REVIEW

Genogenic immunohistochemistry: a new era in diagnostic immunohistochemistry

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Summary

Immunohistochemical studies have traditionally focused on markers of specific cell and/or tumour type as aids in the diagnosis of specific tumours. However, as our knowledge of the molecular basis of tumours has increased, immunohistochemistry is being used with increasing frequency to identify underlying molecular changes or the presence of specific molecular markers in tumours, both as an aid to diagnosis and as a guide to appropriate therapy. There are three major classes of genogenic immunohistochemistry: (a) identification of specific mutations, by documentation of loss of expression of the corresponding protein, exemplified by E-cadherin in lobular breast cancer; (b) identification of proteins expressed as a consequence of specific translocations, such as the t(2;5) translocation of anaplastic large cell lymphoma leading to ALK protein expression; and (c) identification of molecular targets of novel tumour therapies, e.g. identification of overexpression of the HER-2/neu protein to validate the use of trastuzumab (Herceptin™) in the treatment of breast cancer patients. This review offers a snapshot of the current status of genogenic immunohistochemistry, which heralds a new era in diagnostic immunohistochemistry. © 2002 Elsevier Science Ltd. All rights reserved.

INTRODUCTION

Traditionally, the goals of diagnostic immunohistochemical studies have been to explore and certify diagnoses by identifying the pathway of differentiation (sometimes referred to as identifying the ‘cell of origin’) of a given tumour. Immunohistochemistry, the marriage of the precision of immunohistochemistry with the visualization provided by a sensitive detection system, is a science that has its origins in the work of Albert Coons, 60 years ago. Building upon the work of Coons, who initially sought to prove the presence of immunoglobulin molecules in plasma cells, the past half century has seen tremendous refinement in the development of the antibody tools and the detection systems. However, the overwhelming majority of this effort has been directed at further analysis of protein expression as a marker of a particular cell, tissue or tumour type. However, as our understanding of the molecular basis of tumours has increased, it is becoming clear that many tumours have alterations that are best defined at the molecular level.

This article provides a number of examples of ‘genogenic immunohistochemistry’, a phrase I have coined to describe this novel application of immunohistochemistry, where this protein-based technique is employed to answer questions about alterations at the molecular level. There are four major categories of molecular alterations that can be assessed by immunohistochemical methods: (a) identifying the presence of genetic mutations, generally truncation mutations, but also loss secondary to processes such as methylation; (b) identifying the presence of specific chromosomal translocations owing to the identification of novel proteins expressed as a direct consequence of the translocation; (c) identifying the presence of gene amplification; and (d) identifying molecular therapeutic targets for the treatment of cancer and other diseases.

IDENTIFYING THE PRESENCE OF GENETIC MUTATIONS USING IMMUNOHISTOCHEMISTRY

In each of these examples, immunohistochemistry is used to detect the ‘final common pathway’ of loss of
protein expression, which may actually result from mutations, promoter methylation or other mechanisms.

**Lobular carcinoma of the breast**

The 'molecular signature' of this disease is a genetic alteration which results in loss of expression of the E-cadherin protein. E-cadherin mediates adhesion and morphogenesis, and the resulting loss of expression of this protein is, in part, responsible for the loss of cell–cell adhesion, which characterizes this tumour and leads to its distinctive histological appearance of 'single cell' infiltration. Loss of E-cadherin protein expression was first documented almost 10 years ago by Moll et al., in an immunohistochemical study employing cryostat and paraffin sections; noteworthy in their study was the demonstration of E-cadherin expression in all cases of high-grade infiltrating ductal carcinoma, i.e. the loss of expression of this protein was not a function of tumour grade per se but something unique to the lobular subtype. Our more recent studies have shown that virtually all cases of lobular carcinoma, as determined by studies in deparaffinized, formalin fixed tissues, are characterized by loss of immunostaining with antibodies to E-cadherin (Fig. I).

![Figure I](image)

**Figure I** In situ and infiltrating lobular carcinoma of the breast: (A) haematoxylin and eosin and (B) E-cadherin immunostain, showing retention of E-cadherin expression in ductal epithelial cells and complete loss of signal on focus of in situ and surrounding infiltrating lobular carcinoma (original magnification × 200).

