC. Microsatellite alterations in tumour DNA (T) and leucocyte DNA (N). AI is shown at D8S298, D3S192, Rb, D8S261, D9S171 and D9S162. The arrows highlight the altered alleles. *(b)* Relationship between the FAL score and overall survival for 48 cases with primary head and neck SCC.

Table III. Tumour cell detection frequency, comparison of positive and negative immunomagnetic selection and the standard ICC technique (no enrichment) model experiments

	Number of tumour cells identified following immunocytochemistry		
Number of SCC 25 cells mixed with 2×10^7 leucocytes	*Positive immunomagnetic selection*	*Negative immunomagnetic selection*	*No enrichment (2×10^6 cells)**
1000	230 (23%)	530 (53%)	610
500	161 (32%)	320 (64%)	386
100	23 (23%)	48 (48%)	61
10	4 (40%)	7 (70%)	6

* Figure determined by counting 2×10^6 leucocytes, but with results shown as number of cells recovered for 2×10^7 leucocytes to facilitate comparison with the enrichment technique. Results shown are mean of triplicate experiments
Figures in parentheses show percentage of tumour input recovered

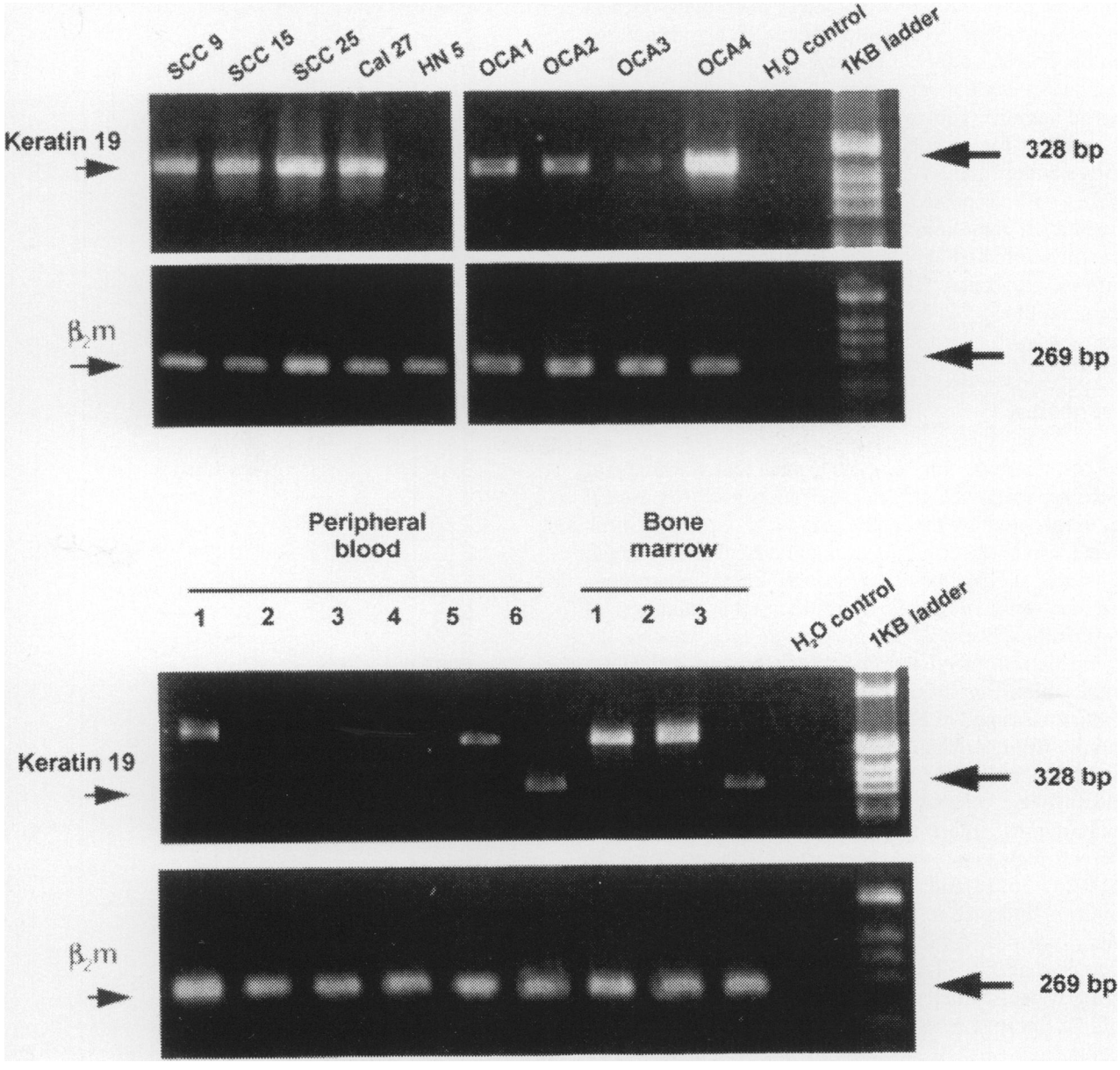

Figure 3. Products of RT–PCR for keratin 19 and β_2 microglobulin mRNA separated by gel electrophoresis and stained with ethidium bromide. Five head and neck cell lines are shown (HN5 does not express keratin 19), four patient venous blood samples (positive signals OCA 1–3), six venous blood samples from healthy volunteers (illegitimate transcripts lane 6), three bone marrow aspirates from healthy volunteers (illegitimate transcripts lane 3), H_2O control and 1 Kb molecular weight markers. (The larger 450bp bands represent amplification of contaminating genomic DNA.)

samples examined, the samples we also screened for expression of the melanoma-associated gene 3 (MAGE 3) by RT–PCR. This target is expressed by 55% of head and neck SCC, but no positive signals were seen when the patient samples were tested (data not shown).

Utilisation of new diagnostics to investigate the reasons for treatment failure for patients with head and neck SCC

Table IV summarises data for three representative cases, obtained using the p53 phage plaque assay (Fig. 4) to screen surgical margins and lymph nodes for malignant cells harbouring the p53 mutation present in the primary cancer, together with ICC to detect micrometastases in the haematopoietic cell compartment. When compared with the findings after conventional light microscopy, the p53 phage plaque assay identified more tumour-positive surgical margins (approximately 0.2–12% mutant plaques per sample) for case 50 and the presence of tumour-positive lymph nodes (0.4–2.0% mutant clones) for cases 49 and 50, reported as being tumour free after conventional light microscopy. All cases were found to have disseminated epithelial cells in bone marrow aspirates, and cases 50 and 51 micrometastases in the peripheral blood.

