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ANATOMIC PATHOLOGY

Comparison of Morphologic Features and Outcome of Resected Recurrent and Nonrecurrent Squamous Cell Carcinoma of the Penis: A Study of 81 Cases

Chaux A, Reuter V, Lezcano C, Velazquez EF, Torres J, Cubilla AL.

Penile squamous cell carcinoma (SCC) is considered a loco-regional disease with a fairly predictable pattern of progression. Widespread dissemination occurs in at least one-third of the patients. Local recurrence (defined as the presence of tumor after a primary treatment affecting any remainder tissue, including skin, erectile corpora, or urethra) present in up to 30% of the patients increases the risk of regional inguinal and pelvic lymph nodes metastases. The aim of this study was to identify adverse pathologic prognostic factors in patients with recurrent tumors. Clinicopathologic features of 81 surgically treated patients (25 with recurrent and 56 with nonrecurrent SCC) were evaluated; 56 patients (19 with recurrent and 37 with nonrecurrent tumors) additionally received groin dissections. Follow-up (2 to 372 mo, mean of 71 mo) was obtained in all patients. Comparison of recurrent tumors at the time of the primary diagnosis and of recurrence showed that histologic subtype and grade were identical in 76% of the cases and converted to a higher grade tumor in 24% of the cases, especially, in patients treated with local excisions and circumcisions. Most of the recurrences (67%) seemed at or before 12 months. Comparison of recurrent and nonrecurrent tumors showed that high grade tumors (basaloid and sarcomatoid) tended to be significantly associated with recurrent tumors, whereas low grade variants (papillary, warty and verrucous) were more frequent in the nonrecurrent group; recurrent tumors invaded into deeper anatomic levels than nonrecurrent tumors. The incidence of inguinal lymph node metastasis was higher in recurrent tumors (79% vs. 49%, $P=0.0272$). Cancer-specific survival was of 46% versus 76% at 3 years of follow-up in recurrent and nonrecurrent tumors, respectively. Patients with recurrent tumors had a median survival of 2.9 years; no major changes in survival were noted after 3 years of follow-up. Mortality was higher in the recurrent group (56% vs. 29%, $P=0.0188$); 80% of patients with high-grade tumors (basaloid, sarcomatoid, and high grade usual or hybrid verrucous SCCs) died from penile cancer. Mortality in patients with usual SCC was higher in the recurrent group, but similar in basaloid and sarcomatoid SCCs. After 3 years there was no survival difference in patients with low-grade recurrent tumors; however, in the high grade recurrent group there was a progressive and gradual decrease in survival from 2 to 10 years (median survival of 2.5 y). In summary, histologic subtypes and grades of SCCs were similar in the majority of original and recurrent carcinomas. Inguinal metastasis and mortality were higher in recurrent than in nonrecurrent carcinomas. Basaloid, sarcomatoid, and mixed usual-verrucous variants and invasion of corpora cavernosa or preputial skin were significant adverse prognostic factors of recurrent carcinomas. Local excision and partial penectomy were not adequate procedures for sarcomatoid and basaloid penile carcinomas. Carcinomas of foreskin had a better prognosis. Conversion from low to high-grade carcinoma was related to significant mortality. The identification of the adverse prognostic factors found in this study should be the base for an aggressive initial therapy to prevent recurrence in a subset of

penile cancers. Re-excision of the recurrent tumor permitted the control of the disease only in one-third of the patients.

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Real Time Intraoperative Confocal Laser Microscopy-Guided Surgery

Nguyen, Nam Q.; Biankin, Andrew et al

Objective: To assess the potential utility of in vivo histologic surface and subsurface imaging in real-time using the Optiscan confocal laser microscope to detect diseased tissue at the time of surgery.

Summary Background Data: The goal of surgical treatment of diseases such as cancer is complete microscopic resection of diseased tissue; however, current methods for the assessment of extent of disease at the time of surgery are inadequate.

Methods: We assessed the potential of the Optiscan confocal laser microscope, a new device developed for real-time in vivo histologic surface and subsurface imaging during surgery.