The molecular correlates of this were first described by Kanai and colleagues, who examined the exons 5–8 (which includes the region essential for E-cadherin specific binding) of the E-cadherin gene using PCR-SSCP as well as direct sequencing; in 10% of the cases of lobular carcinoma examined, a point mutation in exon 7 was found. Berx and colleagues extended these findings, finding E-cadherin mutations in 56% of lobular carcinomas, and not in any other forms of breast cancer (e.g. ductal). These authors also documented the presence of 23 different mutations in a series of lobular breast cancers, of which seven were insertions, 11 deletions, two nonsense mutations and three splice site mutations. All the frameshift and nonsense mutations would be expected to have generated secreted E-cadherin fragments instead of a transmembrane protein with cell adhesion capabilities. In a later paper, these same authors found mutations in the extracellular domain of 55% of lobular carcinomas, with complete loss of plasma membrane-associated E-cadherin expression in 84% of cases. Very recently, Droufakou and colleagues have demonstrated the heterogeneity of genetic alterations that lead to the final common pathway of lobular carcinoma. In this latter study, the overwhelming majority of lobular carcinomas showed methylation of the E-cadherin promoter. In addition, five frameshift mutations, which resulted in downstream stop codons and one splice site mutation were found in a subset of the tumours. It can therefore be concluded that most ILCs show genetic or epigenetic changes, e.g. biallelic inactivation of the E-cadherin gene by promoter methylation, mutation or allelic loss in any combination thereof, affecting the E-cadherin gene and that most of these tumours, as a direct consequence, lack E-cadherin expression.

Interestingly, Vos and colleagues have demonstrated that inactivating point mutations in the E-cadherin gene were found in lobular carcinoma in situ (LCIS) but not ductal carcinoma in situ (DCIS), suggesting that E-cadherin is a very early target gene in lobular neoplasia. More recent studies we have published along with those of Jacobs et al. have extended these findings of E-cadherin alterations in in situ disease, confirming the earlier results of Vos et al. In both of these recent studies, virtually all cases of LCIS were found via immunohistochemistry to be E-cadherin negative, whereas all DCIS cases were found to be E-cadherin positive. In addition, the clinical significance of demonstrating E-cadherin expression in the context of in situ breast carcinoma has recently been demonstrated.

**Microsatellite instability, mismatch repair genes and colonic neoplasia**

The development of the majority of sporadic colorectal adenocarcinomas, as well as adenocarcinomas
developing in the context of familial adenomatous polyps (FAP) has been documented to be a multistep process, as demonstrated in numerous studies from the Vogelstein laboratory.\textsuperscript{11} A smaller subset of sporadic colorectal adenocarcinomas, as well as colonic adenocarcinomas arising in the context of the autosomal dominant disorder known as hereditary non-polyposis colon cancer (HNPCC), arise by a different mechanism that involves alterations in genes that participate in DNA mismatch repair, with approximately 60\% of HNPCC families harbouring mutations in two of these genes, \textit{hMSH2} and \textit{hMLH1}.\textsuperscript{12–14} It has been demonstrated that the tumours from patients with HNPC demonstrate a unique type of genetic instability characterized by alterations within microsatellites, referred to as microsatellite instability (MSI). MSI is now recognized as the biological hallmark of the so-called mutator phenotype.\textsuperscript{15–16} Most of the MSI-positive tumours appear to be directly related to the altered expression of these two genes and can be diagnosed by the examination of protein expression.

Studies such as those of Kim et al.\textsuperscript{17} and Cawkwell et al.\textsuperscript{18} demonstrate that immunohistochemical studies identifying the loss of expression of the mismatch repair gene products hMLH1 and hMSH2 can be effective surrogates for the more expensive and time-consuming genetic studies looking for the presence of microsatellite instability. In the earlier study of Kim et al., in a series of 32 sporadic colorectal carcinomas, MSI was found in six (18\%), of these six cases, five showed immunohistochemically determined loss of expression of one or both of the mismatch repair gene products. In the larger study of Cawkell et al., 66 (of 502 colorectal adenocarcinomas investigated) showed evidence of MSI (13\%). Remarkably, 66/66 (100\%) of these adenocarcinomas with MSI showed complete lack of expression of either hMLH1 or hMSH2 by immunohistochemical analysis. Since patients with mismatch repair defective carcinoma showed improved survival, but a > 5-fold relative risk of developing a metachronous adenocarcinoma, the authors concluded that immunohistochemistry is the recommended method for selecting patients who should be investigated for HNPCC, offered long-term follow-up, and who may not respond to standard chemotherapy modalities. An example of a colonic adenocarcinoma with MSI showing loss of expression of hMLH1 is shown in Figure 2.