Discussion

The results from this analysis reveal that a variety of different patterns of genetic aberration at chromosomal loci, implicated in the pathogenesis of head and neck cancers, can be detected for tumours classified as being of the same TNM stage. Although these aberrations represent only a fraction of the total number of abnormalities likely to be present in each cancer, the varying pattern of AI found for each tumour provides a partial explanation for the different clinical behaviour that tumours, classified as being of the same TNM stage, may exhibit. Summarising the number of loci showing aberrations as a FAL score identified a subgroup of patients likely to show reduced survival, and provided new prognostic information. The finding that AI at three different regions at the short arm of chromosome 3p (3p24–26, 3p21, and 3p13) identifies patients with a significant increase in their mortality rate, relative to a patient with a tumour not showing these changes, suggests that it will be possible to develop molecular staging systems which can help predict outcome, based on profiling the spectrum of genetic abnormalities in a given tumour.

However, we need a more comprehensive description of the events which occur in head and neck tumours if we are to understand them. At this stage these data must be combined with information about abnormalities affecting the oncogenes and sequences which control DNA repair, to build patient-specific mutation databases. These will provide a mechanism for relating genetic events to specific

Table IV. Assessment of the causes of treatment failure for patients with head and neck cancer, as determined by molecular analysis of surgical margins, conventional light microscopy and using immunocytochemistry to detect disseminated epithelial cells

	Outcome			*Light microscopy*		*p53 phage plaque assay*		*Immunocytochemistry with negative immunomagnetic selection*	
Case	*Survival time (months)*	*Locoregional failure*	*Distant failure**	*Surgical margins*	*Lymph node*	*Surgical margins*	*Lymph node*	*Bone marrow*	*Peripheral blood*
49	A (18)	No	Yes	Negative (0/6)	Negative (0/13)	Negative (0/6)	Positive (2/13)	Positive	Negative
50	D (14)	No	Yes	Positive (2/6)	Negative (0/15)	Positive (6/6)	Positive (2/15)	Positive	Positive
51	D (4)	Yes	Yes	Positive (2/6)	Positive (9/43)	ND	ND	Positive	Positive

A = alive. D = dead. ND = not done
* All lung lesions. Figures in parentheses indicate number of margins or lymph nodes positive as a fraction of the total examined

