Methodological issues in the use of tumour markers in cancer epidemiology

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In this chapter, we review major methodological and practical issues associated with the use of tumour markers. At this stage of development, studies with a combination of tumour, susceptibility and exposure markers are needed to illustrate the link between exposure and biological response and to assess the interactive effects of tumour susceptibility markers in this process. Several practical issues related to the application of tumour markers are discussed, including banking of tumour tissue, setting a laboratory strategy and performing etiological heterogeneity analysis.

Recent advances in technology aimed at identifying genetic mutations and abnormally expressed products have led to an interest in incorporating these alterations as tumour markers in epidemiological studies in an attempt to better understand the natural history and the etiology of cancer. There is a concomitant need for research into methodological issues including study design, statistical analysis, and interpretation as well as practical issues such as tumour tissue storage.

Tumour markers have been defined by Schwartz (1989) as: ‘... substances which can be measured quantitatively by biochemical or immunochemical means in tissue or body fluids to identify the presence of a cancer, possibly the organ where it resides, to establish the extent of tumour burden before treatment as well as to monitor the response to therapy.’

The term has been defined more narrowly as (Klavins, 1989): ‘... substances that are produced by cancer cells and present in the circulation of cancer patients at a higher concentration than in individuals without cancer. It can be used to monitor the course of the disease.’

More broadly, tumour markers can be defined as those abnormal biological products or molecular alterations related to any sequence of multistage carcinogenesis, such as tumour initiation and promotion, and may be measured quantitatively or qualitatively by biochemical, immunochemical, cytogenetic and molecular techniques in human biological materials, including tumour tissues, blood and urine samples, etc. These markers may be employed to predict primary or secondary tumour risk, to establish tumour burden, to sub-classify tumours beyond histological classification (in addition to pathological classification), to predict tumour prognosis, to determine treatment strategy and to evaluate chemoprevention or intervention efficacy. They can be employed in primary prevention or etiological research by detecting the relationship between environmental exposure and specific mutations; in secondary prevention studies (i.e. early detection and diagnosis) by identifying a precursor or tumours at the early stage; and in tertiary prevention (i.e. enhanced prognosis) if they predict tumour progression and patient survival.

In this chapter, we will briefly review some tumour markers of relevance to etiological studies of cancer and discuss methodological issues associated with the use of those markers in cancer epidemiology, including issues of study design and statistical analysis. We will also discuss several tumour-marker-related practical issues, including the collection and storage of tumour-marker-related biological materials, the laboratory strategy for measuring tumour markers, and issues associated with the use of tumour markers in prognostic studies.
**Etiological heterogeneity**

The theoretical framework for the use of tumour markers in cancer epidemiology is based on the hypothesis of etiological heterogeneity. The concept of etiological heterogeneity was first suggested by Kreyberg (1937), and was based on the fact that a relationship might exist between histological types and biological or etiological factors. In epidemiological studies of smoking and lung cancer, Kreyberg (1952, 1954) suggested that lung cancer was histologically and biologically heterogeneous and related to particular exposures. The relationships between stage, grade, histology, anatomic location of the tumour and etiological factors have been explored for a number of different tumour types (Palli et al., 1992; Sturgeon et al., 1994; Vaughan et al., 1995). The study of etiological heterogeneity using tumour markers represents a progression from studies of the relationship of environmental exposures at the anatomical and morphological levels to studies at molecular and genetic levels.

