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TITLE: Prevalence of MDS subtypes in Shanghai, China: A comparison of the World Health Organization and French American British classifications

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ABSTRACT

The prevalence of subtypes of the myelodysplastic syndromes (MDS) was determined in a prospective series of 176 patients presenting at 28 Shanghai hospitals. Diagnosis was established in a single laboratory, analyzing morphologic, immunophenotypic, and cytogenetic data, using the World Health Organization (WHO) revised classification and directly compared to the French American British (FAB) criteria. The median age at diagnosis for all cases was 53 years. There was a striking increase in the prevalence of RCMD in younger patients relative to other subtypes (WHO). The overall frequency of clonal cytogenetic abnormalities was 26.5% (WHO) and 31% (FAB). The most frequently encountered lesions were trisomy 8, del(20)q, del(7q), and del(5q). These results are consistent with previously reported age-dependent differences in MDS and a decreased frequency of del(5q) abnormalities between China and the West. These results also indicate that multilineage dysplasia is a prominent feature in MDS developing in younger individuals in Shanghai and suggest distinguishing between RCMD and RA may be important in the design of studies to further understand regional differences in subtype prevalence and to elucidate the pathogenesis of this complex and multifactorial disease.

FULL TEXT

1 Introduction

Myelodysplastic syndromes (MDS) are a heterogeneous group of diseases characterized by ineffective hematopoiesis and abnormal hematopoietic cell morphology, in one or more hematopoietic lineages, and a tendency of progression to bone marrow failure or acute myelogenous leukemia (AML). A central premise in the diagnosis and classification of MDS is the presence of dysplasia in one or more hematopoietic lineages, although other features, such as anemia, cytopenia, bone marrow cellularity, and blast cell count are variously weighted in subtyping the disease. Several reports have suggested an increasing incidence of MDS over the past decade; however, these observations are tempered by difficulties in assessing and discriminating between regional variations in prevalence, differences in classification, the increasing frequency of diagnosis and differences in the invasiveness or sophistication of diagnostic procedures commonly employed [1]. These problems have been further accentuated by the fact that the distinction of MDS as a clinical and nosologic entity, which began with French American British (FAB) classification in 1982 [2], has been evolving over the past 20 years and has only recently been recognized to be part of the same spectrum of neoplastic diseases as the acute myeloid leukemias [3].

The FAB classification places MDS into a single diagnostic framework, primarily on the basis of blast cell count, presence or absence of ringed sideroblasts (RS) and peripheral blood monocyte count, as refractory anemia (RA), refractory anemia with ringed sideroblasts (RARS), refractory anemia with excess blasts (RAEB), refractory anemia with excess blasts in transformation (RAEBT), and chronic myelomonocytic leukemia (CMML). The FAB further distinguishes AML, also primarily on the basis of blast cell count, bone marrow and peripheral blood cellularity as well as lineage involvement [2,4]. The recent World Health Organization (WHO) proposal is an attempt to improve the diagnostic and prognostic utility of MDS subclassification. The evolving clinical experience with the WHO classification system, which has been the subject of ongoing debate [5,6], has recently been reviewed [7,8]. The WHO proposal differs in several aspects from the FAB system that include: (1) the cut-off rate for the number of blast cells in the bone marrow necessary to define AML, which traditionally in FAB has been 30% and is 20% in WHO, and (2) the creation of a new subcategory, "refractory cytopenia with multilineage dysplasia (RCMD)", which distinguishes dysplasia occurring in a single cell lineage (e.g., erythroid) (i.e., RA) from multilineage dysplasia involving two or more lineages (i.e., RCMD) in the absence of an increase in peripheral blood or bone marrow blasts. The reduction in blast cell threshold for classification of AML has the effect of eliminating the FAB subcategory, RAEBT. The addition of RCMD as a category emphasizes the importance of multilineage dysplasia independent of increased blasts which appears to be of prognostic importance [9].

Chen et al. recently reported the results of a retrospective study of MDS which highlighted differences in the age of onset and prevalence of MDS subtypes between Chinese and Western populations diagnosed according to the FAB criteria [10]. We report the results of a concurrent analysis of clinical, morphologic, and karyotypic features of an unselected series of 176 cases of de novo MDS diagnosed in patients at Shanghai hospitals. These cases were diagnosed

according to the WHO classification and results directly compared with those obtained using FAB criteria.

2 Patients, materials, and methods

2.1 Case definitions

All patients, >=18 years of age, presenting at 28 Shanghai hospitals with initial clinical findings consistent with a hematopoietic abnormality between August, 2003 and February, 2005 were candidates for inclusion in this study. Informed consent was obtained according to the Declaration of Helsinki, 2004 and the NIH Common Rule (45CFR46), and together with the protocol, were approved by the Combined Institutional Review Board of the University of Colorado Health Sciences Center in Denver, Colorado and the Internal Review Board at Fudan University in Shanghai, China. Peripheral blood, bone marrow aspirates, and core biopsies were obtained on all individuals using standardized procedures and evaluated in our laboratory using morphologic, immunophenotypic, molecular, and cytogenetic techniques. MDS cases were initially classified according to the WHO as well as the traditional FAB classification systems [2]. Cases of CMML, which are classified as myelodysplastic/myeloproliferative diseases in WHO and as MDS under FAB, were included for comparison purposes. The requisite blast cell percentage for a diagnosis of AML in Shanghai is >=20%. For the purpose of comparison in this study, cases of AML originally diagnosed with blast cell percentages between 20% and 29% were reclassified as RAEBT according to FAB and compared with those classified according to WHO. Patients presenting with concomitant nutritional deficiencies (Vitamin B12, folate, or iron), congenital anemias, viral (including HCV or HIV) or bacterial infections, occupational exposure to benzene or receiving cytotoxic therapy with alkylating or anti-metabolic agents were excluded in this analysis.

2.2 Peripheral blood

Blood samples were collected by venipuncture and processed for routine complete blood count (CBC) (CellDyne 3700, Abbott, Park, IL), viral screen (HCV and HIV) (Imx, Abbott, Park, IL) and clinical chemistry for liver enzymes (LDH, ALT, and AST enzymes) (COBAS, Integra 400 plus, Roche Diagnostics, Shanghai, China). Vitamin B12 and folate were measured by chemical luminescence (Beckman Coulter Dxi800), and total iron binding capacity was measured using a Beckman Coulter LX20. Peripheral blood smears were made from finger sticks.

2.3 Bone marrow

Bone marrow aspirates and core biopsies were obtained by needle extraction (Jamshidi) from the posterior iliac crest. Aspirate cell suspensions where stained with fluorochrome-conjugated antibodies for flow cytometric analysis of bone marrow cellular subsets. Multiparameter analysis was performed using a dual laser flow cytometer (FC-500, Beckman Coulter, Hialeah, FL; Immunotech, Miami, FL) equipped with compensation software (Software CXP, Beckman Coulter). A broad panel of antibodies was used for immunophenotyping of bone marrow cells that included CD4 and CD8. (Beckman Coulter, Immunotech). Morphology and immunophenotype analysis were conducted on both bone marrow aspirate (flow cytometry) and core biopsy (immunohistochemistry) material. Bone marrow aspirate smears were prepared from fresh tissue and evaluated using Wright-Giemsa stained preparations and special stains, including an iron stain. Core biopsy sections were evaluated using sections stained with hematoxylin-eosin, Gomori trichrome, and immunoperoxidase-immunohistochemistry.

2.4 Morphologic analysis

Morphologic diagnoses were subjected to a minimum of two and usually three levels of review. Peripheral blood and bone marrow smears and core biopsy slides were evaluated by two out of three of us (J.M, X.D., R.D.I.), and independently reviewed by one or two of us (R.D.I., J.R.). The threshold for lineage involvement was defined as a minimum of 10% of the cells of a given lineage exhibiting dysplastic changes. Dyserythropoiesis was defined by abnormal nuclear morphology, including internuclear bridging, abnormal budding, multiple nucleoli, and abnormal mitotic figures or megaloblastoid features. Myeloid or granulocytic dysplasia was defined by nuclear hypolobulation as observed in pseudo-Pelger Huet cells, hypersegmentation, abnormal mitotic forms, either hypo- or hyper-granulation of

the cytoplasm as well as the presence of large irregular granules. Megakaryocyte dysplasia was defined by hypo-lobulated, hyper-lobulated or non-lobulated nuclei, multiple nuclei, prematurely segmented or "shedding" cytoplasm or the presence of micromegakaryocytes in the bone marrow or peripheral blood. Microscopic analysis was performed using an Olympus BX51 bright field microscope (Olympus Optical, Ltd., Tokyo, Japan).

All cases were evaluated for RS and iron utilization by morphologic examination of an iron stain. In practice, it is often difficult to distinguish between abnormal iron utilization and iron deficiency on the basis of the bone marrow iron stain alone-a problem that is exacerbated by the relative paucity of RS in the majority of our MDS cases. Similarly, prominent megaloblastic features are often encountered in both MDS as well as folate, iron-folate, and Vitamin B12 deficiencies. Therefore, nutritional deficiencies were independently determined by serum clinical chemistry, and patients with demonstrable deficiencies were excluded from this series.

Myelodysplastic syndrome-unclassifiable (MDS-u) is defined by WHO as MDS lacking findings appropriate for classification as RA, RARS, RCMD, or RAEB and in which blast cells are not increased. We restricted the use of MDS-u to those cases with features that did not meet either the narrow definition of RA (i.e., dysplasia involving only the erythroid lineage), or RCMD (i.e., lacking sufficient evidence of dyserythropoiesis but exhibiting dysplasia in the other two lineages). A total of nine cases fulfilled these criteria for classification as MDS-u, of which seven were hypocellular. Using the FAB criteria, eight of the MDS-u cases would be classified as RA. It is widely appreciated that the differential diagnosis of severely hypoplastic disease as aplastic anemia (AA) or hypoplastic MDS is fraught with difficulty and remains problematic. The use of MDS-u serves to identify cases with evidence of dysplasia but with minimal or inconsistent findings to support further classification.

2.5 Cytogenetic and fluorescence in situ hybridization analysis

Cytogenetic analyses were performed on unstimulated bone marrow cells following culture. Unstimulated bone marrow or peripheral blood cells were cultured for 24-72h, and G-banding analysis performed according to standard techniques. If possible, 20 metaphases were analyzed. Fluorescence in situ hybridization (FISH) analysis was performed on short-term cultures of bone marrow or blood cells. Sample preparation and hybridization were performed according to manufacturer's recommendations (Vysis, Downers Grove, IL). Images were viewed using Olympus fluorescence microscopes (Olympus Optical, Tokyo) equipped with appropriate filters and a PowerGene Macprobe image system (Applied Imaging International, Newcastle, UK). Systematic screening for del(5q), del(7q), +8, and 11q23/MLL rearrangements was performed on each case. In some cases, additional FISH studies were used to either characterize abnormalities observed in banded studies or confirm the presence of cytogenetic aberrations when suggested by other diagnostic work-up. A minimum of 500 cells and 10 metaphases were scored in interphase and metaphase analysis, respectively. All probes used in the FISH analyses were commercially purchased from Vysis (Downers Grove, IL).

2.6 Statistical analysis

The $[chi]^2$, Fisher's exact and Kruskal-Wallis tests were used to examine differences between MDS subtypes employing STATA 8.0 software (StataCorp, College Station, Texas). All *P*-values were two-sided and values less than 0.05 were considered statistically significant.

3 Results

The total number of cases of MDS diagnosed according to WHO was 176, including 101 males (57%) and 75 females (43%). The prevalence of WHO subtypes was: RA: 16 (9%), RARS: 1 (0.5%), RCMD: 122 (69.5%), RAEB: 28 (16%), and MDS-u: 9 (5%). The total number of MDS subtypes diagnosed according to FAB (218 cases) was greater than using WHO (176 cases), in part due to the inclusion of CMML (13 cases) as well as 28 cases of RAEBT and 1 RAEB that met a threshold for diagnosis as AML under WHO (Table 1). The majority of RCMD were reclassified as RA or RARS using FAB criteria. Seven cases of RCMD and one case of RA (WHO) met a threshold for >=15% RS, all of which were classified as RARS (FAB). Eight of the MDS-u cases were classified as RA under FAB. All cases of

AML diagnosed over the same period using WHO criteria that would be classified as RAEBT under FAB criteria were included for purposes of this comparison (Table 2). All had bone marrow blast cell counts less than 30%. Twenty had blast cell counts between 20% and 29%, one had a blast cell count less than 20% (i.e., acute erythroleukemia) and eight independently met criteria for diagnosis as AML with recurring cytogenetic abnormalities according to WHO (i.e., t(8;21), 11q23/MLL rearrangements). Taken together these represent a 20% increase in the number of MDS cases using the FAB compared to WHO criteria. Our findings are consistent with previously reported decreases in the prevalence of CMML and RARS in Asian populations [10-14].

The overall median age for MDS was 53 years (range 19-82) with 25% of the cases <=40 years of age (Fig. 1). In general, cases of RCMD (WHO) demonstrated the largest prevalence in this series independent of age. However, RCMD showed a significant difference in age-dependent frequency in the early age group (second decade) ($[chi]^2$ 92.21, P=0.0001). With the possible exception of platelets, individual bone marrow and peripheral blood parameters did not vary across MDS subtypes diagnosed according to WHO, and none were statistically significant (Table 3). The diagnosis of RCMD requires scoring a minimum of 10% dysplastic changes in two or more myeloid lineages; however, in the majority of these cases the degree of dysplasia was marked.

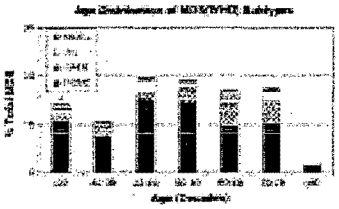

Fig. 1. Age-distribution of MDS subtypes according to WHO in patients grouped by decades. The age-distribution of MDS subtypes is plotted as the percent of total MDS. (*) A higher prevalence of RCMD was observed in all decades relative to other subtypes, but was significant only in the <=29 year age group (P=0.0001).

G-banded chromosome analysis was completed in 163 of 176 total MDS cases diagnosed according to WHO (92.6%) (Table 4). One case for which conventional cytogenetic analysis was not completed was demonstrated to have clonal abnormalities by FISH. Therefore, informative cytogenetic data was obtained from 164 of 176 patients (93.2%). Among those, 43 cases (26.2%) had clonal abnormalities. The most frequently observed chromosome abnormalities were trisomy 8 (20 cases, 12.2%), del(7q) (8 cases, 4.9%), del(20q) (7 cases, 4.3%), and del(5q) (4 cases, 2.4%) (Table 5). Translocations, each of which was unique, were observed in seven cases (i.e., 3 RCMD, 3 RAEB, and 1 MDS-u) with a median age of 63.3 years. A complex karyotype, defined as more than two independent chromosome aberrations, was detected in eight cases (4.90%). Using the definitions outlined by the International Prognostic Scoring System (IPSS) [15], cases with good, intermediate and poor cytogenetic risk features were observed in 76.6%, 12.9%, and 10.4% of patients, respectively. No cases of MDS with del(5q) as the sole abnormality were observed. The highest frequency of clonal cytogenetic abnormalities was found in cases with RAEB (40.7%), followed by MDS-u (37.5%), RCMD (24.6%), and RA (7.1%). Using case definitions for MDS as defined by FAB, 64 cases (31%) had clonal abnormalities.

4 Discussion

Characterization of clonal cytogenetic abnormalities has proven to be important in evaluating prognosis in MDS. However, there is only limited retrospective data on the MDS subtype distribution of cytogenetic abnormalities diagnosed according to WHO [10,11]. The frequency of aberrations in de novo MDS (FAB) has been previously reported to be 35-40% [10,11,16,17]. In this prospective analysis, clonal abnormalities were detected in 26.2% of MDS cases diagnosed according to WHO as compared to 31% diagnosed using FAB criteria. This not withstanding, informative cytogenetic data was available in more than 93% of our patients, which is significantly higher than 40-70% reported in most MDS (FAB) studies [10,11,18-21]. Differences in the frequency of clonal aberrations between our series and previous reports in large part can be attributed to differences in the WHO and FAB MDS classification

criteria. Cases of RAEBT (FAB), which are diagnosed as AML using WHO criteria, and therefore not classified as MDS, usually have a high frequency of chromosome abnormalities [10,11,16,17]. Further, cases diagnosed as MDS (FAB) with t [8,21] and 11q23/MLL rearrangements are diagnosed as AML according to WHO [22].

Cytogenetic abnormalities most commonly described in MDS in the West include (in the order of frequency) del(5q), del(7q), +8, and del(20q) [23]. Several studies, including ours, indicate that trisomy 8 is the most common cytogenetic abnormality in Asian MDS. However, inconsistent results are reported for the prevalence of del(5q), del(7q), and del(20q) [10-12,17,24]. In a retrospective study of Chinese MDS patients diagnosed according to FAB, Chen and colleagues reported the frequencies of del(5q) and del(7q) as 4.6% and 1.6%, respectively [10]. Our findings suggest that Asian patients more frequently present with del(7q) than del(5q) abnormalities, and that the frequency of del(5q) abnormalities is lower than in the West. It is possible that this may be related to the younger median age of MDS cases in Asian versus Western populations. Similar results also have been reported for Japanese patients by Toyama et al. [24] and Matsushima et al. [17] for overall MDS and by Matsuda et al. for RA [25]. According to IPSS, karyotype at diagnosis is an independent prognostic factor to predict survival and leukemic progression in MDS [15]. The frequency of karyotypes in RCMD in our study that are associated with a poor prognosis is low (7.9%) compared to 20% reported by Matsuda in a Japanese population with similar median age [25]. The reason for these differences is not immediately apparent. Using the same criteria, our cases showed frequencies among overall WHO MDS patients that are comparable to other studies (good risk: 76.6%, intermediate risk: 12.9%, and poor risk: 10.4%) [11,15]. Follow-up studies are ongoing to determine if the cytogenetic features defined by IPSS also predict risk in overall MDS or subtypes classified according to WHO.