Results: Intravenous Fluorescein Sodium contrast enabled visualization of cellular and architectural morphology of intra-abdominal organs with magnification equivalent to light microscopy and enabled differentiation between normal organs and disease.

Conclusions: Real time intraoperative confocal microscopy has significant potential application in detecting disease, and influencing decision-making at the time of surgery.

A fundamental principle in the surgical treatment of diseases such as cancer is complete microscopic extirpation of diseased tissue. A current challenge in the surgical management of intra-abdominal malignancy is the accurate determination of the extent of disease at the time of operation so as to successfully accomplish this goal. Confocal endoscopic microscopy is a technique that currently provides real-time in vivo microscopic images of gastrointestinal tract mucosa during endoscopy, and has significant potential clinical utility in colorectal cancer screening, surveillance for cancer in ulcerative colitis, detection of *Helicobacter pylori* infection, diagnosis of gastric cancer, detection of Barrett's associated neoplasia, and in the evaluation of celiac disease. Here, we present the first report of novel technology developed based on endoscopic methodologies, in the surgical management of intra-abdominal malignancy.

The Optiscan intraoperative confocal laser microscope is an adaptation of endoscopic technology to enable real-time in vivo histologic surface and subsurface imaging during surgery. Our aim was to assess the potential utility of this new technology in providing rapid real-time in vivo microscopic histologic information during surgical exploration and resection of intra-abdominal malignancy and assess its potential in assisting the surgeon in decision making at the time of operation, particularly in defining the extent of disease.

Once surgical access to the abdominal organ of interest was established, a fluorescent contrast agent (Fluorescein Sodium, 1 mL of 10% solution) is injected intravenously. With sterile water-based lubricating gel applied to the imaging window of the rigid microscope, the tip of the probe is then placed in direct contact with the organ of interest for imaging. Footswitch controls are used to adjust the depth of the imaging plane and obtain surface and subsurface microscopic views up to a depth of 200 μm .

Fluorescence based confocal laser microscopy was developed and used in this study because of its superior resolution compared with non-fluorophore-based systems, and as a consequence fluorescein is essential for imaging. A laser emitted from a light source along the optical fiber of the probe is focused onto the tissue plane of interest. Upon stimulation by the laser light source, an exogenously administered fluorescent agent reflects the light back at a different wavelength. Through movement of the laser along the x and y axes, a 2-dimensional image is created. The focusing lens, furthermore, can be moved along the z axis to vary the depth of imaging.

This first assessment of intraoperative confocal microscopy for intraoperative examination of abdominal organs suggests that it is feasible to use confocal microscopy to rapidly assess the in vivo histology of various abdominal organs, with recognizable microscopic architecture and cellular features of each organ. Furthermore, the device is ergonomic for use during surgery, not time-consuming, and safe.

This new technology has potential applications that would substantially alter current surgical practice and optimize intraoperative management, particularly for cancer therapy. A central principle in the operative management of malignancy is the complete or maximal extirpation of diseased tissue. Occult metastatic disease is difficult to detect, and the extent of resection is often guided by the probability of achieving clearance, with knowledge concerning resection margins only determined some days after surgery. Examples include assessment of metastatic disease for cancer of the upper gastrointestinal tract, pancreatectomy either for pancreatic cancer, or precancerous lesions such as intraductal papillary mucinous neoplasms, debulking of peritoneal disease in ovarian cancer and resection of liver metastases. Intraoperative sampling with histologic frozen-section examination is commonly used, is often suboptimal and can be time-consuming, adding up to 60 minutes onto operating times. A better understanding of the morphologic appearances on confocal microscopy and correlation with histopathological appearances on light microscopy is required to define its applicability. Foreseeable future applications include the selective targeting of cancer cells using fluorophore-labeled ligands that would selectively bind to the surface of cancer cells and more accurately detect malignancy. Different fluorophores for deeper penetration using longer wavelength, and an ability to differentially label cell nuclei would also facilitate the advancement of this technology.

