Clinipath Pathology Newsletter

- Advances in Histopathology – Immunohistochemistry; the quiet Revolution
- Investigation and Management of Primary Hyperparathyroidism

“we take it personally”
From the CEO

Welcome to the autumn edition of our Newsletter. Dr Paul Glendenning has written an article on primary hyperparathyroidism, which is the most common cause of parathyroid dependant hypercalcemia. This article covers the practical investigations and management of primary hyperparathyroidism. I have written a general article outlining key concepts and the benefits of immunohistochemistry in tumour diagnosis. Haematoxylin and eosin continues as the basic standard stain for histopathology sections and there have been exciting advances with the development of immunohistochemistry and a large array of special stains.

Clinipath Pathology is a medically managed laboratory and we have a commitment to quality and continuing professional development. We enclose a questionnaire to evaluate our newsletters and how we can improve them and our educational program for you. The questionnaire should take no more than a few minutes to complete and we value your feedback. For every completed questionnaire, we will donate $5 to The Cancer Council WA.

Please fax on 9322 9338 or return the questionnaire by courier by 30th June 2008.

Advances in Histopathology – Immunohistochemistry; the quiet Revolution

Whilst the Haematoxylin and Eosin (H&E) stain continues to be the universally accepted routine stain of choice, over the past couple of decades there have been significant advances in immunohistochemistry (IHC) which have revolutionised our diagnostic approach to tumour and to a less extent non tumour histopathology specimens. The immunohistochemistry field is rapidly changing with improving technology and an increasing array of commercially available antibodies are now available. Prior to the immunohistochemistry age, pathologists only had a limited number of histochemical stains (for instance mucin and silver stains) together with electron microscopy to assist in classification of tumours.

**Immunohistochemistry Definition**

Immunohistochemistry combines anatomical, immunological and biochemical techniques for the identification of specific tissue components by means of a specific antigen/antibody reaction tagged with a visible label. Immunohistochemistry makes it possible to visualise the distribution and localisation of specific cellular components within a cell or tissue on microscopic slides. The term immunohistochemistry is often used interchangeably with immunocytochemistry and immunostaining.

**Milestones in Immunohistochemistry**

The concept of immunohistochemistry has existed since the 1930s and in 1942 Coons and his colleagues used fluorescent labeled antibodies to demonstrate pneumococcal antigens in infected tissues. Since this time there have been a series of progressive scientific discoveries and technological improvements which now make immunohistochemistry a routine and essential technique in laboratories. Key advances are summarised in Table 1.

**Development of Antibodies**

Antigens have multiple determinants (epitopes) all inducing the formation of antibodies of differing specificity and affinity. Even a single epitope can induce the formation of several antibodies of differing specificity and affinity.

Polyclonal antibodies were initially available, derived from the serum of laboratory animals which had been exposed to antigens. A major advance was the ability to fuse single immunoglobulin producing plasma cells with non secreting myeloma cells resulting in hybrid cells which produced monoclonal antibodies. The hybridoma clones in cell culture medium produced monoclonal antibodies for research and commercial use. With the advent of molecular biology it is now possible to clone the segment of DNA responsible for a specific antibody into a vector, for instance phage, E.coli or yeast and commercially produce monoclonal antibodies.

**Development of Sensitive Detection and Amplification Systems**

The site of localisation of antibodies (and their corresponding antigens) in tissues required the development of detection systems. Initially antibodies were tagged with fluorescent markers on frozen section slides, which added significant collection and procedural difficulties. Practically this was difficult and not applicable in routine histopathology laboratories with standard formalin fixed tissues. A number of detection systems have been developed which work on routine
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formalin fixed tissues (following antigen retrieval), often used in conjunction with an amplification step to highlight previously undetectable antibody-antigen binding sites. The commonest detection method is the peroxidase antiperoxidase system (PAP), see Figure 1. The PAP reagent comprises antibody against horseradish peroxidase and horseradish peroxidase antigen which gives a brown immunostaining reaction.

Antigen Retrieval Systems

Formaldehyde fixation continues to be the fixative of choice for routine laboratories. Aldehydes fix tissues by crosslinking amino acids with methylene bridges, causing denaturation of molecules and masking antigenic sites (epitopes). The longer the specimen remains in formalin, the greater the degree of antigen masking.