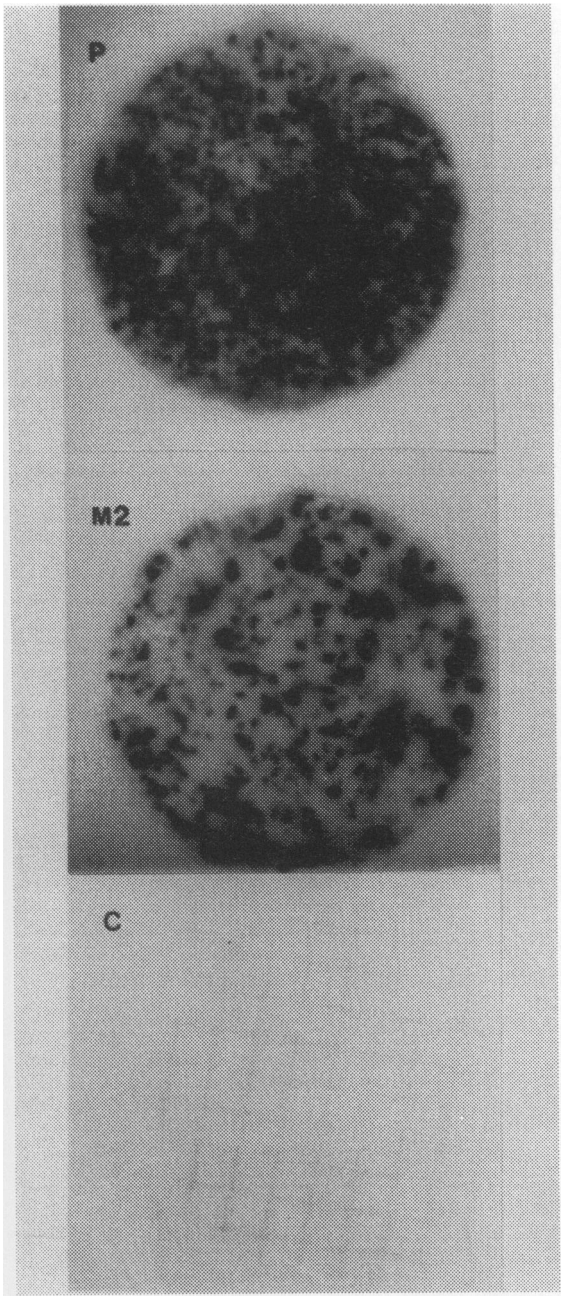

Figure 4. Representative p53 phage plaque assay, case 50. Filters from the primary tumour (P) screened with an oligonucleotide probe designed to recognise the mutation present in the index tumour. The black areas represent the presence of mutant p53 sequences. The surgical margin (M2) contains plaques which hybridise with the mutant-specific probe indicating the presence of malignant cells at this surgical margin. Negative control (C), testing DNA from another primary head and neck SCC harbouring a different p53 mutation with a probe which recognises the p53 mutation in the primary tumour (P), produces no signal.

tumour subtypes and shed light on the genetic aberrations associated with the different clinical presentations.

Several studies have shown that it is possible to use this new knowledge about the key genetic and immunophenotypic differences between normal and cancerous cells to detect disseminated epithelial cells (*6–11*). However, the findings from this study highlight several difficulties which need to be overcome when developing sensitive and specific protocols to detect micrometastases in the haematopoietic cell compartment for patients with head and neck cancer. The sensitivity of the method selected depends on the number of haematopoietic cells examined, and in order to increase the number of leucocytes screened without increasing the number of microscope slides which need to be scored, protocols based on positive or negative immunomagnetic selection of tumour cells from clinical samples have been developed. This investigation used model experiments, spiking leucocytes with tumour cells cultured *in vitro*, to compare the efficiency of positive and negative immunomagnetic selection to enrich clinical samples for tumour. Negative immunomagnetic selection with anti-CD45 Dynabeads was found to result in recovery of a higher percentage of the tumour cell input than protocols based on the use of antibodies recognising the Ber-EP4 epitope. This latter approach has been used successfully in previous studies to enrich samples of blood or bone marrow for patients with breast or colon carcinoma *(10)*. The antibody reacting with the Ber-EP4 epitope recognises two glycopeptides (mol. wt 34 000 and 39 000), present on the surface and in the cytoplasm of some epithelial cells, but does not recognise haematopoietic cells *(17)*. However, whereas 80% of SCC 25 cells were scored as strongly positive for expression of the Ber-EP4 epitope when screened by ICC *(11)*, examination of other head and neck cell lines and 20 primary SCC revealed heterogeneous expression of this epitope, with Ber-EP4 detected principally on the outer basal type and suprabasal cells of clumps of tumour. This heterogeneity of Ber-EP4 antigen on the target cells is likely to account for the poor tumour cell recovery from clinical samples in this study.