In conclusion, real-time “virtual histologic” examination during intra-abdominal surgery using the intraoperative confocal laser microscope is feasible, reproducible and safe. The ability of this technique to differentiate normal and diseased tissue has significant potential in guiding and assisting surgeons in deciding the type as well as the extent of surgery. The potentially significant benefits of this new technology warrant further investigation to define its role in the surgical treatment of cancer and other diseases.

Some Landmark Papers in Surgical Pathology, 1957 to 2007: A Personal Opinion and Some Views

Pai, Sanjay A

From time to time, while signing out our surgical pathology cases, my colleagues and I have noted how certain pathological entities have been described either fairly recently, or at least since we entered the medical field. The best example of such a new disease is, of course, AIDS. In histopathology, lesions such as aggressive angiomyxoma, solid aneurysmal bone cyst, anaplastic large cell lymphoma and gastrointestinal stromal tumour are four lesions that we now see, but which would have been signed out quite differently in a different era.

This, and my interest in history of medicine and pathology got me thinking: which are the 20 most important landmark papers/concepts in surgical pathology over the past 50 years, 1957–2007? I refer to papers which have changed the practice of surgical pathology in one respect or the other. I asked, by e-mail, 83 pathologists around the world what their choice of such papers would be. Of course, the list was likely to be subjective, given that there are obviously much more than 20 important papers. Further, many of those receiving the mail had specialty interests and quite a few have described some of those entities. There was only one condition: the papers must be for the time period 1957 to 2007. Respondents could choose papers which have radically changed the practice of pathology in any respect, or simply their favourite paper (for whatever reason—it may have been elegantly written, it may have had a major impact on their field or changed a concept entirely, it may have been related to the first paper that they had published, etc). Respondents were asked to try and include papers from beyond their current area of interest or specialisation and to state why they had included them in the list, if it were not immediately obvious.

The pathologists included editors of journals, and eminent pathologists and researchers from North America, Europe, Asia, Australia and South Africa. Many of the pathologists had contributed greatly to the pathology literature and some had eponymous diseases or made important, original contributions to the field. Most of the pathologists had specific areas of specialisation—nephropathology, paediatric pathology, haematopathology, neuropathology, etc; some also had an interest in the field of history of medicine and pathology.

Based on an earlier experience when I polled 44 physicians from around the world (to get their lists of favourite books), and received as many as 38 responses, I expected to get a reasonably high response rate. However, only 24 pathologists responded. Thirty-six did not reply; 22 replied, stating that they lacked the time to think on the theme or responded initially and eventually did not find the time to submit their lists. One respondent could not submit a list as he was a basic researcher and not a surgical pathologist.

The 24 who sent in responses, submitted from 0 [an iconoclastic “[horizontal ellipsis][cannot] pinpoint any particular article[horizontal ellipsis]that changed the practice of surgical

pathology[horizontal ellipsis]I do not believe that there has been any radical change in the practice of our craft in the last several decades[horizontal ellipsis]immunohistochemistry might have changed the practice in the US, but in India, by and large, the practice has remained the same” response] to 22 papers. I, too, formed a list of my personal choice of landmark papers (n = 18). In all, including my list, there were 164 papers [mean = 6.2 papers per list], including some concepts, rather than definite papers selected by the respondents. Because of their diverse interests, there were few papers that were common in the different lists.

Some chose specific papers while others suggested new entities or concepts in pathology. Thus, it is difficult, in some cases, to pinpoint a precise research paper on a topic. Despite my request to include papers in different fields, many chose to include papers only from or almost entirely from their fields of interest, clearly a result of subspecialisation. As one specialist put it, “I am so far removed now from surgical pathology for any views I have to be way out of date”. One editor wrote in to say that “there are no modern classics so my tenure as editor has been much of a muchness”, while one respondent had “a great fondness for articles that were conceptual to the diagnostic process”. Yet another chose to only include articles on the history or revisionist history of specific diseases. In six cases, respondents included some of their own papers as among those significant to the field, while one pathologist included a paper which was “the most complete review of a neoplasm of which I had (the previous year) made a very modest, purely histological description, but my humbler paper was followed by the very comprehensive, multidisciplinary and definitive study”.