Table 1

Key Advances in Immunohistochemistry

- Antigen retrieval systems enabling immunohistochemistry to be consistently and reliably performed on routine formalin fixed and paraffin processed tissue. (Previously much of this work could only be done on frozen section material)
- Development of a broad range of polyclonal and monoclonal antibodies
- Development of sensitive detection and amplification systems, enabling demonstration of previously undetectable antigens
- Availability of automated immunostainers

Table 2

Application of Immunohistochemistry

- Diagnosis of primary malignant tumours – some tumours are so poorly differentiated that their histogenesis is not apparent on routine haematoxylin and eosin stained sections
- Determining the likely site of origin of metastatic tumours
- Categorisation of leukaemias and lymphomas e.g. T and B cell markers
- Detection of molecules that have prognostic or therapeutic significance e.g. oestrogen and progesterone receptor protein, HER2 receptor
- Detection of minimal disease – highlighting small numbers of tumour cells, which may be difficult to appreciate on routine sections, small volume residual tumour in resection specimens or sentinel lymph node biopsies
- Use in conjunction with fine needle aspiration cytology – evaluation of cell block material to ascertain nature of primary tumour and or metastasis
- Highlighting proximity of poorly differentiated single tumour cells to surgical margins

The antigenic sites (epitopes) were initially unmasked on formalin fixed tissue sections using proteases (PIER – protease induced epitope retrieval). Whilst this was useful, the technique had its limitations. A significant advance was made when it was discovered that heating formalin fixed paraffin sections in a fluid medium exposed previously masked antigens (HIER – heat induced epitope retrieval). A range of heating systems are used including microwave ovens, autoclaves, pressure cookers, vegetable steamers, ovens and thermal cyclers. The duration of heating required depends upon the maximum temperature reached and the time that the tissue had been fixed in formalin.

Range of Antibodies Available

In general monoclonal antibodies can be developed against virtually any antigen and an extensive range of antibodies are available for routine diagnostic use. Examples include antibodies directed to cell adhesion and surface markers, cytoplasmic elements and structures (for instance intermediate filaments – keratins and vimentin), nuclear proteins, hormone receptors, markers of muscle, neuroendocrine, endothelial and melanocytic differentiation. Cell proliferation markers include proliferating cell nuclear antigen (PCNA) and Ki-67. A number of tumours have chromosomal translocations and these cells have expression of gene products which can be identified with immunohistochemistry (which serves as a surrogate marker for the chromosomal translocation). Tumours with known translocations which can be detected by immunohistochemistry include Ewing’s sarcoma/primitive neuroectodermal tumour, desmoplastic round cell tumour and alveolar soft part sarcoma.

Figure 1 Peroxidase Antiperoxidase system

Peroxidase AntiPeroxidase

Complex

Secondary Antibody

Primary Antibody

Tissue Antigen
Application of Immunohistochemistry in Routine Laboratories

Immunohistochemistry is valuable in the diagnosis and management of tumours and it has applications in a number of scenarios, some of which are listed in Table 2 (previous page).

Immunohistochemistry is an adjunct to the diagnosis of tumours and the findings must be interpreted in the light of the features seen on the routine haematoxylin and eosin sections and the clinical setting. Figure 2. demonstrates positive S100 protein staining, which in conjunction with a panel of other immunostains assisted in the diagnosis of invasive malignant melanoma. Also note that on this section the stain highlights greater numbers of tumour cells, than initially appreciated on the haematoxylin and eosin stained section.

Limitations of Immunohistochemistry and Approach to Diagnosis

There are a number of causes of false negative and positive staining. In addition some tumours may develop aberrant expression of various antigens, not detected in normal mature cells. Other tumours may lose or only partially express antigens which are present in normal mature cells. In view of this and the differing specificity and sensitivity of antibodies a panel of immunohistochemical stains is usually performed in conjunction with control material on the slide. The control material may either be intrinsic to the specimen, for instance normal blood vessels in the specimen, or a section of known positive staining tissue is placed on the same slide as the test section (to ensure the same conditions for the control and test specimen).