Our results are consistent with studies that have reported a much younger age of onset for MDS, as well as a lower prevalence of RS and CMML in Asian populations relative to those found in the West [1,10-14]. The most striking observations in our patients were the increased prevalence of multilineage dysplasia together with the number of cases presenting at an early age. In contrast to other subtypes of MDS in which there is a tendency toward increased frequency with advancing age, cases of RCMD were often encountered in patients 19-29 years old, and the increased frequency of RCMD in this age group relative to total MDS or the other individual subtypes, such as RAEB or RARS is highly significant (P<=40 years of age (Fig. 1). In general, cases of RCMD (WHO) demonstrated the largest prevalence in this series independent of age. However, RCMD showed a significant difference in age-dependent frequency in the early age group (second decade) ([chi]2 92.21, P=0.0001). With the possible exception of platelets, individual bone marrow and peripheral blood parameters did not vary across MDS subtypes diagnosed according to WHO, and none were statistically significant (Table 3). The diagnosis of RCMD requires scoring a minimum of 10% dysplastic changes in two or more myeloid lineages; however, in the majority of these cases the degree of dysplasia was marked.Fig. 1. Age-distribution of MDS subtypes according to WHO in patients grouped by decades. The age-distribution of MDS subtypes is plotted as the percent of total MDS. (*) A higher prevalence of RCMD was observed in all decades relative to other subtypes, but was significant only in the <=29 year age group (P=0.0001).G-banded chromosome analysis was completed in 163 of 176 total MDS cases diagnosed according to WHO (92.6%) (Table 4). One case for which conventional cytogenetic analysis was not completed was demonstrated to have clonal abnormalities by FISH. Therefore, informative cytogenetic data was obtained from 164 of 176 patients (93.2%). Among those, 43 cases (26.2%) had clonal abnormalities. The most frequently observed chromosome abnormalities were trisomy 8 (20 cases, 12.2%), del(7q) (8 cases, 4.9%), del(20q) (7 cases, 4.3%), and del(5q) (4 cases, 2.4%) (Table 5). Translocations, each of which was unique, were observed in seven cases (i.e., 3 RCMD, 3 RAEB, and 1 MDS-u) with a median age of 63.3 years. A complex karyotype, defined as more than two independent chromosome aberrations, was detected in eight cases (4.90%). Using the definitions outlined by the International Prognostic Scoring System (IPSS) [15], cases with good, intermediate and poor cytogenetic risk features were observed in 76.6%, 12.9%, and 10.4% of patients, respectively. No cases of MDS with del(5q) as the sole abnormality were observed. The highest frequency of clonal cytogenetic abnormalities was found in cases with RAEB (40.7%), followed by MDS-u (37.5%), RCMD (24.6%), and RA (7.1%). Using case definitions for MDS as defined by FAB, 64 cases (31%) had clonal abnormalities.4 DiscussionCharacterization of clonal cytogenetic abnormalities has proven to be important in evaluating prognosis in MDS. However, there is only limited retrospective data on the MDS subtype distribution of cytogenetic abnormalities

diagnosed according to WHO [10,11]. The frequency of aberrations in de novo MDS (FAB) has been previously reported to be 35-40% [10,11,16,17]. In this prospective analysis, clonal abnormalities were detected in 26.2% of MDS cases diagnosed according to WHO as compared to 31% diagnosed using FAB criteria. This not withstanding, informative cytogenetic data was available in more than 93% of our patients, which is significantly higher than 40-70% reported in most MDS (FAB) studies [10,11,18-21]. Differences in the frequency of clonal aberrations between our series and previous reports in large part can be attributed to differences in the WHO and FAB MDS classification criteria. Cases of RAEBT (FAB), which are diagnosed as AML using WHO criteria, and therefore not classified as MDS, usually have a high frequency of chromosome abnormalities [10,11,16,17]. Further, cases diagnosed as MDS (FAB) with t [8,21] and 11q23/MLL rearrangements are diagnosed as AML according to WHO [22].Cytogenetic abnormalities most commonly described in MDS in the West include (in the order of frequency) del(5q), del(7q), +8, and del(20q) [23]. Several studies, including ours, indicate that trisomy 8 is the most common cytogenetic abnormality in Asian MDS. However, inconsistent results are reported for the prevalence of del(5q), del(7q), and del(20q) [10-12,17,24]. In a retrospective study of Chinese MDS patients diagnosed according to FAB, Chen and colleagues reported the frequencies of del(5q) and del(7q) as 4.6% and 1.6%, respectively [10]. Our findings suggest that Asian patients more frequently present with del(7q) than del(5q) abnormalities, and that the frequency of del(5q) abnormalities is lower than in the West. It is possible that this may be related to the younger median age of MDS cases in Asian versus Western populations. Similar results also have been reported for Japanese patients by Toyama et al. [24] and Matsushima et al. [17] for overall MDS and by Matsuda et al. for RA [25]. According to IPSS, karyotype at diagnosis is an independent prognostic factor to predict survival and leukemic progression in MDS [15]. The frequency of karyotypes in RCMD in our study that are associated with a poor prognosis is low (7.9%) compared to 20% reported by Matsuda in a Japanese population with similar median age [25]. The reason for these differences is not immediately apparent. Using the same criteria, our cases showed frequencies among overall WHO MDS patients that are comparable to other studies (good risk: 76.6%, intermediate risk: 12.9%, and poor risk: 10.4%) [11,15]. Follow-up studies are ongoing to determine if the cytogenetic features defined by IPSS also predict risk in overall MDS or subtypes classified according to WHO.Our results are consistent with studies that have reported a much younger age of onset for MDS, as well as a lower prevalence of RS and CMML in Asian populations relative to those found in the West [1,10-14]. The most striking observations in our patients were the increased prevalence of multilineage dysplasia together with the number of cases presenting at an early age. In contrast to other subtypes of MDS in which there is a tendency toward increased frequency with advancing age, cases of RCMD were often encountered in patients 19-29 years old, and the increased frequency of RCMD in this age group relative to total MDS or the other individual subtypes, such as RAEB or RARS is highly significant ($P<0.00001$). The increase in frequency of RCMD relative to RAEB probably accounts for the decreased numbers of RAEB in this series. However, the remarkably low frequency of RS remains unexplained. Cases of childhood MDS, independent of defined congenital hematologic disorders, have been recognized in the West [26]. However, neither the origins of the disease nor its clinical significance in such a young age group are understood. It is interesting that the prevalence of childhood AML in Shanghai is more than twice that reported in the West [27]. Whether there is a biological link between the development of childhood AML and the frequency of multilineage dysplasia in young adults in Shanghai remains unexplored. However, there are a number of factors that could possibly influence the development of MDS in general and multilineage dysplasia specifically. Nutritional factors are well known to play a role in the development of anemias. A total of 44 cases were excluded from our series due to confounding by deficiencies involving iron, folate, and Vitamin B12 either singly or in combination. These findings suggest that the frequency of anemia attributable to nutritional deficiency is relatively high in Shanghai and surrounding areas and is an important complicating factor in the evaluation of regional hematopoietic disease.

It previously has been suggested that regional differences in subtype-specific survival might contribute to artifacts in the prevalence of MDS subtypes and age-distribution. However, it is unlikely that these influences could explain the magnitude of disparity observed in this series, and the trend toward RCMD in a younger age group is more likely to be the result of differences in the etiology and/or pathogenesis of individual MDS subtypes. Chen et al. have suggested that differences in the age-dependence of MDS occurring in Asian populations may be explained by exposure to environmental factors including chemicals and infectious agents [10]. However, in our series we excluded 30 cases of MDS with known previous exposure to benzene or alkylating chemotherapy. The features of dysplasia developing in

individuals with previous evidence of benzene poisoning appears to have a discrete clinicopathologic signature that is useful in distinguishing benzene-induced persistent dysplasia from previously characterized forms of MDS [28]. Nevertheless, environmental or dietary exposure to chemicals remains an important question with respect to their potential influence on the prevalence and age-dependence of RCMD in our patients. Moreover, the role of infectious agents in the development of MDS is largely unexplored and may be of importance in light of the fact that a great deal of evidence has emerged to implicate a role for altered immune regulation in the pathogenesis of MDS [29-32]. Finally, the role of genetic polymorphisms that may impact on the susceptibility of developing MDS in this population is unknown. Any of these factors, either singly or in combination, might influence the pathogenesis of MDS. However, at present their role in explaining these results remains undetermined. Our results provide a basis for direct comparison of WHO and FAB criteria in the differential diagnosis of MDS and further identify a marked predisposition toward multilineage dysplasia at a young age in this Asian population. Follow-up of these cases will provide further insight into the role of dysplastic changes in the etiology and pathogenesis of MDS as well as provide a basis for the continuing evaluation of the clinical and biological significance of the WHO diagnostic paradigm.

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TABLES

Table 1

Comparison of WHO and FAB diagnostic criteria in MDS sub-types

MDS (WHO)	Total cases	MDS (FAB)	Total cases
RA	16 (9%)	RA	140 (64.2%)
RARS	1 (0.5%)	RARS	8 (3.6%)
RAEB	28 (16%)	RAEB	29 (13.3%)
RCMD	122 (69.5%)	RAEBT	28 (12.8%)
MDS-u	9 (5%)	CMML	13 (5.9%)
Total	176	Total	218

Re-evaluation of WHO MDS by FAB

Table 2

Comparison of WHO and FAB diagnostic criteria in cases of AML

AML (WHO)	*N*	Blasts (%)	MDS (FAB)
AML not otherwise categorized	10	20-27	RAEBT
AML with recurrent cytogenetic abnormalities	8	21-29	RAEBT
AML with multilineage dysplasia	10	20-25.5	RAEBT
Acute erythroid leukemia[a]	1	11	RAEB

[a]Blasts=22% of non-erythroid.

Table 3

MDS (WHO) Hematologic and immune parameters in patients diagnosed with MDS according to WHO criteria

Bone marrow (BM) cellularity			Bone marrow lymphocytes	Peripheral blood				
Hyper	Normo	Hypo	%Lymph	CD4:CD8	WBC $\times 10^9$L	HgB (g/dL)	PLT $\times 10$

								^{9}L
MDS total	88 (50%)	28 (16%)	57 (32%)	21.7 (1.0-82.6)	1.1 (0.1-5.7)	2.62 (0.52-10.6)	7.68 (2.71-15.5)	80 (1.9-468)
RA	10 (62%)	3 (18%)	3 (18%)	20.9 (3.1-54.9)	1.2 (0.1-4.1)	2.89 (1.75-7.82)	7.55 (4.17-12.2)	119 (5.7-393)
RARS	1 (100%)	0	0	2.8	0.8	4.50	7.58	335
RAEB	17 (60%)	3 (10%)	7 (25%)	26.6 (1.9-63.3)	1.0 (0.3-2.2)	2.73 (0.76-10.6)	6.82 (3.71-11.3)	60 (1.9-260)
RCMD	58 (47%)	22 (18%)	40 (32%)	20.9 (1.0-82.6)	1.2 (0.2-5.7)	2.40 (0.52-6.75)	7.84 (2.71-14.3)	78 (4.5-468)
MDS-u	2 (22%)	0	7 (77%)	21.0 (7.7-33.5)	1.1 (0.2-1.7)	2.14 (0.54-5.39)	8.42 (3.93-15.5)	98 (5.7-395)

Clinical observations in MDS subjects diagnosed by WHO criteria

Bone marrow cellularity was estimated from the trephine core biopsy (hypo, hypoplastic [<40% cellularity]; normo, normal [40-60% cellularity]; and hyper-hyperplastic [>60% cellularity]. Lymphocytes in BM aspirates: % lymph, % lymphocytes; CD4:CD8, ratio determined by flow cytometry in BM aspirate cells. Reference ranges in Shanghai: WBC (4-10×10^9L^{-1}); Hgb (12-16g/dL), PLT (100-300×10^9L^{-1}).

Table 4
Cytogenetic analysis of MDS subtypes

MDS subtypes	Total cases	No. complete cytogenetic analysis	Total percentage of abnormal cases
RA	16	14	1 (7.1%)
RARS	1	1	0
RAEB	28	27	11 (40.7%)
RCMD	122	114	28[a] (24.6%)
MDS-u	9	7	3[b] (37.5%)
Total	176	163 (92.6%)	43 (26.2%)

Analysis in MDS cases by standard cytogenetic criteria

[a]One case with extra Y.

[b]One case with informative FISH without cytogenetic data.

Table 5
Clonal cytogenetic abnormalities in MDS subtypes

Clonal abnormality (Abnl)	No. of abnormality	%Informative (164)	RA (14)	RAEB (27)	RCMD (114)	MDS-u (7)
del(5q)	4	2.4	0	2 (7.4%)	2 (1.7%)	0
del(7q)	8	4.9	0	3 (11.1%)	4 (3.5%)	1[a]
Trisomy 8	20	12.2	1	6 (22.2%)	13 (11.4%)	0
del(20q)	7	4.3	0	1 (3.7%)	6 (5.3%)	0
Monosomy 21	1	0.6	0	1 (3.7%)	0	0
del(9q)	2	1.2	0	1 (3.7%)	1 (0.9%)	0
Complex karyotype >=3 Abnl	8	4.9	0	3 (11.1%)	5 (4.4%)	0

Frequency of abnormal cytogenetics in MDS cases in Shanghai

[a]One case with informative FISH without cytogenetic data.

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[7]. -300×109L-1).Table 4Cytogenetic analysis of MDS subtypesMDS subtypesTotal casesNo. complete cytogenetic analysisTotal percentage of abnormal casesRA16141 (7.1%)RARS110RAEB282711 (40.7%)RCMD12211428a (24.6%)MDS-u973b (37.5%)Total176163 (92.6%)43 (26.2%)Analysis in MDS cases by standard cytogenetic criteriaaOne case with extra Y.bOne case with informative FISH without cytogenetic data.Table 5Clonal cytogenetic abnormalities in MDS subtypesClonal abnormality (Abnl)No. of abnormality%Informative (164)RA (14)RAEB (27)RCMD (114)MDS-u (7)del(5q)42.402 (7.4%)2 (1.7%)0del(7q)84.903 (11.1%)4 (3.5%)1aTrisomy 82012.216 (22.2%)13 (11.4%)0del(20q)74.301 (3.7%)6 (5.3%)0Monosomy 2110.601 (3.7%)00del(9q)21.201 (3.7%)1 (0.9%)0Complex karyotype >=3 Abnl84.903 (11.1%)5 (4.4%)0Frequency of abnormal cytogenetics in MDS cases in ShanghaiaOne case with informative FISH without cytogenetic data.CONTACT: * Corresponding author. Tel.: +1 303 315 7170.[1]. C. Aul, U. Germing, N. Gattermann, H. Minning; Increasing incidence of myelodysplastic syndromes: real or fictitious?; Leuk Res; Vol. 22, (1998), pp. 93-100.[2]. J.M. Bennett,

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ORIGINAL ARTICLE

A prospective study of 728 cases of non-Hodgkin lymphoma from a single laboratory in Shanghai, China

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Abstract The frequency of subtypes of lymphoid neoplasms was determined in a prospective series of 831 patients presenting at 29 Shanghai hospitals over a 4-year period. Diagnosis and classification was established in a single laboratory according to the 2001 WHO classification system. The frequency of non-Hodgkin lymphoma was 87.6% ($n = 728$) and Hodgkin lymphoma was 12.4% ($n = 103$). The most prevalent NHL subtypes diagnosed using WHO criteria were diffuse large B cell lymphoma (DLBCL), precursor B lymphoblastic leukemia/lymphoma and chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL). Although a low incidence has been reported in some Asian populations, CLL/SLL was commonly encountered, indicating that chronic lymphoid neoplasms are not rare in Shanghai. Consistent with previous reports, our findings indicate a decrease in the frequency of follicular lymphoma and an increase in T cell neoplasms compared to the West. Precursor T lymphoblastic leukemia/lymphoma, anaplastic large T cell lymphoma, aggressive NK cell leukemia, angioimmunoblastic T cell lymphoma and peripheral T cell lymphoma were prominent subtypes of T cell NHL.

Keywords Non-Hodgkin lymphoma · 2001 WHO diagnostic criteria · Prospective study

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1 Introduction

Non-Hodgkin lymphoma (NHL) refers to more than two dozen distinct neoplastic diseases of the lymphoid system that involve the malignant outgrowth of B and T lymphocytes. The individual characteristics of different lymphomas are thought to result from the neoplastic transformation of different lymphocytic populations at different stages in their maturation and transformation [1]. Over the last two decades, advances in knowledge of the biology of NHL have resulted in the development of an international consensus for the diagnosis and classification of NHL and its subtypes. The World Health Organization (WHO) Classification of Tumors of Hematopoietic and Lymphoid Tissues [2] was put forth in 2001 in order to provide uniform diagnostic criteria for NHL subtypes on

the basis of morphology, immunophenotype, cytogenetics, pathogenesis and clinical characteristics. The WHO classification is based on and extends the 1994 Revised European American Lymphoma (REAL) classification system [3]. The WHO classification recognizes three major categories of lymphoid neoplasms: B cell neoplasms, T and NK cell neoplasms and Hodgkin lymphoma. However, in contrast to REAL, lymphoid leukemias also are included in the WHO classification which stratifies lymphoid neoplasms on the basis of cellular origin and biology [2].

To date, the use of the WHO classification in studies of NHL in China have been limited by the lack of uniform access to the diagnostic tools (e.g., immunohistochemistry, cytogenetics, fluorescence in situ hybridization (FISH)) required to meet WHO criteria. Reports from previous epidemiologic studies using the REAL classification system have indicated significant differences in the prevalence of NHL subtypes between Asia and the West. For example, follicular lymphoma constitutes 22% of NHL in the US but is reported to represent only 5% of NHL in China [4]. The prevalence of follicular lymphoma and prolymphocytic NHL in Japan are also less than in the West while diffuse large B cell and lymphoblastic NHL are higher [5]. In Korea as well, extranodal diffuse large B cell and angioimmunoblastic T cell NHL are higher than in the West [6]. In contrast to the US, NHL of T cell origin is almost twice as prevalent in China, Japan and Korea [7]. Conflicting results have been reported for the prevalence of CLL/SLL in Asian and Western populations [8]. CLL is the most common chronic lymphoproliferative disorder in the West accounting for approximately 25–38% of all leukemias. Alternatively, previous studies have reported the incidence of CLL in Asian populations (e.g., Chinese, Japanese and Singapore Chinese) to be 12–20 times lower, occurring in only 1.2–2% of Eastern patients. However, Kwong and colleagues [9] found that CLL represents 12.5% of all leukemias diagnosed in Hong Kong over a 3-year period. The etiology of NHL is complex. Genetics, immunologic or other neoplastic diseases, as well as infectious agents, including viruses such as Epstein–Barr virus (EBV), Hepatitis B and C virus, and Human Immunodeficiency virus (HIV), appear to influence differences in the prevalence of NHL worldwide [10–13].