In perhaps one of the earliest studies of this kind to examine etiological heterogeneity at the molecular level, Taylor et al. (1992) conducted a study of the relationship between occupational exposures and the activation of the ras oncogene in the etiology of acute myeloid leukaemia (AML), employing a conventional control group identified by random digit dialling. In this study, in addition to calculating unadjusted and adjusted odds ratios characterizing the association between occupational factors and AML, the investigators also assessed the association between the occupational risk factors and mutations of the ras gene in cases with AML. Several recent studies have now expanded this paradigm by evaluating the etiological heterogeneity of tumours, exploring the relationship between environmental exposures and TP53 mutations/p53 overexpression in tumour samples from a variety of tumour types, including lung cancer (Kondo et al., 1992; Suzuki et al., 1992; Taylor et al., 1994), head and neck cancer (Field et al., 1991), oesophageal cancer (Hollstein et al., 1991a), bladder cancer (Spruck et al., 1993; Zhang et al., 1994b), colorectal cancer (Zhang et al., 1995b; Freedman et al., 1996a, 1996b), prostate cancer (Zhang et al., 1994a), stomach cancer (Zhang et al., 1995a), liver cancer (Bressac et al., 1991; Hsu et al., 1991; Ozturk 1991) and skin cancer (Brash et al., 1991). The study of the etiological heterogeneity of tumours at the molecular level may provide great insight into the mechanisms and causal pathways to carcinogenesis, which may lead to appropriate preventive strategies to reduce the incidence of cancer.

**Tumour markers**

Tumour markers include all of the biological products related to the development and progression of neoplastic disease. Several markers are quantitatively measured and may indicate the burden or extent of the cancer, such as serum carcinoembryonic antigen (CEA), alphafetoprotein (AFP) and prostate-specific antigen (PSA). Since these markers have been discussed frequently in the literature (Chu, 1987; Sell, 1992; Kramer & Srivastava, 1994), we will not discuss them in this chapter. In the following section, we will briefly review three types of tumour markers with etiological implications: cytogenetic markers such as chromosome aberrations, oncogenes such as the ras family, and tumour suppressor genes such as the TP53 gene.

**Cytogenetic markers**

Since the use of cytogenetic markers has been extensively reviewed by Tucker et al. (this volume), we will limit our discussion to the cytogenetic analysis of lymphohaematopoietic malignancies. Cytogenetic markers in this context include chromosome aberrations, sister chromatid exchange and micronuclei, which can be measured in both tumour tissues and peripheral lymphocytes. Specific cytogenetic changes were found to be non-random events involved in multistep carcinogenesis. Similar cytogenetic changes were identified in second primary acute non-lymphocytic leukaemia (ANLL) after chemotherapy (Mitelman et al., 1981; Rowley, 1983) and in patients with occupational exposures (Mitelman et al., 1981; Golomb et al., 1982; Rowley, 1983; Mitelman et al., 1984). A high correlation was observed between sites of cytogenetic aberrations and positions of oncogene or tumour suppressor gene loci (Rowley, 1984).

Environmental exposures, cytogenetic markers and lymphohaematopoietic malignancies. Sandler and Collman (1987) have extensively reviewed issues concerning cytogenetic and environmental factors in the etiology of acute leukaemia. Sandler et al.
(1993) investigated the leukaemia risk associated with cigarette smoking and cytogenetic changes in a multicentre case-control study of acute leukaemia in adults. Smoking was found to be more common among patients with specific chromosome abnormalities in acute myeloid leukaemia (AML) [7 or 7q-, −Y, +13] and in acute lymphocytic leukaemia (ALL) [t(9;22)(q34;q11)]. In a case–case study of 59 patients with newly diagnosed AML (Fagioli et al., 1992), 18 patients had prolonged contact with pesticides and seven patients had exposure to organic solvents. Cytogenetic studies confirmed the frequent occurrence of 5q and/or 7q aberrations in patients with occupational exposure (10 out of 25 cases). These findings revealed that AML in patients occupationally exposed to toxic substances might represent a distinct cytogenetic entity.

In a case–case study of 162 patients with ANLL (Mitelman et al., 1981), 52 patients were occupationally exposed to chemical solvents, insecticides or petrol products, and 110 patients had no history of occupational exposure to potential mutagenic or carcinogenic agents. Clonal chromosomal aberrations were present in 75% of exposed patients, compared with only 32% in the unexposed group. The incidence of certain characteristic karyotypic abnormalities (5q−, 7q−, +8, +21, t(8;21), and t(9;22)) were decidedly more common in exposed than in unexposed patients. In another case–case study of 74 patients with ANLL (Golomb et al., 1982), 25 of the 58 (43%) unexposed patients had a clonal chromosome abnormality, compared with 12 of the 16 (75%) exposed patients (P = 0.02). Either a 5q– or a 7q– was present in 67% of the exposed patients with a chromosome abnormality, compared with 28% of the aneuploid unexposed patients. These studies support the observation that a subset of patients with de novo ANLL have a history of occupational exposure and a unique pattern of clonal chromosomal aberrations.