Clinical Benefits of Immunoperoxidase Tests

- Immunoperoxidase testing can be performed on biopsy samples and cell block material, enabling rapid diagnosis, without the need for frozen section and or excisional biopsy prior to definitive therapy.
- Definitive diagnosis based upon biopsy material enables pre-operative chemotherapy and radiotherapy, decreasing tumour size prior to definitive surgery and improving prognosis for some advanced tumours.
- Diagnosis based upon at times relatively minimal tissue enables accurate prognostication and in some clinical circumstances therapy without definitive surgery e.g. hormone and chemotherapy for breast carcinoma metastases.
- In rare circumstances tumour diagnosis can be made on partially crushed biopsy material which in the past would have been not diagnostic of malignancy; enabling on going patient management, rather than open biopsy.
- Reduction in reports with indeterminate results.
- Advances in tumour molecular biology and immunoperoxidase techniques have improved classification of tumours and new entities have been discovered enabling more accurate prognostication and improved targeted therapy. An example of this is the significant advances which have been made in our understanding and classification of lymphomas.
- More specific diagnosis and prognostic information enabling targeted therapy. The advances in immunohistochemistry and tumour diagnosis coincide with the increasing array of tumour specific chemotherapy agents and therapy regimens that are currently available.
- Ascertaining the likely primary site of origin of metastatic tumour enables a targeted search for the primary, considerable saving of time and cost and allows specific therapy to be commenced with less delay.
Table 3. Panel for the Workup of common Pleomorphic Cutaneous Spindle Cell Tumors, with Expected Immunophenotypes*

<table>
<thead>
<tr>
<th></th>
<th>Cytokeratins</th>
<th>S100 Proteins</th>
<th>Melanocytic Markers (HMB-45, Melan-A)</th>
<th>Smooth Muscle Actin</th>
<th>Desmin</th>
<th>Endothelial Markers (CD31, CD34)</th>
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<tr>
<td>Sarcomatoid SCC</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Melanoma</td>
<td>−/+</td>
<td>+</td>
<td>−/+</td>
<td>−</td>
<td>−/+</td>
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</tr>
<tr>
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<td>−</td>
<td>−</td>
<td>−</td>
<td>−/+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Leiomyosarcoma</td>
<td>−/+</td>
<td>−/+</td>
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<td>+</td>
<td>+/−</td>
<td>−/+</td>
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<tr>
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</tbody>
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SCC = Squamous Cell Carcinoma

AFX = Atypical Fibroxanthoma

+ positive in more than 90% cases

−/+ positive in 50 to 70% of cases

− usual negative but anomalous expression may be seen in up to 25% of cases.

References

2 Enzinger and Weiss’s Soft Tissue Tumours. Sharon Weiss, John Goldblum. Fifth Edition
3 Rosai and Ackerman’s Surgical Pathology. Juan Rosai. Ninth Edition
4 Seminars in Diagnostic Pathology. Immunohistochemistry in Tumour Diagnosis. Volume 17, No 3. August 2000

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Investigation and Management of Primary Hyperparathyroidism

Hypercalcemia is a common laboratory finding. In most cases the differential diagnosis can be separated into parathyroid dependent disease, which is most commonly due to Primary Hyperparathyroidism or parathyroid independent disease, which is most often due to malignancy.

In 2008 in Australia, Primary Hyperparathyroidism (PHPT) is commonly, asymptomatic, long-standing and if symptoms arise, the most common presentation is with either renal stone formation or minimal trauma fractures. Consensus guidelines regarding the diagnosis of PHPT were last published in 2002 and it is worth summarising these recommendations as well as providing some local Australian recommendations (1).

Free ionised calcium
Total calcium, corrected for serum albumin, is routinely used as an estimate of the free or ionised calcium concentration. In several case series, the direct measurement of ionised calcium has been found to be more sensitive than total corrected calcium in the diagnosis of PHPT (2). One other important reason to measure ionised calcium is to confirm an elevated corrected total calcium result. Consequently, at Clinipath Pathology, in patients suspected of suffering from hypercalcemia or who have an elevated corrected total calcium, assessment of ionised calcium is recommended. The main limitation of ionised calcium measurement is the need to measure ionised calcium using a fresh dedicated sample ideally collected after an overnight fast.

Parathyroid hormone (PTH)
Once hypercalcemia has been confirmed, measurement of PTH is advised. An elevated intact PTH result which is 1-2 times elevated is typical of PTH dependent disease. Even an intact PTH result within the upper half of the reference limits is regarded as inappropriate in the setting of hypercalcemia and supportive of the diagnosis of PTH dependent hypercalcemia (3).