**Figure 2** Adenocarcinoma of the colon showing loss of hMLH1 as identified via immunohistochemistry: (A) haematoxylin and eosin and (B) hMLH1 immunostain. In (B), note complete loss of signal in nuclei of tumour cells (lower right) compared with the overlying normal colonic epithelium (top) (original magnification × 200).

### IDENTIFYING THE PRESENCE OF CHROMOSOMAL TRANSLOCATIONS USING IMMUNOHISTOCHEMISTRY

**ALK overexpression as an indicator of the t(2;5) translocation in anaplastic large cell lymphoma**

Anaplastic large cell lymphoma (ALCL) is generally a rapidly progressing disease occurring in the first three decades of life, showing male predominance, and often presenting at extranodal sites, especially skin and bone. ALCL was initially referred as ‘Ki-1 lymphoma’ and ‘CD30-positive anaplastic large cell lymphoma,’ reflecting the morphological and immunophenotypic underpinnings of the original identification of this clinicopathological entity. The earliest suggestions that there was a unique genetic alteration in this tumour were provided by studies such as those of Rimokh and colleagues,\textsuperscript{19} who demonstrated lymphoma cases containing a chromosomal translocation involving the long arm of
chromosome 5. Subsequent studies revealed that this rearrangement fuses the NPM nucleolar phosphoprotein gene on chromosome 5q35 to a previously unidentified protein tyrosine kinase gene, ALK, on chromosome 2p23.\(^{20}\) RT-PCR studies performed by Downing et al.\(^{21}\) provided evidence that there was not a 1:1 correlation between the ‘ALCL’ phenotype and the presence of the (2;5) translocation and the presence of the NPM/ALK chimeric protein. Indeed, the NPM/ALK-expressing cases were not confined to lymphomas with ‘anaplastic’ histological appearance alone, and some of the tumours with this translocation had other morphologies, e.g. diffuse large B-cell lymphoma. Furthermore, only slightly greater than half of the cases with classic histological features of ALCL, or those expressing CD30, were NPM/ALK positive. As subsequently demonstrated by Shiota and colleagues,\(^{22}\) recognition of this NPM/ALK positive subgroup is of great clinical importance: those ALCLs positive for the translocation occurred in a far younger patient age group and the patients showed a far better 5-year survival rate. Most importantly, in a landmark study by Lamant and colleagues, the t(2;5) translocation was detected by using cytogenetic analysis, reverse transcription, polymerase chain reaction (RT-PCR) and immunohistochemistry with an antibody directed against the kinase domain of anaplastic lymphoma kinase (ALK) of the chimeric NPM/ALK protein. In all but three cases, there was concordance among the cytogenetic analyses, RT-PCR, and immunohistochemical staining; the authors concluded that immunohistochemistry is a reliable method for detecting NPM/ALK chimeric protein and hence this subset of lymphomas. A subsequent study by Pittaluga et al.\(^{23}\) confirmed that immunohistochemistry allows the identification of a distinct subgroup of lymphomas that contain the 2p23 chromosomal abnormalities but lack ‘anaplastic’ morphology typical of ALCL. Furthermore, immunostaining proved to be the most sensitive method to identify this group. Through the use of these molecular and immunohistochemical techniques, there has been a complete rethinking of the definition of this tumour: as the histology may not be anaplastic and the cells neither anaplastic nor large, it has been suggested that the tumour should be renamed ‘ALK lymphoma’ or ‘ALKoma’ to reflect this.\(^{24}\) Finally, variants of this disease with different translocations leading to different chimeric protein formation yielded a different pattern of immunostaining using antibodies to the ALK (or NPM) protein.\(^{25}\) This is perhaps the most profound demonstration to date of the power of immunohistochemistry to reflect the diversity of genetic alteration in tumours. An example of a case of ALCL with ALK expression appears in Figure 3.