My own selection of 18 classic papers were (in no particular sequence), “new” diseases such as Kikuchi–Fujimoto disease, Rosai–Dorfman disease, anaplastic large cell lymphoma, cellular schwannoma, hyalinising trabecular adenoma of the thyroid, dysembryoplastic neuroepithelial tumour, MALTomas, Nathan Lane’s 1957 paper in *Cancer* on pseudosarcoma, the Carney triad and the HNPCC paper (as papers which showed how apparently diverse diseases could have a common aetiology); other important papers were the one by CDM Fletcher questioning the existence of malignant fibrous histiocytoma, development of the concept of GIST and that of sentinel lymph node, Gleason’s grading of prostate carcinoma, Kohler and Milstein’s paper on monoclonal antibodies, the Working Formulation paper and the paper describing the existence of the myofibroblast and *Helicobacter* gastritis.

Three papers—those on *Helicobacter*, Gleason’s grading of prostate cancer and the concept of monoclonal antibodies—were the most commonly chosen classics (n = 5). The discovery of apoptosis in particular stands out, as it was the only paper chosen in two separate lists: the pathologists were categorical that it was the most important biomedical discovery in the past half century. Also, the papers by WH Clark Jr on the classification of melanoma were selected by four pathologists. Other papers/concepts which appeared in two lists were: the concept of MALToma by Isaacson and Wright, John Beach Hazard’s description of medullary carcinoma of the thyroid, the paper on Burkitt’s lymphoma by Barr’s group, hyalinising trabecular adenoma, Ellis and Elston’s paper on grading of breast carcinoma, sentinel node biopsy, the discovery of HIV–AIDS, papilloma virus as an oncogenic agent, and the concepts of GIST and the adenoma–adenocarcinoma sequence in colonic carcinoma.

Two of the discoveries—those of monoclonal antibodies by Kohler and Milstein and that of *Helicobacter pylori* by Marshall and Warren—have led to the scientists being awarded the Nobel prize for physiology or medicine. The pathologist who selected historical/revisionist historical papers included the discovery of monoclonal antibodies and papilloma viruses as carcinogens as important—but opted for entirely different papers/authors.

Thus, of the papers that have influenced the practice of pathology the most in the recent past, as judged by this survey, those by Gleason and Clarke are by anatomic pathologists, while the ones on apoptosis and on monoclonal antibodies are by basic researchers, and the discovery of *Helicobacter* is by a team consisting of a physician and a pathologist.

Many journals reprint classic papers or have carried articles on the subject of classic papers in medicine. Eugene Garfield, the guru of citation index and impact factors has written many articles on citation classics over the years. At least two of them were in pathology and include *some articles* directly related to anatomic pathology.

The idea of a book on landmark papers in a subject in medicine or other fields is not new. There are many recent books on classic papers in numerous areas of medicine, including haematology (*Hematology: landmark papers of the twentieth century*, by Marshall A Lichtman, Jerry L Spivak, Laurence A Boxer, Sanford J Shattil and Edward S Henderson), breast disease (*Classic papers in breast disease*, by Michael Baum and Craig Henderson), rheumatology (*Classic papers in rheumatology*, by Paul Dieppe, H Ralph Schumacher and Frank A Wollheim), critical care (*Classic papers in critical care*, by Mitchell P Fink, Michelle Hayes and Neil Soni), clinical chemistry (*Landmark papers in clinical chemistry*, by Richard M Rocco), haematological oncology (*Classic papers in hematological oncology*, by E Donnall Thomas, David G Nathan and John M Goldman) and genetics (*Landmarks in medical genetics*, by Peter S Harper).

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Panniculitis: clinical overlap and the significance of biopsy findings

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Panniculitis is a group of clinicopathologic entities characterized by inflammatory changes affecting primarily the subcutaneous adipose tissue. The clinical distinction between individual panniculitis types is often difficult because of the non-specificity of the appearance of skin lesions, even though the differences between presentation of various panniculitides such as lesion distribution, their number, tenderness and evolution are recognized. Such characteristics can help in the clinical differentiation among panniculitides with typical presentations; however, the atypical presentations of panniculitides, for the same reasons, are difficult to diagnose clinically. In addition to the atypical forms, the occurrence of overlap between various panniculitides can delay or mislead the clinical diagnosis. The existence of such atypical variants and the clinical overlap among various panniculitides has been described before, but any significant information as to the extent of such clinical variance and its significance is still not available. The histological examination of adequate subcutaneous tissue biopsy is diagnostic in most cases of panniculitides.