Although only limited comparisons have been made to date, RT–PCR may be more sensitive than ICC in terms of tumour detection. The molecular approach also has the advantage that the readout is available instantly and is more objective than ICC. Many RT–PCR protocols for tumour cell detection have used keratin 19 as the epithelial cell-specific target, designing primers to avoid amplification of the known processed pseudogene. The PCR primers used for the present study also incorporate mismatches to avoid amplification of a second keratin 19 pseudogene *(16)*. Keratin 19 RT–PCR products were identified for 10/11 (91%) head and neck SCC tumour cell lines tested and some patient samples also gave positive signals (Fig. 3). However, keratin 19 transcripts were also detected for 17% of bone marrow aspirates and 18% of peripheral blood samples obtained from healthy volunteers. Every precaution was taken to minimise contamination and hence false-positive results and MAGE 3 RT–PCR products were not identified when the patient samples were screened for the presence of this target. Normal cells in the peripheral blood and bone marrow do not transcribe keratin 19; however, the findings from this study strongly suggest that rare haematopoietic cells do express illegitimate transcripts owing to incomplete repression of keratin 19 gene transcription. In view of this, protocols based on RT–

PCR to screen for keratin 19 mRNA cannot be considered specific for detection of disseminated epithelial cells in the haematopoietic cell compartment.

Application of phage plaque assay, to screen surgical margins and lymph nodes for malignant cells containing the signature p53 mutation present in the primary tumour, identified a higher number of tumour-positive surgical margins for case 50 (Table IV), and the presence of tumour-positive lymph nodes for cases 49 and 50, considered to be tumour free after conventional light microscopy. All cases were found to have disseminated epithelial cells in the bone marrow and two cases had micrometastases in the peripheral blood. The finding of disseminated epithelial cells in the haematopoietic cell compartment and the presence of tumour-positive draining lymph nodes strongly suggest that the lung tumour (squamous cell carcinoma), which was the cause of death for case 50, arose owing to arrest and subsequent proliferation of metastatic cells in the lung bed. However, it is not possible at this stage to establish conclusively whether this solitary lung lesion was a metastasis from the index tumour or a second primary cancer. Further study, to demonstrate the presence of identical key mutations and clonal markers, is required to establish the origin of the paired tumours.

However, these preliminary findings support the need for further study to compare the sensitivity of light microscopy and new molecular approaches to detect residual disease, and establish the prognostic significance of detection of positive surgical margins and disseminated tumour cells in the haematopoietic cell compartment. It will also be important to determine whether the frequency of micrometastases is greater in the peripheral blood or bone marrow, and whether the number of disseminated epithelial cells falls postoperatively. There is evidence, from study of other tumour types, to suggest that some disseminated tumour cells which are detected preoperatively in the haematopoietic cell compartment are recognised and destroyed by the immune system and cleared from the circulation after surgery *(18)*. Thus, it seems likely that only a few of these disseminated cells will be capable of forming new attachments and proliferating to form a new foci of tumour if the local environment is suitable, an interaction commonly referred to as the seed and soil hypothesis *(19)*.

If prospective studies do show that the presence of disseminated tumour cells and molecular analysis of surgical margins provide additional prognostic information, the results obtained with this new generation of diagnostics can be combined with standard prognostic factors of tumour size, lymph node status and tumour grade to provide a basis for identifying patients likely to benefit from early systemic adjuvant treatment. However, if these micrometastases are shown to be an important cause of distant relapse, more effective systemic treatments will also be needed. In the future, it may also be possible to utilise assays which detect tumour cells in the haematopoietic cell compartment to measure the ability of chemotherapeutic agents to clear disseminated tumour cells, and establish whether or not remission has been achieved. If the current protocols are shown to be ineffective at clearing micrometastases, this will provide a basis for adding new drugs and considering dose escalation regimens.

Thus, at the present time there is a new opportunity to dovetail clinical, histological and molecular diagnostics to achieve better results. John Hunter would be intrigued by the insight into the basic biology of cancer gained using modern genetics, and with the possibility of using this information to make better predictions and improve patient care. He was a forward-looking and innovative surgeon and was always receptive to new ideas. However, he would also hope that his message of the importance of scientific method in surgery would encourage us to evaluate these powerful new diagnostics carefully before changing our current treatment protocols.

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CORRECTION

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Current practice in primary total hip replacement: results from the National Hip Replacement Outcome Project

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