In PTH independent disease, intact PTH results will often be suppressed. PTH related peptide (PTHrP) which is secreted by many tumours, does not cross react in intact PTH assays. At Clinipath Pathology, PTH is ideally measured in a fresh serum sample because the hormone is unstable in stored samples.

Consider the differential diagnosis
The differential diagnosis of PTH dependent hypercalcemia includes primary hyperparathyroidism (PHPT), due to a solitary adenoma or diffuse hyperplasia, lithium therapy, Familial Hypocalciuric Hypercalcemia (FHH) or tertiary hyperparathyroidism associated with renal failure.

Parathyroid cancer is a common concern of patients but this condition is extremely rare (<0.5%). In the absence of a palpable neck mass or markedly elevated intact PTH results (typically > 4-5 times elevated above the upper reference limit in a patient with normal renal function), parathyroid cancer can be excluded and the patient reassured. A review of medication use, assessment of renal function and enquiry regarding a family history of hypercalcemia is advisable to exclude other causes of PTH dependent hypercalcemia.

FHH and urine calcium assessment
Assessment of fasting urine calcium excretion (CaE) or 24 hour urine calcium/creatinine clearance ratio is required to exclude FHH. FHH is due to a mutation in the Calcium Sensing Receptor (CaSR) which results in loss of function and consequently higher circulating calcium levels with a characteristically low urine calcium. Consequently, assessment of either a fasting urine calcium excretion (CaE) or a 24 hour urine calcium creatinine clearance ratio is needed (4). In FHH, CaE is typically less than 30 umol/L GFR (Glomerular filtration rate) and the 24 hour clearance ratio is characteristically less than 0.01. Furthermore, it is rare to see intact PTH results more than 2 fold elevated in FHH. The importance of recognising FHH is to avoid unnecessary surgical neck exploration as this disorder cannot be corrected surgically and is not associated with the same morbidity that is characteristic of PHPT. Occasionally, cases of PHPT can present with very low urine calcium results and a specialist opinion may be advisable (see later).

If screening tests suggest a diagnosis of FHH, it is advisable to measure ionised calcium, intact PTH and fasting urine CaE or 24 hour urine calcium creatinine clearance ratio in first degree family members (parents or siblings) as there is a 1 in 2 chance of siblings harbouring the same mutation and hence being hypercalcaemic and having a low urine calcium excretion.

Since FHH may occur with a variety of CaSR mutations, a single genetic test is not available and therefore genetic testing is not helpful in the initial diagnostic algorithm.

Lithium therapy
In patients taking lithium and where it is safe to cease medication, withdrawal and retesting in 3 months is currently advised. Not all cases of lithium induced
Investigation and Management of Primary Hyperparathyroidism continued

hyperparathyroidism will resolve with this strategy but most cases with persistent hypercalcemia will suffer from PHPT.

Bones, dietary calcium and vitamin D

In PHPT, further investigation with bone mineral density (BMD) is advisable to assess future fracture risk. Fracture rates are increased at all sites and a BMD T score < -2.5 is one of the current criteria for surgical neck exploration. Assessment of 25 hydroxyvitamin D is also advisable as vitamin D deficiency often coexists with primary hyperparathyroidism. In patients with 25OHD < 50 nmol/L, supplementation with cholecalciferol is suggested, although more frequent monitoring of serum calcium is advisable. In such cases, assessment of ionised calcium at 2 weeks, 1 month and then 3 monthly after starting cholecalciferol is one approach. Several short term studies have indicated that this approach appears safe and may result in a fall in intact PTH. For similar reasons, restriction of dietary calcium is unnecessary and may in fact result in worsening parathyroid disease.

Surgery for PHPT

Based on consensus recommendations, surgical treatment for PHPT could be offered if the patient is:

- young (<50 years of age),
- serum corrected total calcium is high (> 3 mmol/L or ionised calcium > 1.5 mmol/L),
- 24 hour urine calcium excretion is increased (>7.5 mmol/day),
- creatinine clearance is reduced (by > 30%),
- bone density is low (T score below -2.5 at hip, spine or forearm), or
- medical surveillance is not desirable or possible.