Background: Panniculitides are well-recognized clinicopathologic entities but the non-specificity of their clinical and pathological features often troubles the diagnostician.

Methods: This study retrospectively evaluates the clinical overlaps and the significance of histological findings among various panniculitides.

Results: The clinical evaluation in 55 panniculitides cases suggested the diagnosis of typical erythema nodosum (EN) in 26 cases, atypical EN in 17 cases, atypical nodular vasculitis (NV) in two cases, soft tissue infection in five cases and five cases remained unclassified. Skin biopsy evaluation provided definite panniculitis diagnosis in 53 cases including EN (28 cases), leukocytoclastic vasculitis (seven cases), NV (four cases), superficial thrombophlebitis (ST) (two cases), eosinophilic panniculitis (EP) (three cases), infection-related panniculitis (five cases), and one case each of erythema nodosum leprosum (ENL), lupus panniculitis (LP), pancreatic fat necrosis and acne conglobata with two cases remaining unclassified. Histologically, 'predominantly septal' and 'mixed panniculitis' were the chief inflammatory patterns in EN cases, while mixed panniculitis was seen in most LCV cases and predominantly lobular and mixed panniculitis in NV cases.

Conclusions: Biopsy evaluation of a panniculitis lesion is usually significant, and the application of a combination of histologic features rather than of a single biopsy finding or an inflammatory pattern is helpful in the diagnosis of panniculitis.

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CLINICAL PATHOLOGY

Improved Method for Assessing Iron Stores in the Bone Marrow

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Background: Bone marrow iron microscopy has been the “gold standard” method of assessing iron deficiency. However, the commonly used method of grading marrow iron remains highly subjective.

Aim: To improve the bone marrow grading method by developing a detailed protocol that assesses iron in fragments, in macrophages around fragments and in erythroblasts.

Methods: A descriptive study of marrow aspirates of 303 children (aged 6–60 months) with severe anaemia and 22 controls (children undergoing elective surgery) was conducted at hospitals in southern Malawi (2002–04).

Results: Using an intensive marrow iron grading method, 22% and 39% of cases and controls had deficient iron stores, and 40% and 46% had functional iron deficiency, respectively. Further evaluation of the iron status classification by the intensive method showed that functional iron deficiency was associated with significantly increased C-reactive protein concentrations (126.7 (85.6) mg/l), and iron stores deficiency with significantly increased soluble transferrin receptor concentrations (21.7 (12.5) μ g/ml).

Conclusions: Iron assessment can be greatly improved by a more intense marrow examination. This provides a useful iron status classification which is of particular importance in areas where there is a high rate of inflammatory conditions.

Functional iron deficiency develops when normal physiological systems for transporting iron to target tissues are impaired in the presence of satisfactory iron stores. This is commonly caused by cytokines released during an acute phase response to infection, leading to impaired erythropoiesis and later anaemia, usually termed “anaemia of inflammation”.

Although mass spectrometry has been recently used to give a definitive determination of iron in tissue, microscopic examination of Prussian blue-stained bone marrow aspirate has been considered the practical “gold standard” for determining iron depleted states. Conventionally, iron has been primarily assessed in marrow fragments which represent iron stores in the form of hemosiderin. Although some studies have shown a reasonable correlation between histological iron grading and chemical iron concentration in bone marrow, others have not, raising questions about the validity of the histological grading.

Iron visualised in marrow fragments is from a meshwork of reticular cells which are usually undistinguishable. However, single “loose” macrophages may be inspected for iron and it is hypothesised to be particularly important when iron in fragments is absent and may signify the lowest level of iron stores depletion.