The object of this study was to diagnose and classify all lymphoid neoplasms that presented over a 4-year period in a single diagnostic laboratory in Shanghai, China. A total of 831 consecutive cases of lymphoid neoplasms met criteria for diagnosis and classification according to WHO. Herein, we present and compare distribution of NHL subtypes obtained in our prospective series with a recent report of retrospective classification of lymphoid tumors in China using WHO criteria [14]. We also include patient demographics, laboratory values, viral serology and cytogenetic data for the major NHL subtypes diagnosed in our laboratory.

2 Materials and methods

2.1 Case series

Patients $\geq$18 years presenting at Shanghai hospitals between August 2003 and May 2007 were referred to our laboratory based on initial clinical presentation. Protocol approval for this case review was obtained by the Combined Multiple Institutional Review Board of the University of Colorado at Denver and Health Sciences Center in Denver, CO and the Internal Review Board at Fudan University in Shanghai, China.

2.2 Sample collection and clinical laboratory analysis

Peripheral blood, bone marrow aspirates, tissue and core biopsies were collected in conjunction with diagnostic procedures and were evaluated in our laboratory. Peripheral blood smears were obtained by finger stick. Blood samples were collected by venipuncture and processed for routine CBC (CellDyne 3700, Abbott, Park, IL), viral serology (HCV and HIV) (Imx, Abbott) and clinical chemistry for liver enzymes (LDH, ALT and AST enzymes) (COBAS, Integra 400 plus, Roche Diagnostics, Shanghai, China).

2.3 Morphology

Bone marrow aspirates and core biopsies were obtained by Jamshidi needle extraction from the posterior iliac crest, while lymph node and tissue biopsies were obtained by surgical resection. Bone marrow smears were prepared and evaluated using Wright–Giemsa stained preparations and special stains. A broad panel of antibodies were used for immunophenotyping of bone marrow and lymphoid cells by either dual laser flow cytometry (FC-500, Beckman Coulter, Hialeah, FL; Immunotech, Miami, FL), immunoperoxidase-immunohistochemistry of tissue sections or both. Biopsy sections were stained with Hematoxylin–Eosin (H&E). Morphology was independently evaluated by three of us (X.-Z. Z., R. D. I., J. R.). Microscopic analysis was performed using Olympus BX51 bright field microscopes (Olympus Optical Ltd., Tokyo, Japan).

2.4 Cytogenetic analysis

Conventional cytogenetic analysis was performed on unstimulated (1–2 day), and B cell mitogen stimulated (3-day) (lipopolysaccharide) (Sigma, St. Louis, MO), cultures

of bone marrow or lymph node preparations. A minimum of 20 metaphases were analyzed in each case. Criteria for a clone and descriptions of karyotype followed the recommendations of the International System for Human Cytogenetic Nomenclature (1995) [15].

2.5 Fluorescence in situ hybridization analysis (FISH)

FISH analysis was performed on bone marrow or lymph node preparations using Vysis probes (Downers Grove, Illinois) to identify *IGH*/14q34, *ALK*/2p23 *CCND1/IGH/* t(11;14), BCL2/IGH/t(14;18) and other abnormalities as required. Sample preparations and hybridizations were conducted following the manufacturer's recommendations. Whenever possible, a minimum of 500 interphase cells were scored for each probe. A clonal aberration was defined as the percentage of cells with any given aberration over the normal cut-off limits that were determined from ten cytogenetically normal individuals.

3 Results

3.1 Total number lymphoid neoplasms diagnosed using WHO classification criteria

A total of 831 consecutive lymphoid neoplasms were diagnosed according to the WHO classification over a 4-year period (Table 1). The percent of B cell neoplasms as compared to T/NK cell neoplasms was 68.4 and 18.2%, respectively. A total of 103 cases of Hodgkin lymphoma were diagnosed, which constituted 12.4% of total lymphoid neoplasms. Using WHO criteria, a total of 728 NHL were diagnosed, including precursor B and T cell leukemias as well as lymphomas (Table 2). B cell NHL composed 78.0% ($n = 568$) of all NHL cases whereas T/NK cell NHL composed 20.7% ($n = 151$) of the total NHL cases diagnosed in our series.

Table 1 Total number of lymphoid neoplasms diagnosed in series by WHO criteria in Shanghai, China over a 4-year period

Total lymphoid neoplasms	$n = 831$	Percent of total (%)
Mature B cell neoplasms	428	51.5
Precursor B lymphoblastic lymphoma	3	< 1
Precursor B lymphoblastic leukemia	137	16.5
Total B cell NHL	568	68.4
Mature T/NK cell neoplasms	105	12.6
Precursor T lymphoblastic lymphoma	21	2.5
Precursor T lymphoblastic leukemia	25	3.0
Total T/NK cell NHL	151	18.2
NHL NOS	9	1.1
Hodgkin lymphoma	103	12.4

3.2 NHL subtypes defined by WHO criteria

B cell NHL was at least 3 times more prevalent than T cell NHL (568 and 151, respectively) (Table 2). The most frequent subtype of B cell NHL diagnosed in our laboratory was diffuse large B cell lymphoma ($n = 212$) followed by precursor B lymphoblastic leukemia/lymphoma ($n = 140$) and then CLL/SLL ($n = 71$), representing 29.1, 19.2 and 9.8% of all NHL diagnosed in our laboratory, respectively. The most commonly diagnosed type of T/NK cell NHL was precursor T lymphoblastic leukemia/lymphoma ($n = 46$) followed by peripheral T cell lymphoma, unspecified ($n = 40$) and angioimmunoblastic T cell lymphoma ($n = 26$). These findings differ somewhat from those of Wang et al. (2005) [14] who reclassified 447 archival specimens of malignant lymphoma presenting at the Shanxi Tumor Hospital in Taiyuan, China, over an 8-year period using the WHO classification. They found diffuse large B cell lymphoma to be the most common NHL subtype (35.1%) followed by peripheral T cell lymphoma, unspecified (12.0%), extranodal marginal zone B cell lymphoma (11.7%) and follicular lymphoma (8.6%). Cases of T/NK cell neoplasms comprised 30.6% of NHL reported for this re-classification.

3.3 Gender and age distribution of NHL subtypes as defined by WHO criteria

The gender and age distribution for NHL cases presenting in our laboratory is displayed in Fig. 1. The overall gender distribution for NHL in our laboratory was approximately 2:1 male to female (443 and 285, respectively). Gender distribution of B cell NHL favored male (60%) over female (40%) and was similar for T/NK cell NHL, male (64%) female (36%). There was a marked male predisposition for CLL/SLL (3:1) and mantle cell lymphoma (3.7:1). Females with precursor B lymphoblastic leukemia slightly outnumbered males (1.06:1). However, when normalized for gender, the prevalence of precursor B lymphoblastic leukemia in females was twice that observed in males. Males with peripheral T cell lymphoma, unspecified (PTCL-u) outnumbered females (1.8:1). However, when normalized for gender the prevalence of PTCL-u was equal in males and females. The median age for all NHL and B cell NHL was 56 and 58 years, respectively. The median age for T/NK cell neoplasms was slightly younger (49 years). Overall there were no obvious differences between males and females for age at presentation with the exception of precursor B lymphoblastic leukemia for which the median

Table 2 Comparison of the distribution of NHL subtypes in China classified according to WHO criteria

	Distribution in Shanghai[a] (n = 728) % (n)	Wang et al.[b] (n = 385) % (n)
B-cell NHL	78.0 (568)	68.3 (263)
T/NK cell NHL	20.7 (151)	30.6 (119)
NHL NOS	1.2 (9)	
Diffuse large B cell lymphoma	29.1 (212)	35.1 (135)
Precursor B lymphoblastic leukemia[a](137)/ lymphoma[a](3)	19.2 (140)	1.0 (4)
Chronic lymphocytic leukemia[a](29)/ small lymphocytic lymphoma[a](42)	9.8 (71)	3.6 (14)
Follicular lymphoma	7.0 (51)	8.6 (33)
Precursor T lymphoblastic leukemia[a](25)/ lymphoma[a](21)	6.3 (46)	7.0 (27)
Peripheral T cell lymphoma, unspecified	5.5 (40)	12.0 (46)
Angioimmunoblastic T cell lymphoma	3.6 (26)	2.3 (9)
Plasma cell myeloma	2.9 (21)	1.3 (5)
Extranodal marginal zone B cell lymphoma (MALT)	2.5 (18)	11.7 (45)
Anaplastic large cell lymphoma	2.1 (15)	4.2 (16)
Mantle cell lymphoma	1.9 (14)	2.6 (10)
Burkitt lymphoma[a](10)/leukemia[a](2)	1.6 (12)	0.3 (1)
Aggressive NK cell leukemia	1.2 (9)	(0)
Nodal marginal zone B cell lymphoma	1.1 (8)	0.5 (2)
Lymphoplasmacytic lymphoma	0.8 (6)	2.3 (9)
Splenic marginal zone lymphoma	0.7 (5)	0.3 (1)
Extranodal NK/T cell lymphoma	0.6 (4)	1.3 (5)
T cell prolymphocytic leukemia	0.6 (4)	0.5 (2)
B-cell prolymphocytic leukemia	0.6 (4)	0.5 (2)
T cell large granular lymphocytic leukemia	0.6 (4)	(0)
Other NHL subtypes represented at <1%	<2.5 (18)	<1.0 (4)

[a] Diagnosed and classified in series at a single diagnostic laboratory in Shanghai China over a 4-year period (n = 728)

[b] Adaptation from Jinfen Wang et al. (2005) [14] (n = 385)

age at presentation for males and females was 38 and 47 years, respectively.

3.4 Distribution of NHL cases from Shanghai and nearby provinces

The vast majority of NHL cases presenting were from Shanghai (78.5%) (Table 3), including cases that constitute the most prevalent NHL subtypes diagnosed in our laboratory. We recorded 18 cases of CLL/SLL and DLBCL in patients from neighboring Zhejiang (4 and 14, respectively). Moreover, 47 of our NHL cases came from Jiangsu province (including 5 CLL/SLL and 12 DLBCL). Other than Shanghai, there were no distinct clusters of NHL subtypes associated with a specific province.

3.5 Clinical features associated with NHL subtypes

Clinical findings for the most prevalent B cell and T/NK cell NHL subtypes are illustrated in Table 4. Abnormal mean hematologic values indicated peripheral blood involvement for 8 out of the 13 major NHL subtypes presented. Predictably, NHL cases presenting as leukemia, precursor B lymphoblastic leukemia and precursor T lymphoblastic leukemia, showed hematologic abnormalities in multiple lineages. The LDH index was elevated in 11 NHL subtypes with the highest levels observed for precursor B lymphoblastic leukemia. Histological grading of follicular lymphoma cases was performed based on the absolute number of centroblasts present in neoplastic follicules. These findings indicate that 43% of the follicular lymphoma cases in our laboratory were classified grade 3. Viral serology for hepatitis C virus (HCV) was performed in 509 NHL cases (data not shown). Only five cases were positive for HCV serology: three cases of precursor B lymphoblastic leukemia, one case of angioimmunoblastic T cell leukemia and one case of anaplastic large cell lymphoma. Serology for HIV was measured in cases of NHL, but because individuals testing positive for HIV in China are routinely quarantined, no HIV+ cases of NHL were encountered in this series.

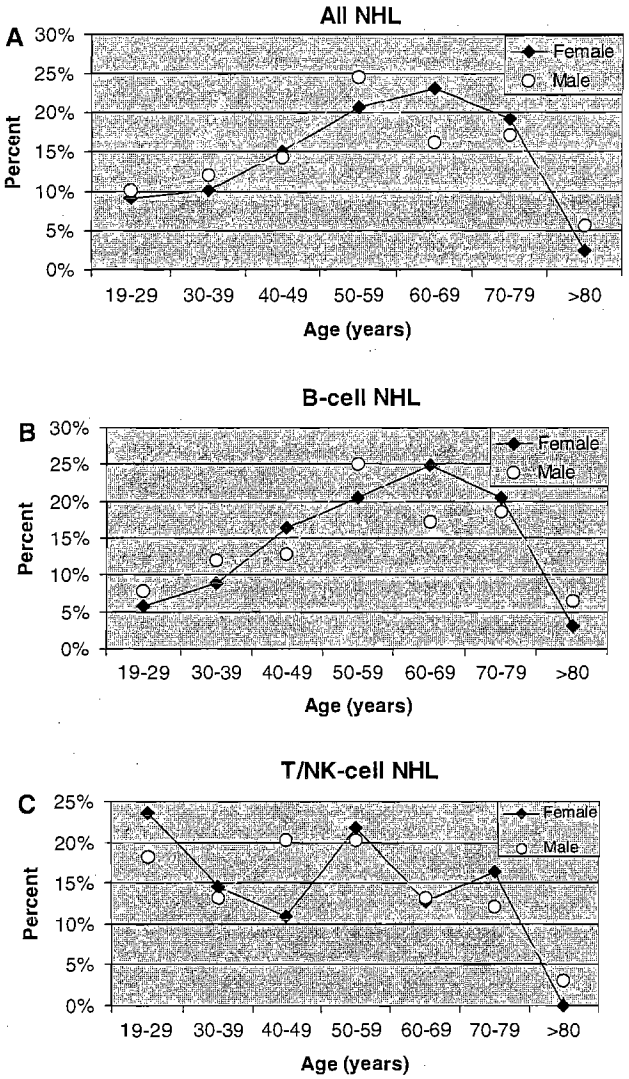

Fig. 1 Age and gender distribution for both males and females diagnosed with **a** All NHL, **b** B cell NHL and **c** T cell NHL in Shanghai, China

3.6 Clonal abnormalities associated with NHL subtypes

Cytogenetic and/or FISH analysis was performed in 724 out of 728 cases of NHL. Informative analysis was obtained in 603 cases and the overall rate for clonal abnormalities in all NHL cases was 81%. Cytogenetic abnormalities for the most prevalent B cell and T/NK cell NHL subtypes are presented in Table 5. The most common cytogenetic abnormality observed in precursor B lymphoblastic leukemia was t(9;22) (37%). Predictably, all follicular lymphomas had clonal cytogenetic abnormalities with complex abnormalities observed in 72% of cases. However, t(14;18) was observed in only 37% of follicular lymphoma cases. Trisomy 12 (+12) was observed in 25% (13/52) of CLL/SLL cases where clonal abnormalities were observed. Overall, complex karyotypes predominated in all NHL subtypes. Whenever possible, genetic aberrations observed in chromosomal analysis were further confirmed by FISH analysis with appropriate probes (Fig. 2).

Table 3 Distribution of NHL cases from Shanghai and nearby provinces

Province	Percent
Shanghai	78.5
Zhejiang	7.5
Jiangsu	6.4
Anhuei	3.3
Jiangxi	1.6
Sichuan	0.7
Other[a]	<2.0

[a] Additional provinces each represented at less than 0.5

4 Discussion

We present 831 consecutive cases of newly diagnosed lymphoid neoplasms evaluated in a single laboratory in Shanghai, China. Patients with suspected lymphoid neoplasms were referred from 29 participating hospitals in the Shanghai area. Prospective diagnosis and disease classification were based on the WHO system (2001) [2]. Consistent with previous reports, our findings indicate that 87.6% of the lymphoid neoplasms are NHL and 12.4% are Hodgkin lymphoma [14]. Of the NHL cases diagnosed in our series, the frequency of B cell NHL is 78.0% and T/NK cell NHL is 20.7%, confirming that although B cell neoplasms predominate, NHL of T/NK cell origin are more frequently encountered in China than in the West [14, 16, 17]. Predictably, there is a general 2:1 predisposition of males over females for all NHL. Notable exceptions were CLL/SLL (male:female = 3:1), mantle cell lymphoma (male:female = 3.7:1) and precursor B lymphoblastic leukemia (male:female = 0.94:1).

Historically, the largest previous Western study of NHL employed the REAL system which classified solid tumors of immature B and T cell origin as lymphoblastic lymphoma. Precursor B and T cell leukemias were evaluated separately as acute lymphoblastic leukemia (ALL). Armitage and colleagues [18] compiled 1,403 cases of NHL diagnosed by REAL from 9 different countries and reported diffuse large B cell lymphoma (31%) was the most common form of B cell neoplasm followed by follicular lymphoma (22%). CLL/SLL, peripheral T cell lymphoma-unspecified, mantle cell lymphoma and extranodal marginal zone B cell lymphoma were all represented relatively equally in the population (5–6%). Lymphoid neoplasms of T cell origin represented only 9–10% of NHL. Similar to

Table 4 Mean laboratory values for the most prevalent NHL subtypes diagnosed in series according to WHO in Shanghai, China in a 4-year period

	ANC (10^9/L)	ALC (10^9/L)	PLT (10^9/L)	Hgb (g/dL)	LDH Index
Diffuse large B cell lymphoma[a](125/212)	3.7	1.4	200.2	12.1	4.7[b]
Precursor B lymphoblastic leukemia[a](137/137)	2.2	18.6[b]	71.0[b]	9.0[b]	4.2[b]
Chronic lymphocytic leukemia/small lymphocytic lymphoma[a](54/71)	2.9	12.7[b]	171.1	11.9[b]	1.1
Follicular lymphoma[a](26/51)	3.7	1.7	207.5	13.3	1.3[b]
Extranodal marginal zone B cell lymphoma (MALT)[a](13/18)	1.9	6.2	135.0	7.7[b]	0.9
Plasma cell myeloma[a](21/21)	2.8	2.7	132.6	6.7[b]	1.2[b]
Mantle cell lymphoma[a](7/14)	3.3	3.9	142.5	11.7[b]	1.3[b]
Peripheral T cell lymphoma, unspecified[a](23/40)	3.1	1.9	176.5	11.2[b]	1.3[b]
Angioimmunoblastic T cell lymphoma[a](16/26)	4.4	1.3	200.0	12.0	1.4[b]
Precursor T lymphoblastic leukemia[a](25/25)	2.2	27.2[b]	124.0	10.2[b]	3.6[b]
Anaplastic large cell lymphoma [a] (8/15)	5.4	6.0	199.0	11.9[b]	2.1[b]
Precursor T lymphoblastic lymphoma [a](5/21)	3.6	2.3	247.4	12.3	1.2[b]
Burkitt lymphoma [a](6/10)	3.8	5.9	240.0	12.0	2.8[b]

Abbreviations and references ranges: absolute neutrophil count (ANC) 2–7 × 10^9 L^{-1}, absolute lymphocyte count (ALC) 1.6–6 × 10^9 L^{-1}, platelet count (PLT) 100–300 × 10^9 L^{-1}, lactate dehydrogenase (LDH) Index = n IU L^{-1}(255 IU L^{-1})$^{-1}$, hemoglobin (Hgb) 12–16 g dL^{-1}

[a] Cases with laboratory values versus total number of cases ()

[b] Indicates abnormal laboratory value

Armitage, diffuse large B cell lymphoma (45%) was the most prevalent NHL subtype diagnosed in our laboratory in Shanghai. However, significant differences were observed between our series and Armitage for CLL/SLL, follicular lymphoma and T cell neoplasms. CLL/SLL was almost 3 times more prevalent in our series (15%), whereas follicular lymphoma represented only 11% in our series compared to 22% in Armitage. Malignant lymphoma of T cell origin was also more prevalent in our series (24%) compared to Armitage (9–10%).