Cytogenetic changes in patients with second acute non-lymphocytic leukaemia after treatment of a primary malignancy. Cytogenetic studies were conducted on 26 patients who developed ANLL or a dysmyelopoietic syndrome after treatment of a primary malignancy (Rowley et al., 1981). Fifteen patients had radiotherapy and chemotherapy, seven had only chemotherapy, and four had only radiotherapy. Twenty-five patients had an abnormal karyotype in myeloid cells. Loss of part or entire chromosomes 5 and/or 7 was noted in 23 of 25 patients with aneuploidy. Loss of chromosome 5 was noted only in patients who previously had malignant lymphoma, whereas loss of chromosome 7 was seen in these patients as well as in those who had other malignancies. Abnormalities of both chromosomes 5 and 7 occurred in 53% of the patients treated with combined therapy and in only 27% of patients treated with either modality alone. Cytogenetic changes were analysed in 76 cases of secondary myelodysplasia (sMDS) and acute non-lymphocytic leukaemia (sANLL) (Johansson et al., 1991). Among the 36 sMDS patients, 23 (64%) displayed clonal chromosomal abnormalities. The most common aberrations were −7, 5q−, −5 and +8. Of the 40 sANLL patients, 30 (75%) cases displayed clonal chromosomal abnormalities. The most frequent aberrations were −7, −5, +8, 7q−, −17 and +21. When the incidences of characteristic cytogenetic abnormalities were correlated with the type of previous therapy, −7 was found to be more frequent in sMDS and sANLL patients who had been exposed to chemotherapy, whereas 5q− was associated with previous exposure to ionizing radiation in sMDS patients. Those results suggest that cytogenetic measures may be employed to predict the risk of second primary tumour after treatment such as radiotherapy and chemotherapy for the first primary tumour.

In summary, many chromosomal abnormalities identified will have diagnostic, prognostic and therapeutic implications. The identification of chromosomal abnormalities directs us to investigate abnormal loci of the genome that harbour the molecular basis responsible for malignant transformation and progression (McClay, 1989). The implementation of interphase cytogenetics by techniques such as fluorescence in-situ hybridization (FISH) will lead to the more frequent use of cytogenetic markers in cancer epidemiological studies.

Oncogenes and tumour suppressor genes
Inherited (germline) or acquired (somatic) gene mutations and altered gene products controlling cell growth, cell death and differentiation are considered to be crucial steps in human carcinogenesis. Molecular studies have defined that aberrations affecting two major types of genes, proto-oncogenes and tumour suppressor genes have a direct role in tumorigenesis and cancer progression.
Oncogenes. Proto-oncogenes are normal cellular genes involved in a wide variety of functions, such as cell growth and signal transduction. The activation of a proto-oncogene into an oncogene yields a gain of function or dominant event. Oncogene activation usually occurs by a somatic mutation, mainly gene amplification or point mutation. These alterations can convert a proto-oncogene from a normal cellular gene to an oncogene and can lead to uncontrolled or neoplastic growth (Taylor, 1989).

Tumour suppressor genes. These genes are also normal cellular genes. Tumour suppressor genes contribute to oncogenicity through their loss of functions, and are considered recessive events. The end result is that the products of these genes are absent or inactivated in the malignant cells. Tumour suppressor gene mutations are the most frequently observed genetic events in cancer. In general, suppressor gene inactivation occurs by a point mutation of one allele and a deletion of the remaining contralateral allele. The loss of both alleles (homozygous deletion) is an alternative but uncommon mechanism. These alterations result in an inability to suppress cell proliferation, and it is followed by tumour development. Since many mutagens are capable of altering tumour suppressor genes, it has been hypothesized that tumour suppressor genes contribute to the development of cancer and may be a critical area in which to study cancer etiology (Hollstein et al., 1991b; Jones et al., 1991; Harris, 1993).