In areas where there is a high prevalence of inflammatory conditions, functional iron deficiency commonly occurs. Erythroblast iron may be indicative of cellular iron utilisation and decreased

in functional iron deficiency; however there has been little research on the use of erythroblast iron as a marker of cellular iron availability. Furthermore, in malaria endemic areas, interpretation of iron status may be confounded by the presence of hemozoin.

Recent studies have suggested that it may be detrimental to mass treat children with iron, so it is important to be able to identify children with iron stores deficiency. The aim of the present study was to determine if a more intensive bone marrow classification can distinguish iron deficiency states in severely anaemic children and their controls living in a malaria endemic area.

METHODS

This was a descriptive study which was part of a large case-control study investigating the aetiology of severe anaemia in Malawi. Children were recruited between July 2002 and July 2004 at Queen Elizabeth Central hospital in Blantyre and Chikwawa District hospital.

All children who presented to hospital with severe anaemia (haemoglobin of less than 5.0 g/dl and no history of being transfused in the preceding four weeks), aged 6–60 months, were eligible for recruitment as cases. A group of children undergoing elective operations with no obvious signs of infection and within the same age range as cases were recruited as “normal” controls. Samples of venous blood and bone marrow aspirate were collected from cases and controls under anaesthesia from either the anterior or posterior iliac crest. The first few drops of a bone marrow aspirate were collected in an EDTA tube for smear preparation.

Written informed consent was obtained from the guardians of the children and the study was approved by the ethics committees of the University of Malawi and the Liverpool School of Tropical Medicine, UK.

Bone marrow smear preparation

Bone marrow smears were air-dried before being fixed in methanol for 5 minutes. Iron staining was done at the Wellcome Trust laboratories, Blantyre, using a commercial kit and according to methods recommended by the manufacturer (HematoGnost Fe, Darmstadt, Germany). Positive controls were included in each batch of slides.

Bone marrow smear iron grading

Bone marrow smears were graded by the conventional Gale’s method and by a new more intensive grading method.

Marrow smears were first assessed by one of the authors (KP) according to Gale’s histological grading method which assesses only marrow fragments. In order to reduce subjectivity, predefined descriptions and sample illustrations of each iron grade were used to grade fragments of all marrow smears. Only iron smears with at least seven fragments were assessed. Deficiency of iron stores was defined as an iron grade of none (grade 0) or very slight (grade 1).

All marrow smears were then systematically assessed using an intensive histological grading method in which iron is assessed in three sites—the fragments (as in Gale's method), macrophages and erythroblasts. Iron assessed in the fragments and macrophages represented iron stores while iron in the erythroblast represented utilisable iron. Additionally, 20 fields around and behind the fragments were examined at high power ($\times 1000$) and all macrophages in these fields were examined for the presence of iron. At high power magnification ($\times 1000$), 100 erythroblasts were examined and the percentage containing iron granules in their cytoplasm (ie, sideroblasts) were enumerated. Erythroblast iron deficiency was defined when $<30\%$ of erythroblasts had visible iron granules.

Results of iron smear assessment using the intensive histological grading method were interpreted as normal status (normal iron stores and normal erythroblast iron); functional iron deficiency (normal iron stores and deficient erythroblast iron); iron stores deficiency (depleted iron stores and normal erythroblast iron); and combined functional iron and iron stores deficiency (depleted iron stores and deficient erythroblast iron).

Other laboratory tests

Bone marrow iron status assessment was compared to peripheral blood iron markers from samples taken at the same time as the bone marrow aspirate. Haemoglobin was measured using a Coulter counter machine (Beckman Coulter, Durban, South Africa). Ferritin, a measure of iron stores deficiency, was determined using the electrochemiluminescence immunoassay (Modular Analytics E170, Roche Diagnostics, Switzerland), and soluble transferrin receptor (sTfR) levels, a measure of cellular iron need, using ELISA (Ramco Laboratories, Texas, USA). Immunoturbidimetric assay (Modular P800, Roche Diagnostics, Switzerland) was used to determine C-reactive protein (CRP) levels (measure of inflammation) in blood.