A total of 81% of CLL/SLL presented with structural cytogenetic abnormalities in our series. The most frequently encountered abnormality was trisomy 12 (25%) which is similar to that reported in Western populations (~20%) [2]. Conflicting reports concerning the prevalence of CLL/SLL in Asia have appeared over the last decade with the prevalence ranging from 3.6% (Wang et al. 2005) to 12.5% (Kwong et al. 1994) [9, 14, 16]. Our findings indicate a relatively high prevalence of CLL/SLL for NHL in Shanghai (9.8 and 15% for WHO and REAL, respectively). A possible explanation for the disparity between these results and some other studies may involve variations in the sampling and/or diagnostic methodologies employed. Historically in China, lymphoid neoplasms presenting as leukemias are more likely to be reported by hematologists, whereas solid lymphoid tumors are more likely to be encountered by pathologists. Therefore, even utilizing WHO 2001 criteria, retrospective studies may have a selection bias based on the source of material sampled for evaluation.

The potential impact of sampling bias is further illustrated by the marked difference in prevalence between precursor B lymphoblastic leukemia in our series (19.2%) compared to Wang et al. (1.0%) [14]. Even though both employed WHO diagnostic criteria, the vast majority of precursor B lymphoblastic leukemia in our series initially presented as hematology cases and not as solid tissue tumors. The majority of studies of precursor B lymphoblastic leukemia focus on children with immature B cell leukemia being the most common form of leukemia diagnosed in the West under the age of 6 years and in youths [19]. However, in the West adult precursor B lymphoblastic leukemia is rarely diagnosed in the general adult population (0.7–1.8/100,000 per year) [20]. Precursor B lymphoblastic leukemia/lymphoma was the second most frequently diagnosed lymphoid neoplasm in our series in Shanghai (n = 140). Taken together, these findings suggest that the adult form of the disease may be under-reported in China. Not only did females predominate in precursor B lymphoblastic leukemia, there was a marked gender difference in the median age at presentation, suggesting possible differences in the etiology or pathogenesis of precursor B lymphoblastic leukemia.

Reflecting the lower prevalence of follicular lymphoma in Asian populations relative to the West, this disease represented only 7% of the NHL in our series based on WHO classification [4, 14, 17, 21–23]. Although, complex cytogenetic abnormalities were observed in virtually all cases analyzed in this series, an explanation for the low frequency of t(14;18) is not immediately apparent.

Table 5 Clonal abnormalities in NHL subtypes diagnosed in series according to WHO in Shanghai, China in a 4-year period

NHL subtype	Patients with informative analysis (%)	Structural abnormalities (%)	Most common abnormality
Diffuse large B cell lymphoma	180/212 (85%)	174/180 (97%)	Abn. 13 (7%)
			t(3;14) (10%)
			6q- (12%)
			t(14;18) (3%)
			IGH rearrangements (26%)
Precursor B lymphoblastic leukemia	114/137 (83%)	87/114 (76%)	t(9;22) (37%)
			+8 (13%)
			−7 (10%)
Chronic lymphocytic leukemia/small lymphocytic lymphoma	64/71 (90%)	52/64 (81%)	+12 (25%)
			IGH rearrangements (21%)
			11q- (11%)
Follicular lymphoma	43/51 (84%)	43/43 (100%)	t(14;18) (37%)
			+7 (6%)
			t(13;14) (8%)
			IGH rearrangements (14%)
			Complex (72%)
Extranodal marginal zone B cell lymphoma	11/18 (61%)	4/11 (36%)	Complex (75%)
Plasma cell myeloma	16/21 (76%)	11/16 (69%)	Complex (82%)
Mantle cell lymphoma	12/14 (86%)	12/12 (100%)	t(11;14) (42%)
Peripheral T cell lymphoma, unspecified	28/40 (70%)	16/28 (57%)	+X (19%)
			del (6) (19%)
			Complex (69%)
Angioimmunoblastic T cell lymphoma	19/26 (73%)	7/19 (37%)	Abn. X or Y (57%)
Precursor T lymphoblastic leukemia	21/25 (84%)	17/21(81%)	Complex (82%)
Anaplastic large cell lymphoma	11/15 (73%)	7/11(64%)	Complex (86%)
Precursor T lymphoblastic lymphoma	16/21 (76%)	11/16 (69%)	Abn. (1)(p36) (27%)
			abn 5 (27%)
			Complex (73%)
Burkitt lymphoma	9/10[a] (90%)	4/9 (44%)	t(8;14) (40%)
			Complex (43%)

Definitions: Abn. Refers to an abnormality in a particular chromosome. Complex refers to a karyotype with greater than two independent cytogenetic lesions. Informative cytogenetic and/or FISH analysis refers to cases with a positive FISH analysis and either a normal karyotype or abnormal karyotype in greater than or equal to 20 metaphases (see Sect. "Materials and methods")

[a] All cases of Burkitt Lymphoma were positive for c-myc by cytogenetics, FISH or immunohistochemistry

Typical of Asia, the frequency of T cell neoplasms in Shanghai is approximately 3 times higher than encountered in Western populations. Surprisingly, rare neoplasms (i.e., anaplastic large T/null cell lymphoma, angioimmunoblastic T cell lymphoma and aggressive NK cell leukemia) together constituted the majority of T cell lymphomas, while peripheral T cell lymphoma was not encountered more often than reported in Western studies [17, 18]. The majority of these tumors exhibited complex karyotypes. EBER positive cells were detected in 63% of angioimmunoblastic T cell lymphoma and in all cases of aggressive NK cell leukemia (a diagnostic criteria for this disease) [24].

NHLs are a heterogeneous group of neoplasms with complex clinical, biological and molecular features. It is becoming increasingly appreciated that significant regional differences in the pattern of NHL subtypes exist that can be attributed to multiple factors, such as infection, family history, genetic predisposition, autoimmune disease, environment, immunosuppression as well as methodologic limitations or artifact. Our findings provide the first prospective basis for comparison of the prevalence of NHL subtypes in Shanghai based on WHO 2001 criteria and serve as a point of departure for future studies to better understand the contribution of multiple influences on the development of these diseases in China.

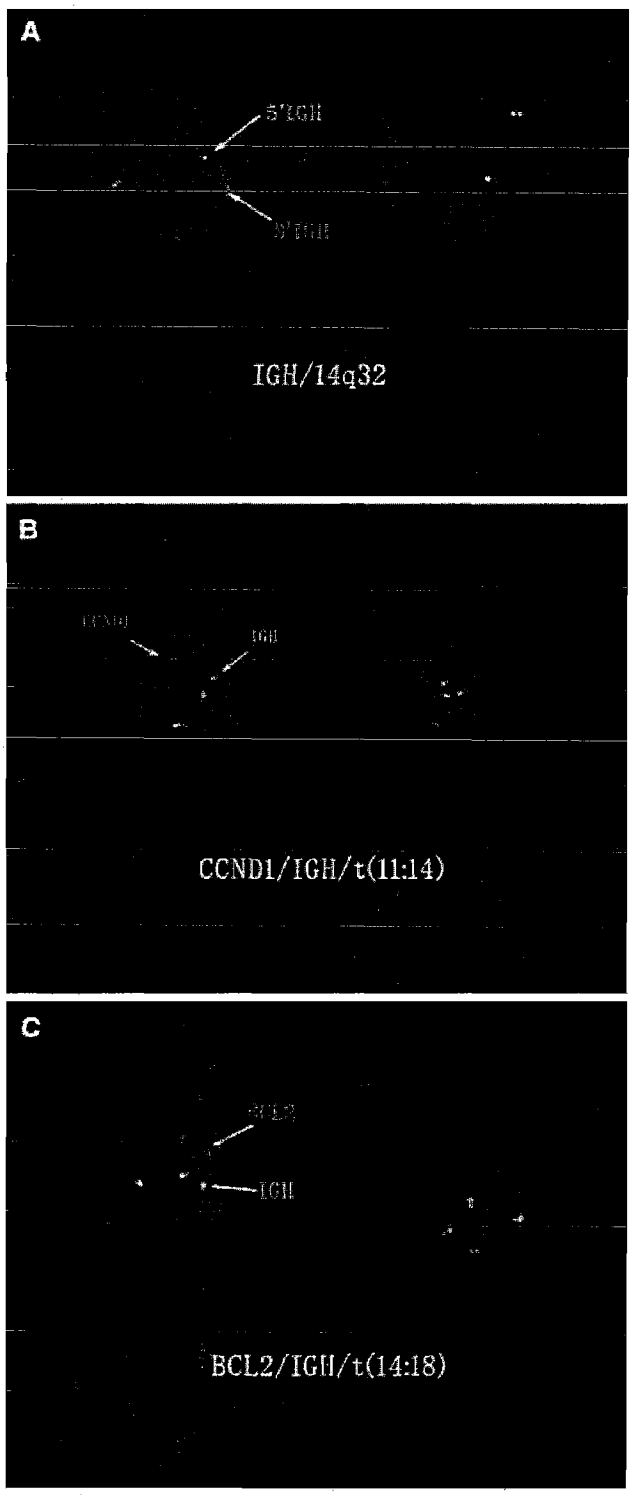

Fig. 2 FISH analysis of IGH fusion genes. **a** FISH using I*GH* BreakApart probe. Fusion signal represents normal; *green* and *red* represent rearranged 5′ and 3′, respectively, of *IGH* locus. **b** FISH with the Dual-fusion and Dual-color *CCND1/IGH* probe. Fusion signals represent fusion genes. **c** FISH with the Dual-fusion and Dual-color *BCL2/IGH* probe. Fusion signals represent fusion genes

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Chronic exposure to benzene results in a unique form of dysplasia

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Abstract

Hematotoxicity following chronic benzene exposure has been recognized for over a century, although the mechanism remains unknown. We describe a novel form of bone marrow dysplasia in 23 workers exposed to high concentrations of benzene. Distinguishing features of benzene-induced dysplasia include: marked dyserythropoiesis, eosinophilic dysplasia and abnormal cytoplasmic granulation of neutrophilic precursors. Hematophagocytosis, stromal degeneration and bone marrow hypoplasia are also seen. Severe bone marrow dysplasia is frequently accompanied by clonal T cell expansion and alterations in T lymphocyte subsets. No clonal cytogenetic abnormalities were observed. These results suggest that autoimmune-mediated bone marrow injury is an early or predisposing event in the pathogenesis of benzene-induced persistent hematopoietic disease.

Keywords: Myelodysplastic syndrome; Hematotoxicity; Benzene; Immunopathology

1. Introduction

Myelodysplastic syndrome (MDS) is a heterogeneous group of diseases characterized by ineffective hematopoiesis, dysplasia in one or more hematopoietic lineages, clonal evolution and a tendency to progress on to bone marrow (BM) failure or acute myelogenous leukemia (AML). The etiology and pathogenesis of MDS are unknown, with genetic, infectious and environmental influences variously suggested to play a role in conferring susceptibility to these diseases. Clonal cytogenetic abnormalities, abnormal cytokine production and immune activation all are prominent features of MDS, and it has been hypothesized that damage to hematopoietic stem or progenitor cells leads to immunologic response directed against antigens in the hematopoietic environment [1–4]. Nevertheless, neither the initial clonal origin of MDS nor the cellular or molecular targets of activated immune cells, or even the order of events in the evolution of the disease have been identified, and a unifying hypothesis for the pathogenesis of MDS in general or for individual subtypes remains elusive.

Chronic exposure to benzene is known to result in bone marrow failure and increase the risk of AML [5–7]. Previous

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descriptions of AML associated with occupational exposure to pesticides or solvents that have contained benzene suggest a pattern of disease similar to therapy-related AML (tAML), although direct evidence linking benzene to tAML is lacking [8–11], and the mechanism of benzene-induced hematotoxicity remains unknown. Chronic exposure to high concentrations of benzene has long been associated with aplastic anemia (AA), diagnosed nearly always on the basis of pancytopenia, with hundreds of cases reported during the first half of the 20th century. However, direct evaluation of BM was almost never performed in these cases [12–14]. Although, AA and MDS are thought to be related conditions, MDS is distinguished from other forms of progressive BM failure by the presence of dysplasia and a tendency to progress or evolve into AML. MDS (non-specific) has previously been reported in a very small number of benzene workers who were generally described as having hypocellular bone marrow and dyserythropoietic changes [15]. In contrast, the majority of cases of de novo MDS present with hypercellular BM and abnormal hematopoietic cell morphology, while a hypocellular BM is observed only in a subset of de novo cases. In 2001, the World Health Organization (WHO) published new criteria for the diagnosis of MDS subtypes [16]. Dysplasia in one or more lineages is a central diagnostic feature of MDS, although cytopenia, anemia, or pancytopenia, together with BM hypercellularity and a blast cell count <20% are prominent characteristics of most subtypes of the disease.

We describe a novel pattern of dysplasia developing in 23 individuals who were previously exposed to high concentrations of benzene. The evaluation of these patients employing current diagnostic and molecular techniques has given us the opportunity to revisit and to further characterize the clinical paradigms associated with benzene-induced hematotoxicity and to examine early events in the pathogenesis of persistent benzene-induced blood disease. Most cases of benzene-induced dysplasia exhibited BM pathology that was characterized by: hypocellularity, multilineage dysplasia including megaloblastic changes, marked dyserythropoiesis and abnormal granulation in the cytoplasm of maturing neutrophilic precursors and neutrophils. A particular striking feature was a severe dysplasia in eosinophilic precursor cells. Additional findings were consistent with altered inflammatory and immune response, including prominent hematophagocytosis, increases in circulating large granular lymphocytes (LGLs) and altered distribution of CD4 and CD8 T cells which were often accompanied by evidence of clonal expansion of T cell subpopulations in BM. Significantly, the peripheral blood CBC was nearly normal in approximately 25% of cases. These findings suggest a distinct form of multilineage dysplasia in individuals chronically exposed to benzene, and provide evidence for a prominent role for altered immune response in the development of benzene-induced dysplasia. These findings further suggest that routine CBC may not be a reliable monitor for BM injury following chronic exposure to benzene.

2. Materials and methods

2.1. Patients

Patients were referred by physicians to Shanghai hospitals based on initial clinical presentation and/or a medical history of benzene intoxication. Participation was voluntary and the framework for consent was obtained according to the Declaration of Helsinki, 2004 and the NIH Common Rule (45 CFR 46). Informed consents were approved by both the Colorado Multiple Institutional Review Board and the Internal Review Board at Fudan University in Shanghai, China. All individuals were administered questionnaires requesting information on medical, occupational and environmental history. A total of 27 subjects employed in the rubber, petrochemical, pharmaceutical, manufacturing or painting industries were referred to our laboratory with a history of benzene poisoning. Previous occupational exposure to benzene was independently confirmed by: review of factory industrial hygiene monitoring records, real time quantitative industrial hygiene analysis, including personal samples ($n = 325$) and breathing zone analyses ($n = 225$), and/or previous evidence of hydrocarbon intoxication and anecdotal descriptions of solvent use and composition. Benzene from personal samples (3M 3500 organic vapor monitors) and area samples were measured according to the National Institute for Occupational Safety and Health (NIOSH) method with minor changes [17] and were performed using a Finnegan Trace Gas Chromatograph Ultra with a Flame Ionization Detector (FID), autosampler and SPB-1 capillary column (30 m × 0.25 mm × 0.25 m) (Agilent Technologies, Wilmington, DE). Individuals for which quantitative data was available ($n = 17$) were estimated to have full shift exposures averaging between 50 and 300 parts per million (ppm) benzene for varying periods of time ranging from 6 to 22 years and were removed from exposure an average of 2.7 years prior to evaluation in our laboratory (Table 1). Patients were evaluated for potential confounding factors, including: vitamin B12, folate or iron deficiencies and viral status (i.e. HCV, HIV). Two patients were excluded due to concurrent iron deficiency, and two patients were excluded because of inability to confirm exposure to benzene. The final study series consisted of 16 females and 7 males with a mean age of 44.4 year (S.D. = 7.8). This represents a small subset of individuals chronically exposed to high concentrations of benzene at the facilities studied.

2.2. Hematology and flow cytometry

Peripheral blood (PB), BM aspirates and core biopsies were collected in conjunction with diagnostic procedures and were evaluated in our laboratory. Peripheral blood smears were obtained by finger stick. Enumeration of LGL was performed by visual examination of Wright-Giemsa-stained smears. Blood samples were collected by venipuncture and processed for routine CBC (CellDyne 3700, Abbott, Park, IL), viral screen (HCV and HIV) (IMx®, Abbott

Table 1
Initial presentation of occupational benzene poisoning[a]

Case	Exposure duration (year)[b]	Time since last exposure (month)[c]	Signs/symptoms[d]	Laboratory findings[d]
1	17	48	Bleeding, fatigue	Pancytopenia
2	6	24	Bleeding, fatigue, dizziness	Thrombocytopenia/leukopenia
3	19	24	Bleeding, fatigue	Pancytopenia
4	13	48	ND	Granulocytopenia
5	ND	96	ND	Leukopenia
6	20	0	Bleeding, fatigue, dizziness	ND
7	8	3	Bleeding	Thrombocytopenia
8	22	36	Fatigue, dizziness	Pancytopenia
9	7	36	Bleeding, fatigue	Granulocytopenia/anemia
10	17	24	Bleeding, fatigue	Leukcopenia
11	ND	24	ND	ND
12	8	48	Bleeding, dizziness	Anemia
13	7	48	Bleeding, fatigue	Pancytopenia
14	16	27	ND	Thrombocytopenia
15	6	56	Bleeding, infection, fatigue	Pancytopenia
16	15	36	Fatigue, dizziness	Pancytopenia
17	10	30	Bleeding, fatigue, dizziness	Leukopenia/thrombocytopenia
18	9	36	Bleeding, fatigue, dizziness	Pancytopenia
19	15	24	ND	ND
20	13	13	Bleeding, dizziness	Leukopenia/thrombocytopenia
21	14	3	ND	Leukopenia/thrombocytopenia
22	18	9	Infection	Leukocytosis
23	16	48	ND	Leukopenia/thrombocytopenia

[a] All subjects were originally diagnosed with benzene poisoning according to Chinese occupational health criteria (i.e. a total WBC count <4000 ($\times 10^6$/l) or 4000–4500 ($\times 10^6$/l) and a platelet count <80,000 ($\times 10^6$/l, employment in a factory with documented benzene exposure for at least 6 months and exclusion of other causes for abnormal blood counts).