**FLI-1 overexpression as an indicator of the t(11;22) translocation of PNET/ES**

Approximately 90% of primitive neuroectodermal tumours/Ewing’s sarcoma (PNET/ES) have a specific t(11;22)(q24;q12), translocation, which results in the fusion of these genes on the der(22) chromosome, resulting in the production of a novel chimeric EWS/FLI-1 message, and overexpression of FLI-1 protein. While cytogenetic studies were originally employed to identify the presence of this chromosomal translocation, more recently identification of this genetic alteration has been possible through the use of appropriate primers using the RT-PCR technique.\(^{26}\) However, most recently it has been found that demonstration of the ‘molecular signature’ of this disease is possible by identifying the ‘neo-expression’ of the FLI-1 gene product using immunohistochemical techniques.\(^{27,28}\) In the study of Folpe and colleagues,\(^{29}\) and confirmed more recently by Llombart-Bosch and Navarro,\(^{30}\) FLI-1 expression has been demonstrated to be present in almost three-quarters of PNET/ES, but not in other small blue round cell tumours with which it can be confused histologically. Immunohistochemical detection of FLI-1 may thus be a valuable technique for identification of PNET/ES in cases in which molecular genetic evaluation is not feasible. FLI-1 protein expression is also helpful in distinguishing PNET/ES from other tumours that may be CD99-positive.

**WT-1 overexpression as an indicator of the t(11;22) translocation of DSRCT**

Another member of the small blue round cell group of paediatric tumours is the desmoplastic small round cell tumour (DSRCT); this distinctive tumour shows a different translocation involving chromosomes 11 and 22: t(11;22)(p13;q12). This results in unique chimeric EWS/WT1 transcripts with expression of the corresponding fusion gene product.\(^{39}\) Since a portion of this fusion protein corresponds to the C-terminal portion of the
WT-1 gene product, it is not surprising that studies have demonstrated that antibodies to the WT-1 gene product can detect the unique fusion product that results from this translocation, and can thus be used to identify desmoplastic small round cell tumour by immunohistochemistry. Indeed, among small blue round cell tumours (with the exception of Wilms tumour, which is generally confined to the kidney), the DSRCT is unique in showing positive immunostaining for the WT-1 gene product, as shown in Figure 4.

USING IMMUNOHISTOCHEMISTRY TO IDENTIFY THERAPEUTIC TARGETS FOR CANCER

Herceptin™ and HER-2/neu

The HER-2/neu (c-erbB-2) oncogene protein is a transmembrane glycoprotein in the epidermal growth factor receptor family. It is expressed at low levels in a variety of normal epithelia, including breast duct epithelium, but is ‘overexpressed’ at significantly higher levels in approximately one-fifth to one-quarter of invasive breast cancers, usually as a direct consequence of amplification of the HER-2/neu gene. Many studies have now demonstrated the importance of HER-2/neu overexpression as an independent prognostic marker of clinical outcome, particularly in node-positive patients. Recent studies also suggest that HER-2/neu status predicts response to adriamycin-based adjuvant chemotherapy. In addition, the predictive value of HER-2/neu overexpression in node-negative patients has been demonstrated, with the additional finding that HER-2/neu overexpression is associated with poor response to tamoxifen, even in the setting of positive oestrogen receptor (ER) expression. Most recently, HER-2/neu protein overexpression in breast cancer has been used to select, among patients with metastatic disease, those eligible for immunotherapy treatment with the new humanized anti-HER-2/neu antibody, trastuzumab (Herceptin™, Genentech, Inc. South San Francisco, CA). Determination of HER-2/neu status has thus taken on great importance in the routine work-up of breast cancer in the pathology laboratory, complementing but not replacing the traditional diagnostic and prognostic markers such as tumour type, tumour size, tumour grade and lymph node status.

Because the alterations of HER-2/neu occur simultaneously at the genomic and protein levels, a number of studies have addressed the issue of comparative sensitivity and specificity of the two techniques. Controversy has ensued over the reproducibility and reliability of the semiquantitative IHC scoring system (0, 1+, 2+, and 3+) employed, and the use of FISH, for which binary results

Figure 4 Desmoplastic small round cell tumour of childhood showing strong, uniform nuclear immunostaining with antibodies to the C-terminal portion of the Wilms tumour gene product (original magnification × 200).