The use of oncogenes and tumour suppressor genes in cancer epidemiology. The use of molecular and genetic alterations of tumour suppressor genes and proto-oncogenes in cancer epidemiology has advanced our understanding of cancer biology and carcinogenesis. Point mutations in tumour suppressor genes (e.g. TP53) and oncogenes (e.g. ras) may be specific for both tumour type and the critical environmental exposure (etiological heterogeneity). Lung tumours from smokers show a high frequency of G to T transversions in both K-ras and TP53, and may reflect the molecular fingerprint of carcinogenic constitution of tobacco smoke (Jones et al., 1991). Such molecular epidemiological evidence supports the well established association between smoking and cancer (Vineis & Caporaso, 1995), although it is important to point out that mutational patterns differ for other smoking-related cancers such as bladder cancer.

The wide range of involvement of TP53 in human tumours and the broad spectrum of mutations make the gene a good candidate for molecular epidemiological studies (Hollstein et al., 1991b; Jones et al., 1991; Harris, 1993; Harris & Hollstein, 1993). TP53 mutations have been suggested as DNA fingerprints of exposure in a variety of tumours. Dietary AFB1 exposures are associated with AGG→AGT mutations at codon 249 in liver cancer (Ozturk et al., 1991; Bressac et al., 1991; Hsu et al., 1991). UV exposure may induce CC→CT mutations in skin tumour (Brash et al., 1991). Radon exposures are related to AGG→ATG mutations in codon 249 in lung cancer (Taylor et al., 1994).

Newly developed molecular biological methods, e.g. polymerase chain reaction (PCR), automated sequencing techniques and comparative genomic hybridization (CGH), will accelerate the process of characterizing DNA alterations. The CGH method represents a recently developed molecular cytogenetic screening technique that can be employed to survey entire genomes for variations in DNA sequence copy number, as well as to map chromosome regions with amplifications or deletions in tumour DNA prepared from fresh or archived materials (Kallioniemi et al., 1993; Thompson & Gray, 1993; Houldsworth & Chaganti, 1994; Kallioniemi et al., 1994). CGH is a powerful adjunct to traditional cytogenetic techniques and a useful tool with which to screen for molecular and genetic defects, which will eventually lead to the identification of tumour suppressor genes and oncogenes in solid as well as haematological tumours. By combining advanced methods for characterizing exposure to carcinogens and measuring tumour markers, there is a great potential for further elucidating the etiology of cancers and for the development of strategies for cancer prevention.

Study designs for the use of tumour markers. Tumour marker studies provide some interesting new challenges in study design and statistical analysis. A detailed discussion of study designs for the use of biomarkers in epidemiology is provided by Rothman et al. (1995), Hulka & Margolin (1992) and Hulka & Garrett (1996).
Case–case study design

It has been suggested that case–case (or case–series or case-only) studies can be employed to evaluate gene–environment interactions. The critical assumption is that the exposure and genetic factors occur independently and the disease is rare. Under these assumptions, the case–case approach is valid and offers better precision for estimating gene–environment interactions than does the case–control approach (Piegorsch et al., 1994). The case–case study design can be employed to assess the association between exposure and tumour markers and to evaluate etiological heterogeneity between marker-positive and marker-negative tumours (Taylor, 1989; Begg & Zhang, 1994). This study design may be used to evaluate the hypothesis that the two categories of cases, distinguished by the presence or absence of the tumour marker, are characterized by etiological heterogeneity, as evidenced by differences in the strengths of effects of the risk factor in the two case groups. The differences could be due to the fact that the causal pathway differs, or they could merely reflect a different magnitude of effect via the same mechanism. Empirical evidence of such etiological heterogeneity with respect to one or more risk factors would provide strong justification for more detailed investigations of the specific mechanisms of action. This study design consists of a series of incident cases. Ideally, this would be a consecutive series of population-based incident cases. If the ascertainment is not complete, or if, for example, the study is hospital-based, we must assume that case selection for the two disease categories is not influenced differentially by the risk factors associated with case ascertainment.