Statistical analysis

Data were double entered using Microsoft Access and Microsoft Excel. All data were exported to SPSS for Windows for analysis. Difference in means was evaluated using Student's t test and difference in proportions, using the $[\chi]^2$ test. Odds ratios (OR) and their 95% CI were used to measure association between categorical variables.

RESULTS

A total of 381 cases and 23 controls were recruited. Cases had an average age of 1.7 years (SD 1.1), and 46.7% (178/381) were male; controls had an average age of 1.8 years (SD 0.9) and 86.4% (19/22) were male. Death in-hospital occurred in 6.3% (24/381) of cases, with no deaths in controls.

Of a total of 381 cases, 334 bone marrow aspirations were attempted, from which 303 marrow smears were prepared. Forty-seven (12%) cases did not have a bone marrow aspiration for the following reasons: refusal by guardian, child being too sick, unsuccessful aspiration. Twenty-two

of 23 (96%) controls had a bone marrow aspirate collected and smear prepared. Therefore 303 case smears and 22 control smears were available for assessment.

Gale's grading method

From the total smears considered for assessment, 66 of 303 (22%) cases and 4 of 22 (18%) controls were not assessed because they contained inadequate bone marrow fragments for proper assessment. Assessment of the remaining smears showed that iron deficiency was present among 33.8% (80/237) of cases and 61.1% (11/18) of controls. According to conventional grading, iron stores deficiency was more frequent among the controls than cases, but this difference was not significant (OR = 1.8, 95% CI 0.8 to 4.25).

Intensive grading method

Staining quality was adequate to enable assessment of macrophage and erythroblast iron in 79% of cases (187/237) and 72% of controls (13/18). Cases and controls were classified into different iron status categories depending on iron assessment in fragment, macrophage and erythroblast. Functional iron deficiency was the most common iron status category among both cases (39.6%; 74/187) and controls (46.2%; 6/13). Iron stores deficiency was less frequent among cases (21.9%; 41/187) than controls (38.5%; 5/13; $p = 0.2$).

Categories of iron status classified by the intensive grading method were compared with values for peripheral blood markers of iron stores. Low levels of ferritin and high levels of sTfR signify deficiency of iron stores in the absence of inflammation. Mean ferritin concentration was lower in children with deficiency of iron stores (1.9 (SD 0.7) $\mu\text{g/l}$) than in those with no deficiency (2.8 (SD 0.5) $\mu\text{g/l}$, $p = 0.05$), or with functional iron deficiency (2.6 (SD 0.6) $\mu\text{g/l}$, $p < 0.001$). Children with deficiency of iron stores had a higher mean concentration of sTfR (21.7 (SD 12.5) $\mu\text{g/l}$) than those with normal iron stores (12.5 (SD 16.2) $\mu\text{g/l}$, $p < 0.001$), or functional iron deficiency (11.4 (SD 6.0) $\mu\text{g/l}$, $p < 0.001$). Children with functional iron deficiency had increased mean levels of CRP (126.7 (SD 85.6) mg/l) compared to those with iron stores deficiency (71.9 (SD 74.7) mg/l , $p < 0.001$), or normal iron status (99.8 (SD 70.1) mg/l , $p = 0.01$).

Ferritin, sTfR and CRP were determined for normal iron and iron deficiency states classified by Gale's grading method. Mean log ferritin was higher in those with normal status cases compared to those who were iron deficient. The converse was true for mean sTfR levels. Mean CRP levels were increased in children with normal iron status compared to those classified as iron deficient ($p < 0.001$).

DISCUSSION

The intensive histological grading method attempts to distinguish four different iron states compared to the two categories using Gale's method. The ability to distinguish states in which there is decreased cellular iron delivery to erythroblasts in the presence of adequate iron stores (termed functional iron deficiency), compared to states with limited availability due to lack of available iron in the reticular endothelial system, is of particular importance in areas of high malaria transmission and infection. Although some of the results of the biochemical tests are

lacking and some of the aspirates were too poor to examine for iron, this study managed to assess a substantive number of bone marrow aspirates. There were no identifiable reasons for selection bias for the missing results.