[b] Duration of chronic exposure to benzene prior to initial complaint.

[c] Time between cessation of benzene exposure and diagnosis of benzene-induced dysplasia in our laboratory.

[d] Signs, symptoms and laboratory findings consistent with hematologic injury documented at the time of exposure. ND indicates information that was not available.

Laboratories, Abbott Park, IL), clinical chemistry for liver enzymes (LDH, ALT and AST enzymes) (COBAS, Integra 400 plus, Roche Diagnostics, Shanghai, China) and serum analysis for nutritional factors. Vitamin B12 and folate were measured by chemical luminescence (Beckman Coulter Dxi800), and total iron binding capacity was measured using a Beckman Coulter LX20. BM aspirates and core biopsies were obtained by Jamshidi needle extraction from posterior iliac crest. Aspirates were stained with fluorochrome-conjugated antibodies for flow cytometric analysis of BM cellular subsets. Antibody panels included: anti-CD45, CD4, CD8, CD3 (Beckman Coulter, Hialeah, FL; Immunotech, Miami FL). Multiparameter analysis was performed on stained BM cells using a dual laser flow cytometer (FC-500, Beckman Coulter) equipped with compensation software (Software CXP, Beckman Coulter).

2.3. Bone marrow morphology

BM smears were prepared and evaluated using Wright-Giemsa stained preparations and special stains. Core biopsy sections were evaluated using sections stained with Hematoxylin-Eosin, Gomori trichrome, and immunoperoxidase-immunohistochemistry. Morphology was independently evaluated by two of us (R.D.I., J.R.). Dyserythropoiesis was scored on the basis of abnormal nuclear morphology, including internuclear bridging, abnormal budding, multiple nuclei and abnormal mitotic figures or megaloblastoid features. Myeloid or granulocytic dysplasia was defined by nuclear hypolobulation, (i.e. pseudo-Pelger Huet cells, hypersegmentation, abnormal mitotic forms) either hypo- or hypergranulation of the cytoplasm as well as the presence of large irregular granules. Megakaryocyte dysplasia was defined by non-, or hyper-lobulated nuclei, multiple individual nuclei or the presence of prematurely segmented or "shedding" cytoplasm. Eosinophilic dysplasia was defined by the presence of megaloblastoid features and abnormal cytoplasmic basophilic and eosinophilic hypergranulation. Microscopic analysis was performed using an Olympus BX51 bright field microscope (Olympus Optical, Ltd., Tokyo, Japan) equipped with a Sony EXwave HAD color video camera (Sony Ltd., Tokyo, Japan). Images were processed using software custom designed for the purpose (Vision Image Technology, Shanghai, China), and photomicrographs were cropped and edited using Adobe Photoshop (San Jose, CA). Cases of benzene-induced dysplasia were compared to MDS subtypes diagnosed according to the WHO classification system

2.4. Cytogenetic analysis

Fluorescence in situ hybridization (FISH) and cytogenetic analyses were performed on unstimulated BM cells

following 24–72 h of culture. Chromosomes were prepared and G-banding after trypsin was performed according to standard techniques. If possible, 29 metaphases were analyzed. Interphase or metaphase FISH analyses were performed on the short-term cultured cells or metaphases prepared from the BM or PB samples. All probes used in the FISH studies were purchased from Vysis (Downers Grove, IL). Sample preparations and hybridizations were performed according to the protocols provided by the manufacturer. Slides were viewed using an Olympus fluorescence microscope (Olympus Optical, Ltd., Tokyo, Japan) equipped with the appropriate filters and the PowerGene Macprobe image analysis system (Applied Imaging International Ltd., Newcastle, UK). Whenever possible, 1000–2000 nuclei were analyzed for each probe and scored by two readers. Signal patterns for each probe were compared against a reference range established from analysis of 100 samples from individuals with no evidence of clonal disease or previous benzene exposure.

2.5. Analysis of FLT3 mutations

FLT3 mutations are often encountered genetic abnormalities in AML, frequently involving an internal tandem duplication (ITD) or activation loop mutations in the tyrosine kinase domain (TKD) of the *FLT3* gene [18,19]. *FLT3* mutations were determined by PCR analysis and sequencing using DNA isolated from BM cells. Genomic DNA was isolated from blood and BM samples using A Qiagen QIAmp DNA mini Kit (Chatsworth, CA) according to the manufacturer's directions. Exons 14 and 15 and the intervening sequence intron of the *FLT3* gene were amplified by PCR to detect ITD. The PCR products were run on both 2% agarose gel and 5% polyacrylamide gel. Exon 20 of the *FLT3* gene was also amplified by PCR to detect TKD using primers as reported by Yamamoto et al. [20]. These PCR products were subjected to digestion by EcoRV and analyzed on a 3.5% agarose gel. Finally, PCR products were isolated from the gels using the DNA Recovery Kit (Biologic Technology Co., Ltd., Shanghai, China) and directly sequenced on an ABI 377 DNA Sequencer (Applied Biosystems, Foster City, CA).

2.6. T cell receptor (TCR) rearrangement analysis

Detection of clonal and oligoclonal expansion of T cell populations was determined by analysis of rearrangements of TCR beta (TCRβ), TCR gamma (TCRγ) and TCR delta (TCRδ) genes by multiplex PCR using BIOMED-2 kits (InVivoScribe Technologies, San Diego, CA) according to the manufacturer's protocol. For the *TCRβ* gene, the kit comprised of three individual master mix reactions, the *TCRγ* kit utilized two master mixes and *TCRδ* kit utilized a single PCR master mix. Amplification of *TCRβ, γ and δ* genes was performed in a thermocycler and the PCR samples were loaded unto a 6% non-denaturing polyacrylamide TBE gel, stained with ethidium bromide, visualized by UV illumination and documented by digital photography. Clonal rearrangements were determined according to published guidelines with exclusion of nonspecific bands [21].

3. Results

Patients diagnosed with benzene-induced dysplasia exhibited a distinct set of characteristic features that both overlap with and can be distinguished from standard classifications of MDS. At the time of diagnosis in our laboratory, PB findings in these cases included pancytopenia ($n = 1$), and cytopenias, singly or in combination ($n = 16$). The most consistent abnormal finding was lymphocytopenia ($n = 14$). Six subjects presented with normal or marginally normal blood counts (Table 2). All patients exhibited significant BM pathology including evidence of multilineage dysplasia which was characterized by macrocytic megaloblastic changes in granulocytic, eosinophilic as well as erythroid lineages and at all stages of development. Stromal degeneration was also present in 12/23 cases. Most cases of benzene-induced dysplasia were hypocellular (17/23) with residual hematopoietic cells unevenly distributed throughout the BM. Dyserythropoiesis was commonly found and often severe (Figs. 1 and 2). Several features distinguished benzene-induced dysplasia from other previously defined subtypes of MDS including therapy related (t)MDS [16,22,23]. Prominent among these were the presence of abnormal BM eosinophilic precursor cells (22/23 cases). These cells are reminiscent of those found in AML with the 16(p13;q22) chromosome abnormality [16] (Fig. 3). Hematophagocytosis, which is commonly associated with a poor prognosis in BM failure, was a particularly frequent and striking observation (16/23 cases) (Fig. 4). Maturing myeloid cells and granulocytes also typically exhibited megaloblastic alterations and abnormal cytoplasmic morphology (18/23 cases) (Fig. 5). Finally, for a significant subset of cases there was a lack of concordance between the severity of pathology observed in the BM and the relatively mild or moderate abnormalities encountered in the PB (6/23 cases). This suggests that monitoring of the peripheral blood CBC may not accurately reflect the progression of hematopoietic disease in patients previously exposed to benzene.

3.1. Clonal cytogenetic abnormalities in benzene-induced dysplasia

Reoccurring clonal genetic abnormalities are frequently encountered in MDS, and have been suggested to play an early or predisposing role in the development of the disease. Therefore, we analyzed BM cells from each subject for evidence of clonal hematopoietic lesions. In contrast to the overall frequency of cytogenetic abnormalities observed for MDS in our laboratory (26%; $n = 100$), cytogenetic analyses revealed no clonal abnormalities in any benzene poisoning case in this series. The most frequent cytogenetic abnormalities encountered in tMDS/tAML are −5, −7, del(5q)−, and del(7q−) which are found in greater than 70% of reported

Table 2
Characteristics of PB and BM in benzene-induced dysplasia

Case	ANC (10^6/l)	ALC (10^6/l)	LGL (%)	PLT (10^9/l)	Hgb (g/dl)	Bone marrow						
						Cellularity	CD4/CD8 Ratio	Stro	Dysplasia				HP
									Gran	Ery	Meg	Eo	
1	1240	730	27	93.7	11.5	Norm	0.24	N	+	ND	ND	+	+++
2	1920	1620	15	122	11.9	Hypo	1.62	N	ND	+	++	++	+++
3	1250	1390	28	176	13	Hypo	0.92	N	+++	+++	0	+	++
4	1520	980	20	200	13.5	Norm	0.76	N	+++	++	++	++	+++
5	930	1530	24	61.8	15.9	Hypo	2.42	N	++	+++	0	+++	ND
6	2980	190	25	77.8	13.9	Norm	0.60	SA	0	+++	0	+++	++
7	1760	340	32	124	8.3	Hyper	0.64	N	+++	+++	+++	+++	+++
8	1590	2220	40	146	13	Hypo	0.59	SA	ND	+	0	++	+
9	3390	1740	41	203	13.3	Hypo	0.71	SA	+++	+++	+++	+	+++
10	1650	1440	46	168	12.9	Hypo	0.45	FN	ND	+	+	++	+
11	1510	1160	30	122	12.7	Hypo	0.43	SA	+	+	0	ND	ND
12	3140	823	42	92.7	11	Hypo*	0.42	QNS	+++	0	0	+++	ND
13	2020	2300	41	133	11.8	Hypo*	0.97	QNS	0	0	0	+++	ND
14	2380	1600	28	174	15.2	Hypo	0.69	SA	+	+	0	+++	ND
15	2080	1710	30	65.9	12	Hypo	0.83	SA	+++	+	0	+++	ND
16	2270	1780	32	137	13.8	Hypo	0.71	N	+++	+++	0	+	+
17	2370	1350	28	71.1	11.8	Hypo	0.37	SA	+++	+	0	++	ND
18	2950	710	18	113	12.8	Hypo	0.91	SA	+++	+	0	+++	+
19	2240	870	18	33.5	11.4	Hypo	0.20	SA	+++	+++	0	+++	+
20	1400	600	32	84.1	12.9	Hypo	0.59	SA	+++	+++	0	++	+++
21	2090	810	22	99.8	11.9	Hypo	1.39	SA	+++	+	0	++	+
22	4000	2850	33	275	14.7	Hyper*	0.88	0	+++	+	0	++	+
23	1520	990	ND	147	13.2	Norm	0.50	SA	+	+	0	+	+

ANC, absolute neutrophil count; ALC, absolute lymphocyte count; LGL, large granular lymphocytes (%ALC); PLT, platelet count; Hgb, hemoglobin; bone marrow cellularity was estimated from the core biopsy or the aspirate* (e.g. hypo-hypoplastic [<40% cellularity], norm-normal [40–60% cellularity] and hyper-hyperplastic [>60% cellularity]; BM lymphocyte CD4/CD8 ratios as determined by flow cytometry; Stro, stroma; SA, focal serous atrophy; FN, fibrinoid necrosis, 0, stromal cells were absent or too rare to evaluate; dysplasia was estimated for granulocytic (Gran), erythroid (Ery), megakaryocytic (Meg) and eosinophilic (Eo) lineages and scored based on the percentage of lineage-specific cells involved, ND [none detected], + [10–50%], ++ [50–70%], or +++ [>70%]). 0, cells of individual lineage were absent or too rare to evaluate for dysplastic changes. HP, hematophagocytosis, was scored as not detected (ND) present (+), moderate (++) or severe (+++). Reference ranges: ANC (2000–7000 × 10^6/l); ALC (1600–6000 × 10^6/l); LGL (5–15%); PLT (100–300 × 10^9/l); Hgb (12–16 g/dl); CD4/CD8 (0.72–2.56) [55].

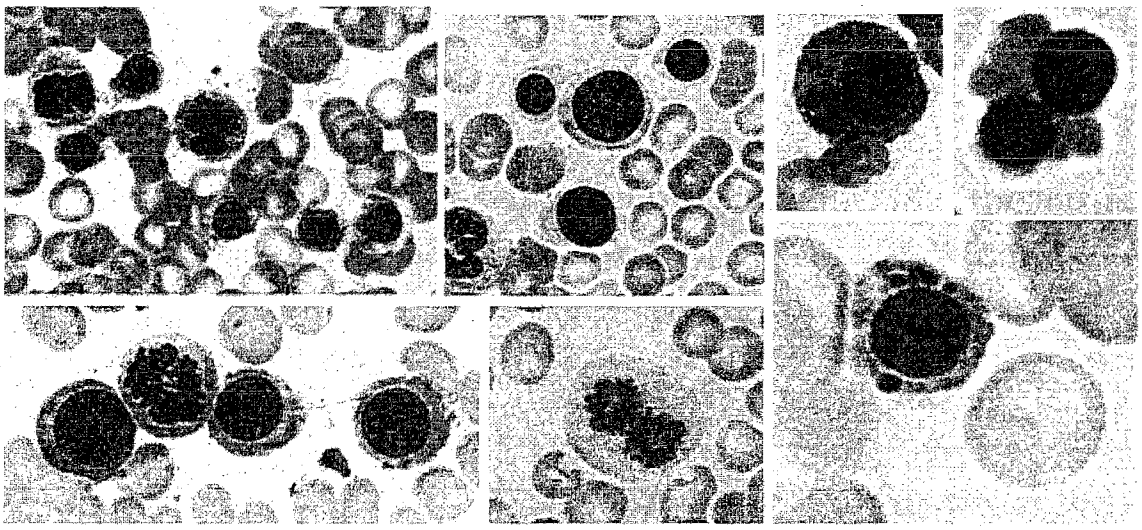

Fig. 1. Erythroid dysplasia and dyserythropoiesis in benzene-induced dysplasia. Abnormal erythroid cells exhibit megaloblastic abnormalities and abnormal nuclear morphology including nuclear bridging. BM aspirate slides stained with Wright-Giemsa (original magnification 1000×).

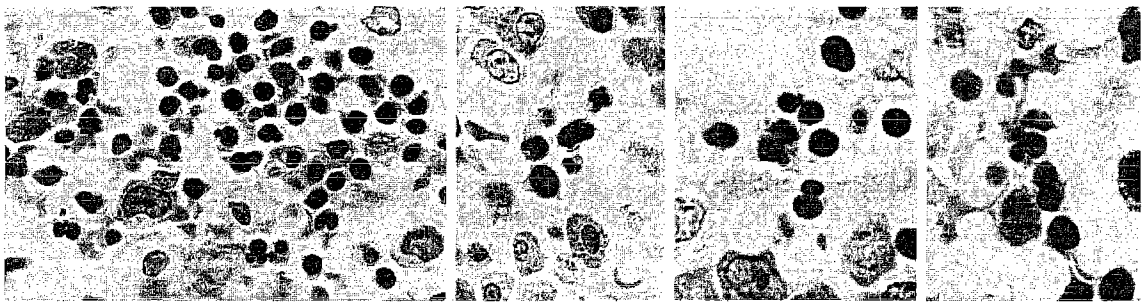

Fig. 2. Prominent dyserythropoiesis in a core biopsy from a representative patient with benzene-induced dysplasia. The most frequent dyserythropoietic changes in H&E stained sections are bizarre budding of nuclei in erythroid precursor cells. Core biopsy section stained with Hematoxylin-Eosin (original magnification 1000×).

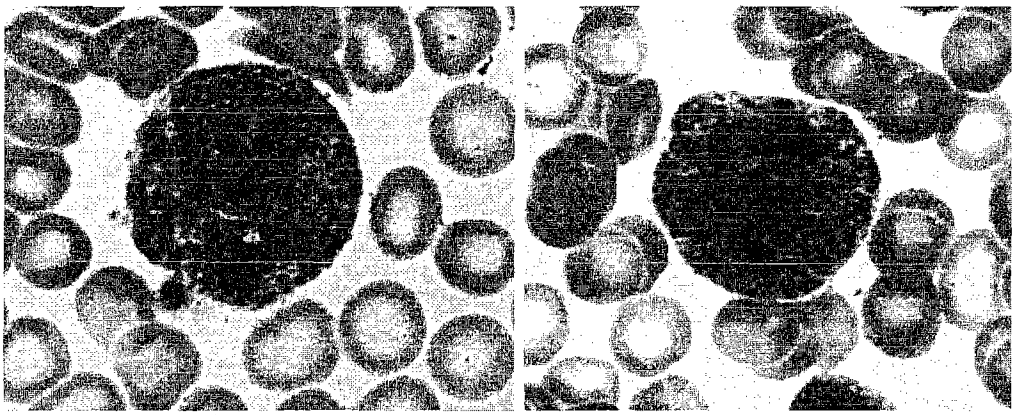

Fig. 3. Abnormal eosinophilic precursor cells in benzene-induced dysplasia. Abnormal eosinophils exhibit megaloblastic nuclear abnormalities, nuclear hypersegmentation and atypical giant basophilic and eosinophilic cytoplasmic granulation. BM aspirate slides stained with Wright-Giemsa (original magnification 1000×).

cases [24,25], and some studies have suggested a similar pattern might exist for AML developing after chronic benzene exposure [10,26–28]. We also performed additional analyses for −7, 5q−, 11q23 and +8 structural abnormalities using FISH and observed no signals above background (data not shown). *FLT3* mutations are the most frequently encountered genetic abnormalities in AML, commonly involving ITD or activation loop mutations in the TKD of the *FLT3* gene [18,19,29]. However, no *FLT3* mutations were observed in this series. These findings suggest that acquisition of cytogenetic or molecular abnormalities in benzene-induced dysplasia may be relatively late events in the neoplastic progression of the disease.