Suppose that Y is the risk factor of primary interest, assumed for simplicity to be binary, and that W denotes the set of remaining risk factors, where Y+ indicates the presence of the risk factor and Y− indicates its absence. Let X+ (X−) denote the presence (absence) of the tumour marker. Furthermore let \( \psi(W) \) be the odds ratio relating Y and X, conditional on W. In the context of our study of p53 overexpression and bladder cancer (Zhang et al., 1994b), Y represents smoking status, X represents the presence or absence of TP53 mutations in the tumour samples, and W represents the remaining risk factors. We can evaluate \( \psi(W) \) using standard statistical methods such as the Mantel–Haenszel procedure or logistic regression. A test of the null hypothesis that \( \psi(W) = 1 \) is a test of the hypothesis of etiological heterogeneity, i.e. that the strength of Y as a risk factor is different for the two case groups (e.g. \( p53+ \) and \( p53− \)).

Case–control study design

In case–control studies, etiological heterogeneity has traditionally been evaluated by two separate analyses: marker-positive cases versus controls and marker-negative cases versus controls. The analytic strategy is to use polychotomous logistic regression (Dubin & Pasternak, 1986). In this model, the relationships between marker-positive cases and controls, and between marker-negative cases and controls are both modelled concurrently using two separate (logistic) regression functions. Let \( \beta_i \) be the coefficient of the primary risk factor in the logistic regression relating marker-positive cases and controls, and let \( \beta_j \) be the corresponding parameter relating marker-negative cases and controls. If there are no interactions between Y and W, then \( \beta_i \) is the conditional log odds ratio of the risk factor on marker-positive disease, and \( \beta_j \) is the conditional log odds ratio of the risk factor on marker-negative disease. To test the null hypothesis that the two diseases possess etiological heterogeneity with respect to the risk factor, one can test the null hypothesis that \( \beta_i = \beta_j \), i.e. that the two odds ratios are equal. Such a comparison can be accomplished by using, for example, a likelihood ratio test. Quantitative evidence of the degree of departure from the hypothesis can be characterized by the difference in these coefficients, \( \beta_i - \beta_j \). This is the logarithm of the ratio of the two adjusted relative risks of the risk factor, i.e. the relative risk with respect to marker-positive cases and with respect to marker-negative cases, respectively.

Case–case versus case–control study design

It has been shown by our group (Begg & Zhang, 1994) that the odds ratio from the case–case study is theoretically equivalent to the parameter \( \beta_i - \beta_j \) in the polychotomous logistic regression model, and thus evaluation of etiological heterogeneity does not require a conventional control group.

We illustrate the method using data from our own case–case study of the relationship between smoking and TP53 mutations in patients with bladder cancer (Zhang et al., 1994b). The raw
Table 1. Frequencies by TP53 mutations and smoking status

<table>
<thead>
<tr>
<th>Smoking status</th>
<th>Cases</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>p53+</td>
</tr>
<tr>
<td>Smoker</td>
<td>34</td>
</tr>
<tr>
<td>Non-smoker</td>
<td>10</td>
</tr>
</tbody>
</table>

frequencies are contained in Table 1. For illustrative purposes we have employed a control group consisting of patients with other cancers believed to be unrelated to smoking, although this would not be an ideal control group for a case–control study in general. The odds ratios and confidence intervals are presented in Table 2. The unadjusted odds ratios are calculated directly from the cross-products, as usual, i.e. $\psi = (34 \times 21)/(10 \times 43)$, $\theta_1 = (34 \times 64)/(10 \times 81) = 2.69$, and $\theta_2 = (43 \times 64)/(21 \times 81)$. Note that $\psi = \theta_1/\theta_2$.

We have shown that the odds ratio relating an environmental risk factor to the presence of a biological marker is an appropriate measure for characterizing the degree of etiological heterogeneity between the disease groupings defined by the marker. These observations legitimize the common recent practice of exploring gene–environment associations in case–control studies (Piegorsch et al., 1994). This parameter has been shown to be the ratio of the relative risk of the factor in causing marker-positive disease to the relative risk in causing marker-negative disease. Moreover, it can be estimated directly from an appropriately designed case–case study without the need for a control group (Zhang et al., 1994a, 1994b, 1995a, 1995b; Freedman et al., 1996a, 1996b). The odds ratio obtained from case–case study needs to be interpreted with great caution, since this measure does not indicate the directions of individual relative risk of marker-positive or marker-negative disease. Therefore, the use of a control group is necessary if we wish to estimate the actual relative risks for marker-defined tumour subtypes.