Functional iron deficiency, classified using the intensive histological grading method, was based on marrow findings alone. Although levels of CRP, a marker of an acute phase response, were mostly increased in all children, the finding of significantly raised levels among children with functional iron deficiency supports the hypothesis that these children have anaemia of inflammation. These children appeared also to have adequate iron stores as they had similar levels of ferritin and sTfR to children with normal iron status.

The use of erythroblast iron to assess iron status has been used in other studies and has certain limitations. Marrow smears were counter-stained with Safranin, giving a uniform pink background colour, which makes visualisation of cell types difficult, and hence may affect erythroblast iron assessment. The use of haematoxylin, or May–Grünwald–Giemsa, for counter-staining smears has been recommended as this provides improved cellular detail. Tham and Macon demonstrated that use of a silver stain to visualise erythroblast iron was more sensitive than Perls' stain. However, the precise chemical basis for the silver staining is still unclear.

Some researchers have described erythroblast iron assessment in comparison to simply counting erythroblasts with iron. Baumgartner-Staubli and Beck developed a “sideroblast score” which assigned an arbitrary value, ranging from 1 to 4 depending on the amount and morphology of iron granules, to each erythroblast with iron. This gave a poor correlation between the sideroblast score and the marrow iron stores assessed using Gale's grading method.

The Malawi Ministry of Health does not have guidelines on whether to give iron in the management of children with severe anaemia. In practice, most children are prescribed iron, however it is often unavailable and the compliance is poor. This study has shown that approximately 30% of severely anaemic children had deficiency of iron stores requiring iron treatment. Methods to identify these children are required in poor-resource settings that are based on simple, less invasive procedures than detailed marrow examination. Additionally this study observes a lower prevalence of deficiency of iron stores among severely anaemic children than controls. This phenomenon is not fully understood, but may support the hypothesis that iron deficiency is associated with decreased risk of infection.

This study of a large sample of bone marrow aspirates demonstrates that using an enhanced bone marrow slide assessment provides more detailed information which allows a more precise iron status classification.

Take-home messages

- Differentiation between functional iron deficiency and quantitative deficiency of iron stores is difficult, especially in areas of high infection pressure.
- A new method of grading of iron content of fragments, macrophages and erythroblasts in the bone marrow is able to distinguish between functional and quantitative iron deficiency in anaemic children.

- Severely anaemic children have less quantitative iron deficiency than controls without severe anaemia.

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MICROBIOLOGY

Immunoblot Analysis of Sera in Uncomplicated Typhoid Fever & With Typhoid Ileal Perforation

Madhulika Nambiar, Belgode Narasimha Harish, V. Mangilal, Dinker Pai & Subhash Chandra Parija

Background & objectives: Ileal perforation is a serious complication of typhoid fever. The exact reasons for the development of perforation in only a few of those infected with *Salmonella* Typhi is unknown, and it is likely that immunological factors are involved. Therefore we undertook this study to compare the antibody profile in patients with uncomplicated typhoid fever with those having ileal perforation by immunoblotting.

Methods: Two groups of patients were included in the study. Group II comprised patients with uncomplicated typhoid fever (n=47), and group I with typhoid ileal perforation (n=33). The flagellar (H), lipopolysaccharide (LPS) and outer membrane protein (OMP) antigens of *Salmonella* Typhi were extracted and used to test patient sera for antibodies by immunoblotting.

Results: Immunoblotting using *S. Typhi* antigens enabled the detection of *S. Typhi* antibodies in the two groups of patients. A significant difference was seen in the response of these two groups of patients with respect to antibodies to flagella, lipopolysaccharide and outer membrane proteins. Antibodies to flagella were more pronounced among patients with uncomplicated typhoid fever, while anti-OMP antibodies were significantly associated with typhoid ileal perforation.

Interpretation & conclusions: A comparison of antibodies in patients with uncomplicated typhoid fever and with ileal perforation revealed the differences in the antibody profiles of the two groups. Our study suggests that the difference in antibody response may in some way play a role in the pathogenesis of typhoid ileal perforation which can also potentially be exploited to develop suitable diagnostic tests.

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