3.2. Benzene-induced dysplasia may involve an autoimmune process

The frequency and severity of lymphocytopenia, hematophagocytosis and eosinophilic dysplasia in benzene-induced dysplasia led us to examine additional immune cell parameters. Patients with benzene-induced dysplasia exhibited relative increases in LGL (mean = 29.6%, $n = 22$) in PB and decreases in the ratio of CD4+/CD8+ lymphocytes in the BM (0.77 ± 0.48, $n = 23$) (Table 2). These observations coincided with demonstration of clonal and oligoclonal proliferations in BM T lymphocytes (14/23 cases), including clonal rearrangements in Vβ TCR gene segments ($n = 4$), Vδ TCR gene segments ($n = 6$) and both Vβ and Vδ TCR segments ($n = 4$).

4. Discussion

The role of autoimmune mechanisms in the development of MDS remains largely unexplored, although, evidence is accumulating to implicate immunologic abnormalities in the pathogenesis of the disease. Frequent findings in MDS include lymphocytopenia, inverted CD4/CD8 T cell ratios, increases in cytotoxic CD8+ T cells (CTL) and serum levels of inflammatory cytokines such as tumor necrosis factor α (TNF-α), and interferon γ (INF-γ) [2], abnormal expression of HLA-DR [30] and evidence of TCR gene rearrangements [31–34]. Positive clinical responses to immunosuppressive therapy with anti-thymocyte globulin (ATG) or cyclosporin A also have been reported in some patients with MDS [3,33,35], together with treatment-related decreases in both CD8+ T cell clones and T-LGL [32,36,37]. The role of environmental exposure in the evolution of MDS is unknown, although it has been hypothesized that the pathogenesis of MDS may involve

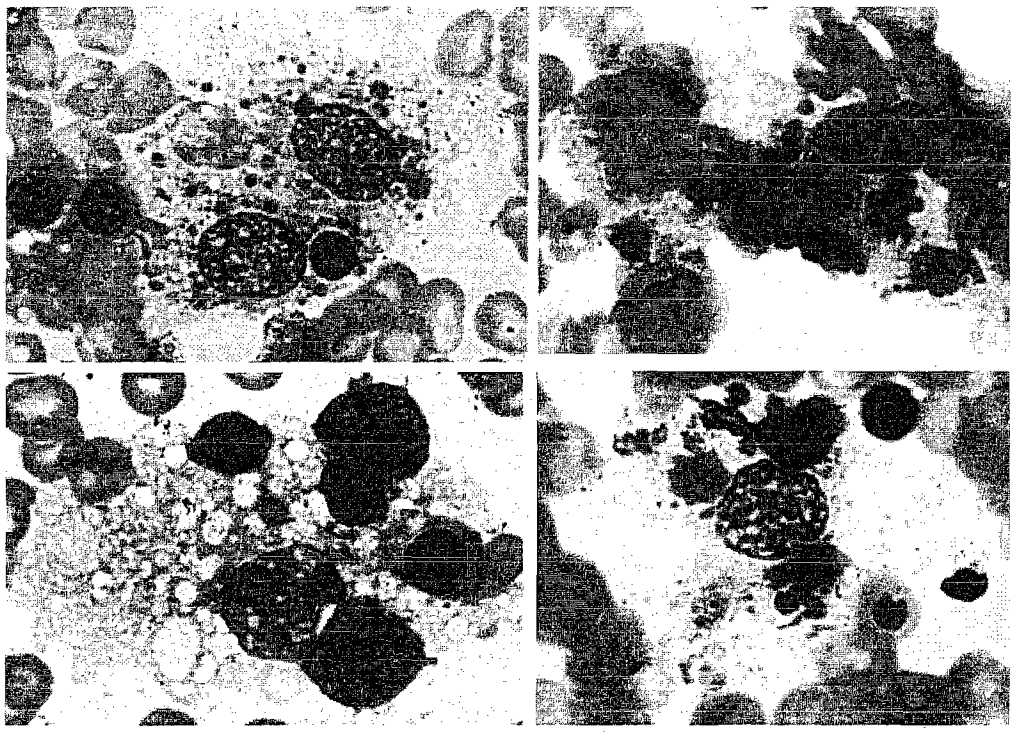

Fig. 4. Hematophagocytosis in benzene-induced dysplasia. Activated histiocytic cells within the BM exhibit prominent phagocytosis of degenerating erythroid and granulocytic cells which is indicative of an immuno-reactive inflammatory process. The histiocytes themselves do not appear atypical and generally exhibited a low nuclear cytoplasmic ratio with small nuclei containing condensed chromatin. BM aspirate slides stained with Wright-Giemsa (original magnification 1000×).

damage to hematopoietic progenitor cells exposed to intrinsic or environmental toxic agents that might lead to immunologic suppression of cell growth and maturation [2,38]. Transient anemia, cytopenias and dysplasia frequently occur in BM toxicity during or immediately following exposure to toxic agents. This usually is not associated with persistent disease and therefore, is not generally considered to be MDS [39–41]. However, our findings show that prolonged chronic exposure to high concentrations of benzene results in the development of a distinct form of dysplasia that differs from commonly defined subtypes of MDS and that can persist for years after cessation of benzene exposure.

Previous proposals to explain benzene carcinogenesis, including those from our laboratory, have presumed a role for genetic injury in the origin of benzene-induced persistent toxicity and the development of AML [28,42–44]. Alternatively, our current results suggest the possibility that an autoimmune process may precede the acquisition of frank structural cytogenetic abnormalities and may be a predisposing event in the development of persistent BM disease following benzene exposure. Histological manifestations of benzene-induced dysplasia include multilineage dysplasia including severe dyserythropoiesis, stromal degeneration, alterations in BM T lymphocyte subsets, abnormal eosinophilic precursors and hematophagocytosis. Hematophagocytosis is associated with severe viral infection or inflammatory response and activation of immune cells [45]. Benzene-induced dysplasia is also accompanied by clonal and polyclonal proliferation of T

Fig. 5. Dysplastic changes in granulocytes in benzene-induced dysplasia. The BM and PB in the case series typically contain maturing myeloid cells with megaloblastic, hypo-segmented (pseudo Pelger-Huett) or hypersegmented nuclei. In some instances abnormal mitotic figures are present in granulocytes with mature cytoplasm. Cytoplasmic abnormalities include protruding pseudopodia which often trails cell bodies and abnormal cytoplasmic granulation in which large granules were unevenly distributed in clusters or marginated directly beneath the plasma membrane. BM aspirate slides stained with Wright-Giemsa (original magnification 1000×).

lymphocytes in the BM which is indicative of an active immune process and has been described in a variety of immune-related conditions involving suppression of hematopoiesis including MDS [4,46,47]. The function of γδ T cell subsets is largely unknown, although increases in γδ T cells also have been observed in patients with BM failure, have been shown to modulate eosinophilic inflammation in other tissues and are known to be activated by TNF-α [35,37,48,49]. In this study, *TCRδ* expansions were accompanied by evidence of corresponding expansion of *TCRγ* gene rearrangements. However, confirmation of γδ T cell subsets by heteroduplex analysis is required.

These observations indicate that previous chronic exposure to benzene is associated with the development of BM dysplasia, and suggest that a reactive inflammatory process may be involved in the suppression of hematopoiesis in persistent BM failure following chronic benzene exposure. These findings further suggest the possibility that markers of immune activation or inflammation may prove useful in monitoring the development or progression of benzene-induced dysplastic disease. Nevertheless, the role of altered immune regulation in the pathogenesis of benzene-induced dysplasia, as well as the identity of any putative antigens remain a mystery. The mechanisms of benzene-induced hematopoietic cell injury are not completely understood. However, a subpopulation of CD34+ BM cells that are responsive to granulocyte-macrophage colony-stimulating factor (GM-CSF) have been implicated as targets. Studies in our laboratory have shown that the benzene metabolite, hydroquinone, enhances cytokine-dependent clonal proliferation of a subpopulation of GM-CSF-responsive human CD34+ BM cells which appears to be mediated via the extracellular signal-regulated kinase/activation protein-1 signaling pathway (ERK/AP-1) [50–53]. Independently, hydroquinone also synergizes with TNF-α to produce apoptosis in human CD34+ hematopoietic progenitor cells (HPC) via a mechanism that involves the inhibition of NF-κB [54].

In recent years occupational exposure to benzene in China has been significantly reduced with the current Chinese occupational exposure limit being 1.9 ppm as a time weighted average (TWA) with a short-term exposure limit (STEL) of 3.2 ppm. Despite these changes, exposures to high concentrations of benzene continue to occur, and subjects diagnosed with persistent BM dysplasia in this report represent only a small fraction of the individuals exposed to high concentrations of benzene in the facilities studied. Previous reports have suggested that genetic polymorphisms in metabolizing and detoxification enzymes may play a role in conferring susceptibility to benzene toxicity. However, preliminary results in our laboratory do not suggest a prominent role for these genetic variants in the development of benzene-induced BM dysplasia. Evaluation of the influence of polymorphisms in other genes, such as TNF-α, on susceptibility to benzene-induced BM dysplasia are ongoing in our laboratory. Continued characterization and follow-up of these subjects with benzene-induced dysplasia may provide insights into early events associated with the pathogenesis, clonal selection and progression of persistent BM disease, including MDS and its potential progression to AML.

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ORIGINAL ARTICLE

Adult precursor B lymphoblastic leukemia in Shanghai, China: characterization of phenotype, cytogenetics and outcome for 137 consecutive cases

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Abstract Acute lymphoblastic leukemia (ALL) accounts for 20–30% of adult leukemia in the West. However, detailed studies of B-cell-specific ALL in adult Asian populations are lacking. We diagnosed and characterized 137 consecutive cases of precursor B lymphoblastic leukemia (precursor B-cell ALL) presented to our laboratory in Shanghai using the WHO 2001 classification system. Patient clinical, phenotypic and cytogenetic characteristics were correlated with outcome. In contrast to Western studies, females (71) outnumbered males (66) partly due to an increased prevalence of the CD10− pro B-cell phenotype. Females with a CD10− pro B-cell phenotype exhibited significantly better overall survival than males. The most common cytogenetic abnormality was the Philadelphia chromosome (PH/BCR/ABL) which was found in approximately 37% of the cases. Cases of precursor B cell ALL lacking the PH/BCR/ABL genotype exhibited a pronounced age-dependent, gender prevalence with a modal age in the sixth decade for females compared to the second decade for males. These findings suggest significant geographic heterogeneity in precursor B-cell ALL which may be of both etiological and therapeutic significance.

Keywords Precursor B-cell ALL · Ph chromosome · *BCR/ABL* · Cytogenetics · FISH · CD10

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1 Introduction

Acute lymphoblastic leukemia (ALL) represents approximately 20–30% of all leukemias in adults with a well-recognized male predominance worldwide [1, 2]. The incidence of ALL increases around the age of 50 years affecting 2 cases per 100,000 people above 65 years of age [1]. The vast majority of adult ALL are of B cell lineage and are classified as precursor B-cell lymphoblastic leukemia (precursor B-cell ALL) by the World Health Organization [3]. Studies of adult precursor B-cell ALL in China are rare, with reports published as recently as 2005 indicating an extremely low prevalence of precursor B-cell ALL in Chinese populations [4]. Nevertheless, we report on a series of 137 consecutive cases of precursor B-cell ALL diagnosed in a single laboratory in Shanghai over a 4-year period. Our findings indicate that precursor B-cell ALL is not a rare disease in China and, in fact is second only to diffuse large B cell

lymphoma in prevalence among lymphoid neoplasms [5]. Characteristics of precursor B-cell ALL in Shanghai that differ from the Western experience include a female predominance and a relatively high proportion of complex cytogenetic abnormalities.

Although it is defined as a single disease entity under the WHO 2001 classification system, precursor B lymphoblastic leukemia is a heterogeneous disease that can be divided into the following four subgroups based on phenotypic differentiation: progenitor B-cell lymphoblastic leukemia (pro B-cell ALL); precursor B-cell lymphoblastic leukemia (precursor B-cell ALL); common B-cell lymphoblastic leukemia (common B-cell ALL); and mature B-cell lymphoblastic leukemia (mature B-cell ALL). Pro B-cell ALL constitutes about 11% of ALL in adults in the West and predominately exhibit a CD19+ and CD10− phenotype. Together, precursor B-cell ALL and common B-cell ALL comprise the vast majority of adult cases and co-express CD19+ and CD10+. Mature B-cell ALL is thought to represent a more mature cell type, 80% of which also express CD20+ [1, 6, 7]. The t(9;22) translocation, resulting in the Philadelphia (PH) chromosome and the formation of BCR/ABL fusion transcripts, is the most common cytogenetic abnormality occurring in adult precursor B-cell ALL with an incidence of 13–33% in Western studies [1, 6, 8–15]. A subset of PH chromosome positive (PH/BCR/ABL+) precursor B-cell ALL frequently co-express the myeloid antigens CD13 or CD33 [16]. Patients with PH/BCR/ABL+ precursor B-cell ALL usually do not respond well to conventional chemotherapy, but newer therapeutic alternatives employing tyrosine kinase inhibitors (e.g. imatinib mesylate) used in combination with stem cell transplantation have been shown to improve outcomes [17–20]. The *t*(4;11) translocation involving 11q23 also occurs in a small subgroup of adult precursor B-cell ALL cases, and is associated with a high leukocyte count and a CD10− pro B-cell ALL phenotype [21]. Geographic heterogeneity in the incidence of genetic aberrations in leukemia has been reported [20, 22], but has not been investigated previously in precursor B-cell ALL in China [23].

Patients in our series were diagnosed in a single laboratory according to WHO 2001 criteria, and routinely analyzed by morphologic, immunohistochemical, flow cytometric, cytogenetic and molecular genetic techniques. Our results reveal significant differences for male:female prevalence in precursor B-cell ALL and a distinct female predisposition toward pro B-cell ALL relative to Western reports. Predictably PH/BCR/ABL+ precursor B-cell ALL responded poorly to conventional chemotherapy and had shorter overall survival (OS) than PH/BCR/ABL− precursor B-cell ALL.

2 Materials and methods

2.1 Patients

A total of 137 patients were diagnosed in series with precursor B-cell ALL in our laboratory in Shanghai, China, between July 2003 and July 2007 according to the WHO 2001 classification criteria [3]. The patients were at least 18 years old and referred from 28 hospitals in Shanghai. Informed consent was obtained according to the Declaration of Helsinki, 2004 and the NIH Common Rule (45CFR46). This study was conducted as part of the Shanghai Health Study and approved by the Colorado Multiple Institutional Review Board, the Cincinnati Children's Hospital Medical Center Institutional Review Board and the Ethic Committee of Fudan University in Shanghai, China.

2.2 Sample collection and clinical laboratory analysis

Peripheral blood, bone marrow aspirates, tissue and core biopsies were collected in conjunction with diagnostic procedures. Peripheral blood smears were obtained by finger stick, and bone marrow aspirates and core biopsies were obtained by Jamshidi needle extraction from the posterior iliac crest. Blood samples were collected by venipuncture and processed for routine CBC (Cell Dyne 3700, Abbott, Abbott Park, IL), viral serology (HCV and HIV) (Imx, Abbott) and clinical chemistry for liver enzymes (LDH, ALT and AST enzymes) (COBAS, Integra 400 plus, Roche Diagnostics, Basel).

2.3 Morphology

Bone marrow and peripheral blood smears were prepared and evaluated for morphology using Hematoxylin-Eosin (H&E), Wright-Giemsa and Gomori trichrome stain preparations. Peripheral blood blast cell number was obtained from the peripheral blood smear and bone marrow blast cell number was obtained from a bone marrow aspirate smear. Blast cells in both smears were enumerated and presented as a percentage of the total number of cells observed. Morphology was independently evaluated by three of us (M.J., R.D.I., J.R.). Microscopic analysis was performed using Olympus BX51 bright field microscopes (Olympus Optical Ltd, Tokyo).

2.4 Cytogenetic and FISH analysis

Banding chromosome and FISH analysis were performed on diagnostic bone marrow or blood. Clonal criteria and descriptions of karyotype followed the recommendations of the International System for Human Cytogenetic

Nomenclature (2005) [24]. For FISH analysis the dual-fusion, dual-color *BCR/ABL* probe was used (Vysis). Sample preparation and probe hybridization were processed following the manufacturer's recommendations. In each case, 500 cells were analyzed. The cut-off was determined using samples collected on 20 healthy individuals.

2.5 PCR analysis

Total RNA was extracted from diagnostic bone marrow or blood using the Qiagen QIAamp RNA Blood Mini Kit (Qiagen, Strassel, Germany) and cDNA was synthesized with the oligo(dT) and Moloney-murine leukemia virus reverse transcriptase RNase H (Promega, San Luis Obispo, CA). The conditions for PCR were modified from the method reported by Cross et al. [25]. Primers for the first round PCR were 5′GGACTTCTCCTCTGGCCAGTCCA3′, 5′GGAGCTGCAGATGCTGACCAAC3′ and 5′CACAGG CCCATGGTACCAGGAG3′, and for the second PCR were 5′ACCGCATGTTCCGGGACAAAAG3′, 5′ACAGA ATTCCGCTGACCATCAATAAG3′, and 5′TGTTGACTG GCGTGATGTAGTTGCTTGG3′. The PCR program started with an initial denaturing at 94°C for 3 min, then underwent 35 cycles at 94°C for 40 s, 59°C for 40 s, and 72°C for 40 s. A final extension reaction followed at 72°C for 5 min. The PCR products were analyzed by gel electrophoresis.

2.6 Immunophenotyping

Bone marrow aspirates were stained with a panel of fluorochrome-conjugated antibodies against CD45, CD34, CD117, DR, CD33, CD14, CD64, CD7, CD10, CD19, CD16, CD13, CD4, CD8, CD3, CD2, CD56, CD11c, cCD3, cCD79a and cTdT. (Beckman Coulter, Hialeah, FL and Immunotech, Miami FL). Criteria for positive antigen expression included $\geq$20% for CD34, and $\geq$10% for CD10, CD13 and CD33 for cells gated in the blast cell population. Positive selection for CD34 antigen expression was set at $\geq$20% since the blast cell cut-off for AML is set at 20% using World Health Organization 2001 criteria. As for positive expression of CD10, CD13 and CD33, we selected a $\geq$10% cut-off based on our flow cytometric analysis for gating of these antigens. Abnormal cells expressing $\geq$10% either CD13 or CD33 were considered positive for myeloid markers.