**Practical issues in tumour tissue banking**

The research strategy to incorporate exposure, susceptibility and tumour markers for a case–control study includes collection and storage of all related biological specimens, such as blood samples (for cases and controls), tumour and normal tissues (for cases only), in addition to the collection of epidemiological exposure data. By collecting and storing blood specimens for all cases and controls, certain tumour susceptibility markers, such as polymorphism in GSTM1, NAT2 and CYP1A1, can be assessed. Other markers such as mutagen sensitivity, DNA repair capacity and haemoglobin/DNA adducts can also be measured. Proper collection and storage of tumour and normal tissues for cases allow the characterization of tumour suppressor genes and oncogenes, as well as the DNA replication repair defect phenotype, and microsatellite instability. By collecting epidemiological data, exposure history can be evaluated and other potential confounding factors can be controlled.

Since the issue of blood specimen banking has been discussed by Landi & Caporaso (this volume)

Table 2. Odds ratio estimates

<table>
<thead>
<tr>
<th>Design</th>
<th>Parameter</th>
<th>Unadjusted</th>
<th>Adjusted*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case–control</td>
<td>$\theta_1(W)$</td>
<td>2.69 (1.23, 5.87)</td>
<td>3.61 (1.41, 9.29)</td>
</tr>
<tr>
<td></td>
<td>$\theta_2(W)$</td>
<td>1.62 (0.87, 3.00)</td>
<td>2.11 (0.98, 4.54)</td>
</tr>
<tr>
<td></td>
<td>$\psi(W)/\theta_2(W) = \psi(W)$</td>
<td>1.66 (0.69, 4.01)</td>
<td>1.71 (0.66, 4.43)</td>
</tr>
<tr>
<td>Case–case</td>
<td>$\psi(W)$</td>
<td>1.66 (0.69, 4.01)</td>
<td>1.71 (0.63, 4.66)</td>
</tr>
</tbody>
</table>

*Adjusted for age using logistic regression for the case–case approach, and polychotomous logistic regression for the case–control approach. $\theta_1 = \text{value positive}; \theta_2 = \text{value negative}.$
in this monograph, we will briefly review here issues related to the storage of tumour and normal tissues.

With the increasing demands for tumour tissue samples for biological, clinical or epidemiological studies, tissue banking has become a very important practical issue (Lee et al., 1995). Limited resources, untrained personnel and absence of uniform protocols for tumour tissue banking have created obstacles for the proper and rapid collection, processing and storage of tumour tissues (Grizzle, 1994). In addition, lack of information on diagnostic quality control, histological classification (stage and grade), treatment and outcome may further jeopardize the optimal usage of normal and tumour samples in cancer research.

There is a rapidly increasing ability to evaluate a range of tumour markers in formalin-fixed, paraffin-embedded tissues, such as p53 nuclear protein accumulation measured by immunohistochemistry (IHC) (Sarkis et al., 1993a, 1993b, 1994, 1995) as well as TP53 point mutations measured by PCR-SSCP and sequencing techniques (Taylor et al., 1996). The development of assays that can use DNA extracted from formalin-fixed, paraffin-embedded tissue will enhance molecular epidemiological investigation. There are some limitations associated with the use of formalin-fixed, paraffin-embedded materials, mainly due to the fragmentation of DNA. Fresh or frozen samples are preferred, if available, for molecular assays at the DNA or RNA level. Since the storage of formalin-fixed, paraffin-embedded tissues is a standard procedure in medical centres or hospitals, we will focus our discussion on the storage of frozen tissues.

In order to perform epidemiological studies and to share the specimen resource with other investigators in biological and clinical sciences, a centralized programme for research specimen banking is needed in any large research medical centre. Biological specimens can be collected and evaluated for research suitability by trained personnel
Figure 2. p53 overexpression and prognosis of superficial bladder cancer.