Where surgery is advisable and this is the initial presentation, preoperative localisation imaging studies are not required. Assessment of thyroid function and thyroid ultrasound imaging is advisable if focal enlargement of the thyroid is detected clinically and pragmatic if surgical neck exploration will proceed. The risks of laryngeal nerve injury and post surgical hypoparathyroidism should be explained and it is advisable to refer the patient to an experienced endocrine surgeon to minimise such risks. Postoperative hypocalcemia may occur due to hypoparathyroidism or hungry bone syndrome which is due to rapid skeletal uptake of calcium. Both conditions are best managed by an expert in the management of bone and calcium disorders and often require in patient management if symptomatic.

Surveillance in non-surgically managed patients

In patients that decline surgical neck exploration or do not meet the above criteria, routine surveillance with 6 monthly assessment of serum ionised calcium, PTH, creatinine/eGFR and annual BMD plus 25 OHD can be offered (see Table 1 over page).

Reasons to refer for a specialist opinion

- Doubt regarding diagnosis, especially where FHH is a possibility.
- Family history of hypercalcemia: other causes such as multiple endocrine neoplasia, jaw tumour syndrome etc should be considered in such cases.
- Doubt regarding surgical management options.
- Advice on medical management in patients suffering from symptomatic disease (renal stones, low BMD) and surgery is not advisable/possible.
- Postoperative hypocalcemia after surgical neck exploration (urgent/immediate referral advised).
- Recurrent PHPT which will require preoperative localisation studies (see Image A & B over page).

Fig 1 Parathyroid adenoma with adjacent rim of compressed parathyroid tissue arrowed (low power insert)
Investigation and Management of Primary Hyperparathyroidism

Medical management of PHPT

Medical management options include encouraging sufficient dietary calcium intake to attain current Australian recommendations (1000mg in adults and 1300mg in postmenopausal women and men over age 70), supplementation with cholecalciferol if 25 OHD is less than 50 nmol/L, and consideration of bisphosphonates or raloxifene (in postmenopausal women) if fracture risk is increased.

Overview

The typical patient suffering from PHPT in Australia is asymptomatic. Approximately one in five patients with PHPT suffer classical symptoms with kidney stones or overt bone disease. Some patients report other symptoms which are non-specific, may not relate to the presence of PHPT and do not figure in decisions regarding surgical treatment. The link between cardiovascular disease (hypertension), gastrointestinal disease (peptic ulcers) or metabolic disease (diabetes mellitus) and PHPT is not clear or proven to be causal. The differential diagnosis of parathyroid dependent hypercalcemia should be considered, a review of surgical indications should be performed and in those patients not subjected to surgery, biannual surveillance should be instituted.

Table 1. Pathology and Radiology Investigations

<table>
<thead>
<tr>
<th>Investigation</th>
<th>Pathology Abbreviation</th>
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<tbody>
<tr>
<td>Ionised Calcium</td>
<td>ionCa</td>
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<tr>
<td>Parathyroid hormone</td>
<td>iPTH</td>
</tr>
<tr>
<td>Creatinine</td>
<td>Cr</td>
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<tr>
<td>Fasting urine calcium excretion</td>
<td>urine CaE</td>
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<tr>
<td>25 hydroxyvitamin D</td>
<td>Vit D</td>
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<td>Bone Mineral Density</td>
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<td>Ionised Calcium</td>
<td>ionCa</td>
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<td>Parathyroid hormone</td>
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<td>Creatinine</td>
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<td>25 hydroxyvitamin D</td>
<td>Vit D (pref winter end)</td>
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<tr>
<td>Bone Mineral Density</td>
<td>BMD</td>
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References


Haemochromatosis Testing and Medicare Rebates

We would like to clarify the Medicare Australia rules that govern patient rebates for Haemochromatosis testing. For your patients to be able to claim a Medicare rebate they must fulfil one of the following criteria:

- a) the patient has an elevated transferrin saturation or elevated serum ferritin on at least two occasions; or
- b) the patient has a first degree relative with haemochromatosis; or
- c) the patient has a first degree relative with homozygosity for the C282Y genetic mutation, or with compound heterozygosity for recognised genetic mutations for haemochromatosis.

Information supporting these criteria should be included on the request form, for example:
- Sister with Haemochromatosis
- Mother C282Y/H63D positive
- High TF sat or high ferritin x2.

If we perform haemochromatosis testing on patients who do not meet any of these criteria, they will receive a private bill and will not be eligible for a medicare rebate.