2.7 Treatment regimen

Patients diagnosed as precursor B-cell ALL were given a reduction regimen using predominantly conventional VDLP (vincristine 2 mg/day, daunomycin 40 mg/day, L-asparaginase 10,000 U/day, prednisone 60 mg/day then 30 mg/day) or VDCP (vincristine 2 mg/day, daunomycin 40 mg/day, cytoxan 1,000 mg/day, prednisone 60 mg/day then 30 mg/day) regimens. Once remission was achieved, patients received consolidation therapy consisting of either VDLP, VDCP, Methotrexate 2–3 g/24 h or VP16 + AraC (Etoposide 150 mg/day plus cytosine arabinoside 150 mg/day). Bone marrow transplantation is not routinely employed for treatment of B-cell ALL in Shanghai, and only two patients had bone marrow transplant following reduction chemotherapy.

2.8 Statistical analysis

Differences in clinical and biological variables were measured using the Fisher's exact or chi-square test. In some analyses, two-tailed hypothesis testing was used to determine differences between variables. OS distributions were estimated using the Kaplan–Meier method with OS determined from the interval between the date of initial diagnosis and death or loss of contact. The 95% confidence intervals (CI) for survival probability estimates were obtained by the method of Simon and Lee [26]. The differences between survival curves were analyzed by log-rank test. $P < 0.05$ is considered statistically significant.

3 Results

One-hundred and thirty-seven consecutive cases of precursor B-cell ALL were diagnosed in adult Chinese patients according to WHO 2001 criteria. More females presented with precursor B-cell ALL than males (71 and 66, respectively) which resulted in a male:female ratio of 0.92. The median age for females at presentation was 50 years (range 19–82) whereas the median age for males was 38 years (range 19–83). Conventional cytogenetic analysis was successful in 112 of 137 (81.8%) of precursor B-cell ALL cases. A total of 87 (77.7%) patients had clonal abnormalities, of which 42 (48.4%) had at least 3 aberrations and 25 (28.7%) presented with multiple clones. A total of 46 (41.1%) cases were positive for the PH chromosome, of which 18 (36.7%) exhibited multiple clones, all due to karyotypic evolution. Seven cases without the PH chromosome possessed multiple clones: 4 due to karyotypic evolution and 3 with multiple independent clonal abnormalities. An additional 4 cases (3.6%) were positive for the *t*(4;11) translocation.

FISH analysis was performed on 127 (92.7%) patient samples including cases in which cytogenetic analysis was not informative and/or failed. A total of 47 (37.0%) were positive for *BCR/ABL* rearrangements by FISH including two cases where cytogenetic analysis showed a normal

karyotype. PH/BCR/ABL status was assessed successfully in 134 of 137 patients either by cytogenetic analysis and/or FISH (Table 1). Combining both cytogenetic and FISH results, the incidence of PH/BCR/ABL+ was 36.6% in our series. Three additional cases, where both analyses failed, were considered unknown with regard to the genetic aberration and not included in the comparisons in Table 1. PCR analysis for *BCR/ABL* was positive for 50 (37%) of 135 patients tested. Four PCR positive cases which did not concur with FISH and/or cytogenetics studies were considered inconclusive and were excluded from further analysis of PH/BCR/ABL+ cases. Subsequent analysis of the 46 PH/BCR/ABL+ precursor B-cell ALL cases revealed that 28 (60.9%) patients possessed the p190 isoform, 17 (37.0%) patients had the p210 isoform and one (2.2%) patient had both p190 and p210. The p230 isoform was not found in our series. Clinical characteristics were compared between cases exhibiting either the p190 or p210 *BCR/ABL* isoform but no significant differences in clinical or biological parameters were observed between these two groups.

There was no difference in median age between PH/BCR/ABL+ and PH/BCR/ABL− precursor B-cell ALL patients, 43 and 45 years, respectively (Table 1). However, there was a significant difference in gender distribution between PH/BCR/ABL+ and PH/BCR/ABL− patients. The PH/BCR/ABL+ abnormality was observed in more male patients than in female patients (ratio 1.45). However, for patients with PH/BCR/ABL− precursor B-cell ALL, females outnumbered males (ratio 0.7). Patients with PH/BCR/ABL+ precursor B-cell ALL also had lower platelet counts and higher percentage of blood and bone marrow blasts ($P < 0.05$). Patients with PH/BCR/ABL+ precursor B-cell ALL had a higher mean WBC. Most of our precursor B-cell ALL patients showed blast cells with CD34+ expression but a higher proportion was observed among the patients also exhibiting PH/BCR/ABL+ expression than in patients with PH/BCR/ABL− precursor B-cell ALL (98.0 vs. 74.1%). Similar to the Western experience, patients with PH/BCR/ABL+ precursor B-cell ALL had more cells expressing either of the myeloid markers, CD13 or CD33, and were more likely to have multiple clones than patients with PH/BCR/ABL− precursor B-cell ALL. All cases were examined for CNS infiltration and there was no significant difference between PH/BCR/ABL positive and negative genotypes (data not shown).

Table 1 Clinical and biological features of Ph/*BCR/ABL*+ and Ph/*BCR/ABL*− precursor B-cell ALL

Features	PH/BCR/ABL+	PH/BCR/ABL−	*P*
No. of patients	49	85	NS
Age (years)[a]	43 (19–82)	45 (19–81)	NS
Sex (male/female) (ratio)	29/20 (1.45)	35/50 (0.73)	0.054
WBC count (10^9/l)	16.8 (0.4–384)	3.4 (0.4–422)	NS
Platelets (10^9/l)	24.6 (9.4–352)	86.3 (10–242)	0.0367
Hemoglobin (g/l)	8.8 (3.2–14.2)	7.7 (3.1–13.2)	0.049
Serum LDH (IU/l)	546 (122–6812)	365 (15–5347)	NS
Blood blast (%)	56 (1–96)	20 (1–95)	0.0008
Bone marrow blast (%)	85 (22–99)	76.5 (20–96)	0.034
Immunophenotype[b]			
CD34+	48/49 (98.0%)	63/85 (74.1%)	NS
Myeloid markers	39/49 (79.6%)	38/85 (44.7%)	0.0001
Cytogenetic abnormalities[b]			
Complex karyotype	26/49 (53.1%)	16/38 (42.1%)	NS
Multiple clones	18/49 (36.7%)	7/38 (18.4%)	0.01
t(4;11)(q21;q23)	0/49 (0%)	4/38 (10.5%)	0.02

Myeloid markers: expression of CD13 and/or CD33

NS not significant

[a] Median (range), b percent on available data

Eighty-five patients with precursor B-cell ALL who lacked the PH/BCR/ABL genotype were further sub-divided into stages of phenotypic differentiation and compared for clinical and biological features (Table 2). Sixty-five cases were identified as common B-cell ALL (CD19+/CD10+) and 20 cases were classified as pro B-cell ALL (CD19+/CD10−). Although there was no difference in median age between these two groups, there were significant differences in both gender and age distribution. Females presented with CD10− pro B-cell ALL twice as frequently as males (ratio 0.43) ($P = 0.004$) with a median age at presentation of 48 years for females and 39 years for males (NS). The translocation *t*(4;11)(q21;q23) was observed in four cases of the CD10− pro B-cell ALL subtype and all four cases were female. Males and females were nearly equal in representation (ratio 0.8) for the CD10+ common B-cell ALL subset (Fig. 1), but there was a significant difference in age at presentation between males and females in this group ($P = 0.008$). The median age for females presenting with CD10+ common B-cell ALL was 55 years, whereas the median age for males presenting with this disease subtype was 34 years. No additional distinguishing clinical or biological features were observed between these two subsets of precursor B-cell ALL.

Treatment responses were assessed by OS with information available on 106 precursor B-cell ALL patients (38 patients PH/BCR/ABL+ and 65 patients PH/BCR/ABL−). The median survival time for cases of PH/BCR/ABL+ and PH/BCR/ABL− precursor B-cell ALL were 10 and 14 months, respectively ($P = 0.036$; Fig. 2). Predictably, PH/BCR/ABL+ precursor B-cell ALL with the p210 had significantly shorter OS than PH/BCR/ABL+ precursor B-cell ALL with p190 isoform (median survival 6 vs. 10 months, respectively; $P < 0.05$; data not shown). The

Table 2 Clinical and biological characteristics of PH/BCR/ABL− CD10+ and CD10− precursor B-cell ALL

Features	CD10+	CD10−	*P*
No. of patients	65	20	
Age (years)[a]	44 (19–81)	43 (21–79)	NS
Sex (Male/Female)	29/36 (0.8)	6/14 (0.43)	0.009
Median age (male/female)	34/55*	39/48	0.008*
WBC count (10^9/l)	16.2 (0.3–348)	35.6 (0.07–130)	NS
Platelets (10^9/l)	76.2 (0–242)	89.5 (0–324)	NS
Hemoglobin (g/l)	8.2 (3–15.7)	11.8 (5.8–87)	NS
Serum LDH (IU/l)	953 (15–18683)	1185 (147–3,568)	NS
Blood blast (%)	32.5 (0–95)	38.5 (1–90)	NS
Bone marrow blast (%)	63.8 (12.5–96)	69.3 (38–93)	NS
Immunophenotype[b]			
CD34+	42/65 (65%)	12/20 (60%)	NS
Myeloid markers	29/65 (45%)	9/20 (45%)	NS
Cytogenetic abnormalities[b]			
Complex karyotype	13/26 (50%)	3/12 (25%)	NS
Multiple clones	7/26 (23%)	0/12 (0%)	0.0467
t(4;11)(q21;q23)	0/26 (0%)	4/12 (20%)	0.002

Myeloid markers: expression of CD13 and/or CD33

NS not significant

*Significant for females

[a] Median (range), [b] percent on available data

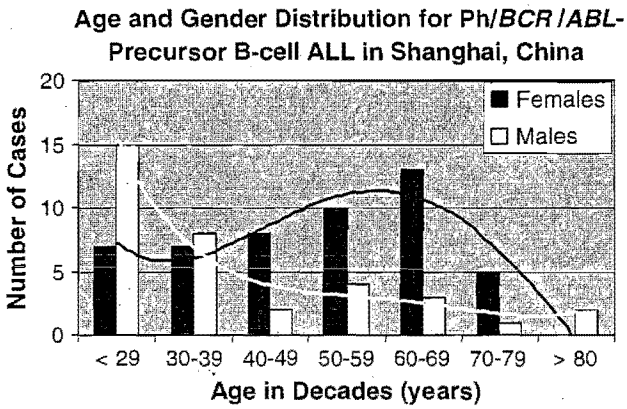

Fig. 1 Age and gender distribution for PH/BCR/ABL− precursor B-cell ALL

median survival for females over the age of 40 years with CD10+ common B-cell ALL was 22 months compared to males under the age of 40 years was 11 months, although this was not statistically significant. In spite of the fact that the total number of PH/BCR/ABL− CD10− pro B-cell ALL cases was relatively small, the median survival for males was well below that of females (4 vs. 18 months, respectively; $P = 0.0003$; Fig. 3).

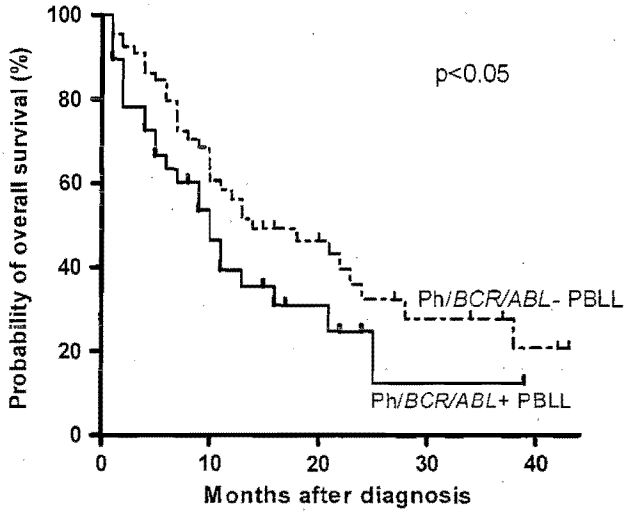

Fig. 2 Probabilities of overall survival in PH/BCR/ABL+ precursor B-cell ALL (*solid line*) and PH/BCR/ABL− precursor B-cell ALL (*dotted line*)

4 Discussion

When viewed in the context of previous epidemiology studies in China, the prevalence of adult precursor B-cell ALL in Shanghai was unexpected. Although this may in part be due to the WHO 2001 criteria used in diagnosis, the pattern of case referral is also likely an important factor. Because of disciplinary compartmentalization in China, study results based on diagnoses obtained either primarily or exclusively from histopathologic analysis would be expected to demonstrate a significant selection bias relative to those based on hematologic diagnosis. In contrast, our laboratory performed integrated hematopathology diagnoses, and the 28 participating referral hospitals represented a large cross section of the Shanghai medical community.

Our findings also revealed significant differences in the features and presentation of precursor B-cell ALL in Shanghai relative to Western studies. Given the common wisdom that there is a male predominance for ALL worldwide, the slight female predominance in Shanghai is surprising. This can be attributed, in part, to a marked increase in prevalence for CD10− pro B-cell ALL in females. Equally unexpected was the decreased probability of survival in males with this subtype. A significant increase in the median age of CD10+ common B-ALL among women was also observed. However, this does not appear to correlate with a significant difference in survival at least in patients receiving conventional chemotherapy. Taken together, these results suggest significant geographic heterogeneity in precursor B-cell ALL which may be of both etiologic and therapeutic significance.

The majority of studies of PH/BCR/ABL in precursor B-cell ALL have been conducted on Western patients with

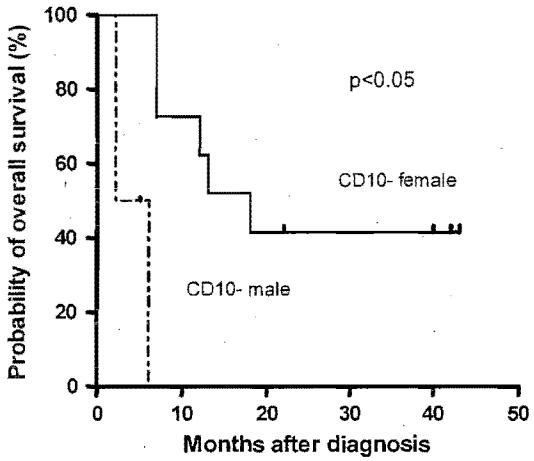

Fig. 3 Probability of overall survival in males (*dotted line*) and females (*solid line*) with CD10− Pro B-cell ALL

only a few reports of Asian ALL patients appearing in the literature [12, 27, 28]. In this series PH/BCR/ABL expression was detected in approximately 37% of our patients which is higher than previously reported in Asian ALL patients [12, 27, 28]. Patient age distributions and geographic heterogeneity might contribute to some of these differences since the frequency of PH/BCR/ABL+ precursor B-cell ALL increases demonstrably in elderly patients [29].

Several clinical characteristics observed among PH/BCR/ABL+ precursor B-cell ALL patients in this series, such as lower platelet counts, higher blood and marrow blasts, higher hemoglobin level, and a male predominance are similar to those reported elsewhere [11, 27, 30]. High CD34 expression (98%) present in PH/BCR/ABL+ precursor B-cell ALL also has been observed in previous studies [27, 30], implying that the PH/BCR/ABL+ cells may be derived from early progenitor cells. The proportion (18%) of the precursor B-cell ALL cases in our series that were positive for both myeloid antigens (CD13 and CD33) is also comparable with others [16]. However, we observed a significant increase in the percentage of PH/BCR/ABL+ precursor B-cell ALL expressing either myeloid antigen than in PH/BCR/ABL− precursor B-cell ALL (79.6 vs. 44.7%).

In this series patients were treated using predominantly conventional VDLP (vincristine, daunomycin, L-asparaginase, prednisone) or VDCP (VDLP + cytoxan) regimens. Consequently, the median OS for PH/BCR/ABL+ and PH/BCR/ABL− precursor B-cell ALL (10 vs. 14 months, respectively) were similar to those reported prior to the introduction of the use of imatinib in combination with stem cell transplantation [11, 16] and unfavorable relative to those reported in recent studies in the West [18]. The development of effective therapeutic paradigms for precursor B-cell ALL in China remains a challenge. Imatinib mesylate has not been commonly included in the treatment of precursor B-cell ALL in China, while haploidentical HLA-mismatched stem cell transplant is only available to a limited number of adult ALL patients in China.

5 Conclusions

The aim of this study is to characterize cases of adult precursor B-cell ALL presented to our laboratory over a 4-year period. We diagnosed and characterized 137 consecutive cases using the WHO 2001 classification system and compared them with respect to clinical, phenotypic and cytogenetic characteristics with outcome. Although adult precursor B-cell ALL in China has rarely been reported, we found it to be the second most common lymphoid neoplasm diagnosed in our laboratory. The therapeutic significance of PH/BCR/ABL+ precursor B-cell ALL is well established in the West. However, no previous studies of the prevalence of PH/BCR/ABL+ precursor B-cell ALL have been reported in the Chinese literature. In marked contrast to Western studies, females outnumbered males primarily due to an increased prevalence of a subset of precursor B-cell ALL that express a CD10− phenotype. Cases of PH/BCR/ABL− precursor B-cell ALL exhibited a pronounced age-dependent gender prevalence with a modal age in the sixth decade for females compared to the second decade for males. These findings provide valuable insight into the previously unrecognized but significant prevalence of adult precursor B-cell ALL in China. Moreover, in the absence of cytogenetic characterization for PH/BCR/ABL+ precursor B-cell ALL in China, tyrosine-kinase-based therapeutic modalities have not been implemented. This report provides the first description of the prevalence of adult precursor B-cell ALL in a Chinese population and highlights therapeutic challenges to the management of this disease that are unique to China.