At Memorial Sloan Kettering Cancer Center, the tumour banking programme assumes the responsibility of delivering all routine specimens from the operating rooms to pathologists on a regular schedule. This ensures that specimens available for research are as fresh as possible. Pathologists are responsible for determining whether diagnostic requirements have been satisfied before giving the portion of the residual tissue for banking. The specimen is then transferred in an iced specimen container. Normal and tumour tissues are stored in plastic cassettes labelled with a coded number to
protect patients' confidentiality. The tissue is embedded in frozen section support media (OCT) and stored at -70°C. A clinical abstract of the patient's history with an accompanying pathology report will be provided for each specimen only if IRB approval has been obtained to conduct such a study. In general, patients will be informed of the ongoing protocol and sign a informed consent form. A data management system needs to be implemented and a relational database system needs to be established so that tissue bank database can be linked to pathological, epidemiological and clinical data.

Laboratory strategy for the use of tumour markers
A protocol for immunohistochemistry and for genetic analysis of formalin-fixed, paraffin-embedded tissues has kindly been provided by Drs William Bennett and Curtis C. Harris, NCI Laboratory of Human Carcinogenesis (see Appendix). A strategy has been suggested (Cordon-Cardo et al., 1994) whereby, from a single tissue sample, different techniques can be performed to examine immunophenotype and genotype. This strategy is illustrated in Fig. 1. Briefly, using consecutive tissue sections cut at different thicknesses and deposited either on microslides or microtubes, one can: (1) evaluate morphology (i.e. haematoxylin and eosin (H&E) staining), (2) perform immunohistochemistry procedures, and (3) characterize molecular alterations (i.e. Southern blot, restriction fragment length polymorphism (RFLP), and PCR-SSCP and sequencing). Antigen expression and/or its modulation can be analysed by immunohistochemistry on tumour tissue samples. Finally, specimen identification can provide the correlation of laboratory data with epidemiological, pathological and clinical follow-up data (Fig. 1).

Issues concerning the use of tumour markers in prognostic studies
The study of the prognostic value of tumour markers raises several special issues concerning subject selection and adjustment for other known prognostic factors such as stage and grade of the disease. For example, study populations should usually be limited to incident cases diagnosed within a year in order to reduce selection bias from differential survival. Known prognostic factors such as age, sex, grade, stage and treatment need to be controlled in the data analysis when assessing the effect of a tumour marker.

We have conducted a series of studies to evaluate the association between p53 nuclear overexpression and progression/survival in a group of patients with superficial bladder cancer. p53 nuclear overexpression was evaluated in tumours of 164 patients (T1 = 77, Ta = 54 and Tis = 33) with superficial bladder cancer by immunohistochemistry using the mouse monoclonal antibody PAB1801 on deparaffinized tissue sections. Antibody 1801 detects both wild-type and mutant p53 proteins. Due to the prolonged half-life of the mutated p53 products, they accumulate in the nucleus and can usually be detected by immunohistochemical assays. We studied 42 primary bladder tumour tissues to estimate the sensitivity of immunohistochemical (IHC) methods in the prediction of TP53 mutations (Cordon-Cardo et al., 1994). We found that the highest sensitivity was reached when the cut-off (in terms of percentage of cells with nuclear immunoreactivity) was 20%, and so we have employed 20% as the cut-off point for IHC results in our analysis. The data were first correlated with conventional prognostic parameters, including stage, grade, vascular invasion, age and sex. Various univariate and multivariate analyses were performed. In the study, none of the normal urothelial and stromal cells showed p53 nuclear overexpression. Patients with bladder tumours were stratified into two groups according to the percentage of cells with nuclear immunoreactivity. Ninety-three patients (56.7%) had none or less than 20% tumour cells with positive nuclear staining (group A), while the remaining 71 (43.3%) had more than 20% tumour cells with nuclear immunoreactivity (group B). Separate analyses of progression and survival were performed for the three stages of superficial bladder cancer (Tis, Ta, T1) after adjusting for age, sex, grade, vascular invasion and adjuvant therapy. Patients in group B had consistent significantly lower progression-free intervals and survival (P < 0.001) at all three stages (Fig. 2). These results suggest that superficial bladder cancers exhibiting p53 nuclear overexpression have a higher rate of disease progression and short survival, and may be useful in selecting appropriate therapy (Sarkis et al., 1993a, 1993b, 1994, 1995).
Appendix. Histology protocol: paraffin sections for immunohistochemical and genetic analyses

1. Background: genetic analysis of archival human tissues
Access to archival human tissues provides many opportunities for both prospective and retrospective studies. Informative assays based on immunohistochemistry and PCR technology are available. Specialized protocols are needed for these studies, and essential elements include confidentiality, proper selection of adhesive coatings and precautions for tissue carry-over in PCR-based studies.