Figure 5 Infiltrating ductal carcinoma of the breast showing amplification of the HER-2/neu gene with concomitant protein overexpression. (A) Fluorescence in situ hybridization, showing multiple orange signals (corresponding to HER-2/neu gene) per tumour cell nucleus, with average of two green signals (corresponding to chromosome 17 centromere) per nucleus. (B) Immunohistochemical demonstration of 3+ level of immunostaining for HER-2/neu gene product (original magnifications ×1000 (A), ×400 (B)).
are reported (amplified/non-amplified), has been recommended by some.48 While 2+ and 3+ immunostain scores have been considered as positive results, questions have persisted, particularly with respect to the significance of the 2+ level of immunostaining.49 Recent studies attempting to compare detection of protein overexpression (measured by IHC) to gene amplification (measured by FISH or PCR) showed concordance rates ranging from 78% to 98%,48–57 with most studies showing over 90% concordance rates. The recent study by Paulet et al.52 is unique in finding poor concordance, but there is reason to believe that there were problems with the IHC component of that study, in which only 17% of all cases were either 2+ or 3+ positive. In a more recent study from our laboratory, involving a series of 306 invasive breast cancer cases, we performed parallel IHC and FISH studies. Among the 173 cases negative for protein overexpression via IHC, only five cases (2.9%) showed gene amplification. Among the 21 cases showing a 3+ positive overexpression score, 100% showed gene amplification. Among the 112 cases showing a 2+ positive immunostaining score, 21 (18.8%) showed gene amplification. However, among the 91 (81.2%) 2+ IHC cases negative for gene amplification, 46 (41%) showed an amplification score between 1 and 2. This suggests that immunohistochemical scores (i.e. 2+) may correlate with low levels of gene amplification, and provides further evidence to suggest that IHC, when employed with optimal antibodies and a scoring system, provides a highly accurate assessment of HER-2/neu gene status. Figure 5 compares HER-2/neu assessment by FISH and immunohistochemistry.

**STI571 (Gleevec™; imatinib mesylate, formerly known as STI571) and gastrointestinal stromal tumours**

In the early 1990s, a drug, eventually called STI571, was developed that targeted the ‘kinase pocket’ of the BCR-ABL protein, the fusion gene product characteristic of chronic myeloid leukaemia (CML) cells.58,59 The BCR-ABL gene product manifests deregulated tyrosine kinase activity, which is instrumental in the pathogenesis of CML, and Gleevec™ acts by blocking this tyrosine kinase activity. Gleevec™ targets not only the BCR-ABL gene product, however, but also is effective in blocking the ‘kinase pocket’ of the platelet-derived growth factor receptor60 and c-kit.61 The latter is expressed in (and highly characteristic of) gastrointestinal stromal tumours (GIST)), and recent published and presented data suggest that Gleevec™ is a highly effective reagent for the treatment of GISTs, a tumour heretofore without effective therapy.61 Thus, immunohistochemical detection of the c-kit gene product can identify tumours that might be treatable with Gleevec™.62

**CONCLUSION**

We are about to enter a new era of immunohistochemistry testing, moving from the current era in which this technique is used solely to determine the cell or tumour type. In this new era, which Albert Coons could not have foretold, immunohistochemistry will be used for the assessment of unique phenotypic aspects of the cell or tumour which directly reflect the underlying genetic alterations of a given tumour and which will, with increasing frequency, determine the optimal treatment for the patient.

**PRACTICE POINTS**

- The range of diagnostic immunohistochemistry is being expanded to include the identification of changes at the protein level which reflect molecular alterations thought to underlie specific tumours.
- Novel immunohistochemical tests now exist to identify, by virtue of the loss of immunostaining, lobular carcinoma of the breast and colonic adenocarcinomas such as those occurring in the context of HNPCC, which exhibit the molecular phenomenon of microsatellite instability.
- Novel immunohistochemical tests now exist to identify the specific chimeric proteins expressed as a consequence of chromosomal translocations characteristic of anaplastic large cell lymphoma, PNET/Ewings sarcoma, and desmoplastic small round cell tumour.
- Novel immunohistochemical tests now exist to identify the targets of new therapies for cancer, e.g. the identification of the HER-2/neu gene product in a subset of breast cancers.

**RESEARCH DIRECTIONS**

- Development of antibodies to other proteins deleted as a consequence of tumour-specific genetic mutations.
- Development of antibodies to chimeric proteins expressed as a consequence of chromosomal translocations of other tumours.
- Development of antibodies to other targets of novel cancer therapeutics.

**REFERENCES**

23. Pittaluga S, Wodarska I, Pulford K, Campo E, Morris S W, Van d-B-H et al. The monoclonal antibody ALKI identifies a distinct mor-