2. Tissue specifications
The optimal tissue sample is a paraffin block containing at least 1 cm² of tissue, including both tumour and non-tumour tissue. The non-tumour tissue is used for an internal negative control and for germline analysis. Five-micron sections are used for immunochemistry, and 20-micron sections for microdisssection of non-necrotic tissues for genetic analysis.

3. Glass slide specifications: silanation and DEPC treatment
Glass slides must be coated to promote tissue adherence, otherwise tissue sections will be lost during multiple washings and incubations of the immunochemistry protocol. Several coating agents are commonly used, including poly-L-lysine, glue, silane and others; non-biological preparations (i.e., silane) are less commonly contaminated with DNase or RNase than poly-L-lysine and represent a better choice for PCR-based studies. In addition, RNase precautions must be used for sections intended for microdisssection; essential elements include DEPC treatment and baking to inactivate RNase and DNase. Specifications and protocols are listed below:

i. Coated or charged slides. Either commercial or locally prepared slides will be used. Commercially coated slides are available, specify RNase-free.

ii. DEPC treatment protocol. To destroy any RNase or DNase attached to the glass slides, load the slides into a metal rack and place in water containing 0.1% DEPC (diethyl procarboante) for 15–30 min; wrap the entire rack in aluminum foil and bake at 180°C for at least 2 h. Cool to room temperature and store wrapped in foil at room temperature indefinitely.

Caution: DEPC is a potent protein denaturant and is a suspected carcinogen; it should be handled with care. Wear gloves and safety glasses and work in a chemical fume hood. Point the bottle away from you when opening; internal pressure can lead to splattering.

4. Paraffin section guidelines: RNase precautions

i. Gloves are worn during microtomy.

ii. Disposable blades are used and replaced between blocks.

iii. The block holder is cleaned with xylene between blocks.

iv. DEPC-treated water is used in the tissue flotation bath.

v. Coated slides (i.e., silane or similar) are prepared with sterile DEPC water and handled only with gloved hands.

vi. Cut 25 sections from each block according to the following specifications. Section nos 1–16 must be 5 microns thick; nos 1 and 16 are stained by haematoxylin and eosin (H&E), nos 2–15 must be unstained. Section nos 17–25 must be 20 microns thick and unstained; put two 20-micron-thick sections on each slide. Coated slides and DEPC water must be used for all sections.

For small tissues, cut only 5-micron sections and place two 5-micron-thick sections on slide nos 17–25.

vii. If the tissue is exhausted during sectioning, make an H&E stain of the last (or close to last) section.

viii. Bake sections at no more than 60°C for no more than 2 h.

ix. Each slide will be labelled with the block number from the paraffin block, the sequential number of the section (i.e., 1–25) and the section thickness (i.e., 5 or 20 microns).

5. Trouble-shooting guide for using this protocol
It is advisable to test the sections produced by a laboratory using this protocol. A common problem is that tissue sections fall off the slide during microwave antigen-retrieval procedures. Usually this is caused by inadequate or improper silanation of sections, although some tissues are more likely than others to fall off the slides (i.e., tissues containing fat or bone). Therefore, investigators are encouraged to send slides from the first 10–20 cases to a histology laboratory, and to request a routine immunostain (i.e., p53 or Ki67) using 30 min of microwave antigen retrieval (this is the longest interval commonly used). If tissue sections fall off the slides, then ask the laboratory to remake their silane solutions and re-check their protocol, and then repeat the pilot immunostains until the problem is resolved.
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Application of Biomarkers in Cancer Epidemiology


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