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BODY:

ABSTRACT

Epstein-Barr virus (EBV) is implicated in the development of a number of human malignancies including several subtypes of non-Hodgkin lymphoma (NHL) [G. Pallesen, S.J. Hamilton-Dutoit, X. Zhou, The association of Epstein-Barr virus (EBV) with T cell lymphoproliferations and Hodgkin's disease: two new developments in the EBV Field, Adv. Cancer Res. 62 (1993) 179-239]. Lymphoproliferative disease and NHL occurring in severely immunosuppressed individuals almost always involve EBV and have been extensively studied and modeled in vitro. EBV has also been causally associated with some cases of NHL occurring in otherwise immunocompetent individuals. However, a direct role for EBV in the pathogenesis of neoplasms developing in the presence of an otherwise competent immune system has not been established. We investigated potential interactions between dithiocarbamates (DTC), an

important class of thiono-sulfur compounds, and EBV leading to immortalization of human B lymphocytes and evasion of cell-mediated immune response in culture. Primary lymphocyte cultures employing wild-type and recombinant EBV mutants were used to assess the respective roles of DTC and viral genes in lymphocyte transformation and survival. Pretreatment of EBV-infected human B lymphocytes with DTC directly enhanced transformation in the absence of T cells (5nM) and independently increased survival of transformed cells in the presence of competent autologous T cells (10nM). Both DTC-induced transformation and immortalization of EBV-infected B lymphocytes were dependent on the expression of viral IL-10. These results provide a biological basis for studying collaborations between chemical and virus that alter lymphocyte biology, and provide a rationale for further molecular epidemiology studies to better understand the potential influence of these interactions on the development of NHL and perhaps other viral-associated malignancies.

FULL TEXT

1 Introduction

Epstein-Barr virus is a [gamma]-herpes virus that infects and transforms B lymphocytes both in culture and in vivo, maintaining a lifelong latent infection in humans that is controlled by cytotoxic T lymphocytes. EBV is virtually always involved in the pathogenesis of lymphoproliferative disease (LPD) and immunoblastic B cell lymphomas associated with severe immunosuppression, for which EBV-induced transformation of B lymphocytes in culture in the absence of competent T cells is recognized to be the in vitro counterpart [2,3]. Early events in the infection and transformation of B lymphocytes by EBV are not completely understood, although EBV-induced transformation is thought to involve activation of signaling pathways in normal B lymphocyte proliferation including the up-regulation of Bcl-2. [4-8]. EBV is also implicated in the development of a subset of sporadic malignancies occurring in otherwise immunocompetent individuals, including: nasopharyngeal carcinoma, leiomyosarcoma, gastric carcinoma, Hodgkin disease (HD), and several subtypes of NHL including those of B, T and NK cell origin [1,9]. However, the precise role of EBV in the development and evolution of these neoplasms is unknown and there are no satisfactory paradigms to explain the direct role for EBV in the pathogenesis of neoplasms developing in the presence of an otherwise competent immune system.

The EBV genome encodes for 94 proteins, only about 10 of which are expressed in immortalized B cells, and with the possible exception of a single nuclear protein, EBV nuclear antigen-2 (EBNA2), there is no general agreement that expression of any particular gene is essential for B lymphocyte transformation [10]. Newly infected B lymphocytes express a set of viral genes under the control of EBNA2 [11-13]. However, in latent infected B lymphocytes, EBV gene expression is under the control of EBNA1 and may be restricted to only a single latent membrane protein, LMP2 [14]. The EBV BamHI C fragment open reading frame (BCRF1) encodes for a viral homologue of IL-10 (vIL-10) that is reported by most but not all groups to be differentially expressed late in the EBV lytic cycle and occasionally in EBV-transformed or immortalized B lymphocytes [15-18]. The BCRF1 protein can be detected 9-15h post infection, rapidly decreases thereafter [19] but is not usually expressed in latent EBV-infected B cells or most EBV-transformed tumor cell lines [20]. Human IL-10 (hIL-10) is a pleiotropic cytokine that is effective in the suppression of primary immune response and the development of immunological tolerance. hIL-10 directly stimulates proliferation of EBV-infected B cells [21,22], prevents spontaneous death of germinal center B cells [23], induces peripheral anergy in T lymphocytes, increases expression of MHC Class II and co-stimulatory molecules on B lymphocytes and antigen presenting cells and mediates evasion of local immune surveillance in EBV-associated neoplastic diseases [18,21,24,25]. With the exception of the N-terminal region of the peptide, vIL-10 shares considerable structural homology with its human counterpart [16]. However, vIL-10 is far less effective in stimulating MHC Class II expression on B lymphocytes and inhibiting IL-2 production by activated CD4+ cells [26,27]. Although antisense nucleotides for vIL-10 mRNA are reported to block B lymphocyte transformation by EBV [18], studies utilizing EBV recombinants indicate that the BCRF1 gene does not play an obligatory role in maintaining latent EBV infection or inducing lymphocyte transformation, nor does it appear necessary for the continued growth of transformed cells in vivo or in vitro [28].

Dithiocarbamates (DTC) are an important class of low molecular weight thiono-sulfur compounds with diverse

biological activity that have been used in agriculture and the clinic to control fungi and bacteria and as accelerating agents in rubber and polymer manufacturing. DTC are employed worldwide as fungicides in agriculture, for the control of mold in a variety of products, including paint, leather, paper and fabrics, and are trace constituents of residues found in fruits, vegetables and other food products [29]. Epidemiology studies have reported generally inconsistent results for an association between occupational exposure to dithiocarbamates or their breakdown products and lymphoid neoplasms [30-33]. However, to date, no studies have evaluated specific multifactorial associations, including influences such as microbial infection, drug or chemical exposure, on the development of NHL.

The biochemistry of DTC is remarkably complex. They exert a variety of striking molecular changes in cell systems, the significance of which is not fully understood. These include the ability to: (1) oxidize protein thiols and inhibit hydroxyradical formation, (2) inhibit nuclear factor-kappa B (NF-[kappa]B) activation via mechanism(s) that notably do not involve either oxidation-reduction dependent modification of the NF-[kappa]B protein or interference with NF-[kappa]B-DNA binding, (3) induce AP-1-dependent cell differentiation and gene expression via *de novo* transcription of *c-fos* and *c-jun*[34] and (4) result in profound changes in some cells via a mechanism that involves alterations in the intracellular transport of copper ions [35-40]. The sensitivity and concentration-response between individual cell types differ widely but are remarkably consistent among various substituted DTC for which the dimethyldithiocarbamate (DMDTC) is a prototype.

Herein, we tested the premise that exposure of a virus-infected cell to a biologically reactive molecule can alter cell survival and transformation under conditions that independently do not result in immunosuppression. We used in vitro cellular, molecular and recombinant approaches to analyze the effects of DTC on naïve and EBV-infected peripheral blood mononuclear cells (PBMC), isolated B lymphocytes and reconstituted mixed autologous B and T lymphocytes. We found that treatment of EBV-infected PBMC or purified B lymphocytes with DMDTC results in a marked increase in transformation and immortalization of lymphocyte clones apparently via independent processes that are both dependent on vIL-10. These experiments also demonstrate that EBV-transformed clones from cells initially treated with DMDTC continue to be capable of inducing unresponsiveness and inhibiting reactivation of autologous T lymphocytes in the absence of chemical.

2 Materials and methods

2.1 Production of EBV stock

The EBV producing cell line, B95-8 (American Tissue Culture Collection, Rockville, MD), was cultured in complete media consisting of RPMI 1640 containing 100IU/mL penicillin, 100[mu]g/mL streptomycin, 2mM l-glutamine and 10% (v/v) heat inactivated fetal bovine serum (FBS) (Gibco/BRL, Grand Island, NY). Cells were maintained at a density of 1×10^6cells/mL in a 37°C incubator with a humid atmosphere of 5% CO_2. For the production of viral stocks, cell were kept at a starting density of 5×10^5cells/mL and left for 14 days without the replacement of media. Supernatants from cultures were subsequently isolated and filtered through a 0.45[mu]m pore filter and 1mL aliquots were stored in liquid N_2.

2.2 Cell isolation and purification

Mononuclear cells used for the EBV infections were isolated from heparinized blood from healthy donors obtained with informed consent. Peripheral blood mononuclear cells (PBMC) were isolated by separation on a Ficoll gradient and washed twice with phosphate buffered saline containing 1% bovine serum albumin (PBS/BSA). Flow cytometric analysis of preparations of freshly isolated PBMC indicated that the B:T lymphocyte ratio ranged from 1:2.0 to 1:2.3. B and T lymphocytes were purified from the ficoll isolated mononuclear cells by incubating with an anti-CD19 (for B cells) or an anti-CD3 (for T cells) microbead conjugated antibody (Miltenyi Biotec, Auburn, CA) and passed through a magnetic separator column (Miltenyi Biotec). Percent purity of CD19 or CD3 positive cells was measured by flow cytometry (Epics 752, Coulter Electronics, Hialeah, FL) using anti-CD19 (HIB19, Pharmingen San Diego, CA) or anti-CD3 (UCHT1, Pharmingen) monoclonal antibody (mAb) and determined to be greater than 95%.

2.3 EBV infection and dithiocarbamate exposure

Mononuclear cells or B lymphocytes (1×10^7) were suspended in 1mL of complete RPMI 1640 media and 1mL of EBV stock was added. Cultures were incubated at 37°C for 2h in 5% CO_2 with occasional agitation. Cells were then pelleted and resuspended in complete media at a density of 1×10^6cells/mL and then treated with various concentrations of DMDTC, DEDTC or PBS (Sigma, St. Louis, MO). For IL-10 neutralization experiments, 1[mu]g/mL goat anti-human IL-10 antibody (AF-217-NA, R&D Systems, Minneapolis, MN) was added.

2.4 Transformation and immortalization of EBV-infected cells

EBV-infected and/or DTC-exposed cells were plated using complete media into 96 well plates in triplicate at 1×104cells/well or 1×10^3cells/well for B lymphocytes and mononuclear cells, respectively. Plates were incubated at 37°C, 5% CO_2 and half of the media changed weekly. Wells were counted for transformed clones by light microscopy after 4 weeks of culture. For the determination of immortalized clones, 12 positive wells were randomly selected, re-plated under limiting dilution conditions, subcultured for an additional 4 weeks and scored. Continual passage and subcloning of these selected clones beyond the additional 4 weeks were also performed to ensure that these LCLs were truly immortal.

2.5 ELISPOT assays for determination of IL-10 producing cells

EBV-infected and DTC-exposed cultures were plated at 1×10^4cells/well onto 96-well Multiscreen HA nitrocellulose plates (MAHAS4510, Millipore, Bedford, MA) previously coated overnight with sterile PBS containing JES3-9D7 mAb (1[mu]g/mL) to capture both hIL-10 and vIL-10 producing cells. After overnight incubation, plates were washed with sterile PBS at room temperature and blocked by incubation with complete RPMI for 2h at 37°C. Plates were then washed with PBS containing 0.05% Tween 20 to remove unbound cells. vIL-10 producing cells were detected with a biotinylated JES3-6B11 mAb, and total hIL-10+vIL-10 producing cells were detected using a biotinylated JES3-12G8 mAb after overnight incubation at 4°C (Pharmingen). Cells were visualized using a peroxidase-labeled streptavidin-HRP secondary reagent (Sigma). Cells secreting cytokine were enumerated following the color reaction using light microscopy by the appearance of spots. The IL-2 ELISPOT assay used the same procedure as above using an anti-IL-2 capture and detector antibody (Pharmingen).

2.6 T cell add-back assay

B lymphocytes were purified and infected with EBV as previously described. Donor matched CD3+ T cells were purified and added in increasing concentrations to the EBV-infected B lymphocyte culture wells. Mixed cultures were either assayed shortly after the addition of T cells by ELISPOT or propagated for an additional 3 weeks and then assessed for transformation and subsequent immortalization as previously described.

2.7 Recombinant vIL-10 EBV cultures

Lymphoblastoid cell lines containing recombinant wild type or vIL-10-deleted EBV were obtained from Swaminathan et al. [28] and maintained in complete RPMI (RPMI 1640 supplemented with 10% FBS, 100IU/mL penicillin, 100[mu]g/mL streptomycin and 2mM l-glutamine). Lytic virus infection was induced by the addition of 20ng/mL 12-tetradecanoyl phorbol acetate (TPA), cells were incubated for 3 days, washed, irradiated with 90Gy via a cyclotron and plated with purified B lymphocytes in 96-well plates at a ratio of 5×10^5 irradiated cells to 1×10^4 B cells (150[mu]L/well). Co-cultivations were carried out in either complete RPMI media or media containing various concentrations of DMDTC. Transformed colonies were scored after 4-6 weeks.

2.8 Reverse transcription-PCR (RT-PCR)

Total RNA from primary B lymphocytes was isolated after 12, 24, 48 and 72h post infection. RNA preparations were treated with RNase-free DNase (Roche Diagnostics, Indianapolis, IN) for 30min at 37°C. After inactivation of the

enzyme, 1[mu]g RNA was reverse transcribed (+RT; Superscript Plus; Gibco/BRL) with random hexamers for 60min at 37°C. Reverse transcription was omitted for control purposes where indicated (-RT). PCR with one tenth of the volume (2[mu]L) was performed in buffer containing 1.5mmol/L $MgCl_2$, 100pmol of each primer, 0.2mmol/L final concentration of each dNTP, and 0.5[mu]L Ampli-Taq-polymerase (Fisher Scientific, Pittsburgh, PA) at a final volume of 50[mu]L in a 9600 GeneAmp thermal cycler (PerkinElmer, Norwalk, CT). The following PCR primers (IDT, Coraville, IA) were used:

* BCRF1: 5'-TGGAGCGAAGGTTAGTGGTCAC-3' and 5'-ATGGTCTTTGGCTTCAGGGTCC-3'
* LMP1: 5'-GTGATTCTGACGAAGCCAGAG-3' and 5'-CGTGGGGCGCCCCAGGCACCA-3'
* EBNA2: 5'-AGAGGAGGTGGTAAGCGGTTC-3' and 5'-TGACGGGTTTCCAAGACTATCC-3'
* [beta]-actin: 5'-CG TGGGCCGCCCTAGGCACCA-3' and 5'-TTGGCC TTAGGGTTCAGGGGGG-3'.

Amplified bands were analyzed by electrophoresis through a 1.5% agarose gel and ethidium bromide stain.

2.9 Flow cytometric analysis

EBV-infected B cells (2×10^6) were isolated at 24, 48 and 72h post infection and subjected to flow cytometry analysis. Cells were washed in PBS/BSA and incubated with FITC conjugated antibodies for EBNA2 (clone PE2, Dako, Carpinteria, CA), BZLF1 (clone BZ1, Dako) and viral capsid antigen p120 (VCA) (clone 2G2, Biogenesis, Kingston, NH) and analyzed using an Epics 752 (Coulter Electronics). A total of 10,000 cells were counted per individual analysis. For FCM analysis of EBNA2, incubation of anti-EBNA2 antibody was carried out in the presence 0.1% Tween 20. For FCM analysis of accessory molecule expression, human B lymphocytes were isolated at daily intervals over 1 week of culture and mean fluorescence intensity measured using anti-B-7.1 (CD80) [clone BB1], anti-B-7.2 (CD86) [clone 2331(FUN-1)], and HLA-DR [clone G46-6(L243)] (Pharmingen).

3 Results

3.1 Increased proliferation and immortalization of EBV-infected B lymphocytes by DMDTC

Growth and transformation of B lymphocytes by EBV in vitro have long been used as a surrogate for the study of immunoblastic transformation in vivo [41-44]. Therefore, we measured the influence of DMDTC on transformation of EBV-infected lymphocytes in cultures of human PBMC and purified B lymphocytes. PBMC (containing both B and T lymphocytes) or purified B cells were infected with EBV, treated with DMDTC or phosphate buffered saline (PBS) and the outgrowth of colonies compared after 4 weeks. Pretreatment with DMDTC resulted in a significant dose-dependent increase in the transformation of B lymphoblasts in PBMC containing autologous T lymphocytes and in purified B lymphocytes at concentrations as low as 10 and 5nM, respectively (Fig. 1). To determine the effects of DMDTC on lymphocyte immortalization we assessed the cloning efficiency of these colonies by subculturing cells under conditions of limiting dilution for an additional 4 weeks and again scoring colony formation. Immortalization of EBV-transformed lymphocytes was enhanced 16 fold in PBMC and over 40 fold in purified B lymphocytes. These results demonstrated that DMDTC induced a marked increase in immunoblastic transformation in EBV-infected B cells and suggested that while treatment of EBV-infected cells with DMDTC increased lymphocyte proliferation it is arguably more potent in inducing immortalization.

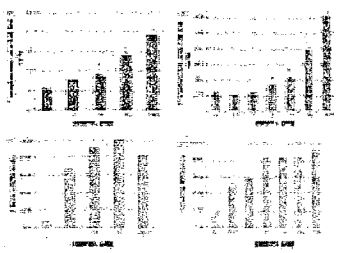

Fig. 1. Transformation and immortalization of EBV-infected B lymphocytes treated with DMDTC. Human Ficoll-purified PBMC (A) or isolated B lymphocytes (B) were infected with B95-8 EBV supernatants, treated with DMDTC or buffer, cultured for 4 weeks and colonies enumerated. Cells from resulting colonies were then subcultured under limiting dilution conditions for another 4 weeks and cloning efficiency expressed as the percent of immortalized colonies (%) for PBMC (C) or purified B lymphocytes (D). Results represent the average number of colonies per treatment group from triplicate cultures ±S.E.M. Asterisks indicate significant difference from buffer-treated cultures using Student *t*-test (*p*'.Amplified bands were analyzed by electrophoresis through a 1.5% agarose gel and ethidium bromide stain.2.9 Flow cytometric analysisEBV-infected B cells (2×106) were isolated at 24, 48 and 72h post infection and subjected to flow cytometry analysis. Cells were washed in PBS/BSA and incubated with FITC conjugated antibodies for EBNA2 (clone PE2, Dako, Carpinteria, CA), BZLF1 (clone BZ1, Dako) and viral capsid antigen p120 (VCA) (clone 2G2, Biogenesis, Kingston, NH) and analyzed using an Epics 752 (Coulter Electronics). A total of 10,000 cells were counted per individual analysis. For FCM analysis of EBNA2, incubation of anti-EBNA2 antibody was carried out in the presence 0.1% Tween 20. For FCM analysis of accessory molecule expression, human B lymphocytes were isolated at daily intervals over 1 week of culture and mean fluorescence intensity measured using anti-B-7.1 (CD80) [clone BB1], anti-B-7.2 (CD86) [clone 2331(FUN-1)], and HLA-DR [clone G46-6(L243)] (Pharmingen).3 Results3.1 Increased proliferation and immortalization of EBV-infected B lymphocytes by DMDTCGrowth and transformation of B lymphocytes by EBV in vitro have long been used as a surrogate for the study of immunoblastic transformation in vivo [41-44]. Therefore, we measured the influence of DMDTC on transformation of EBV-infected lymphocytes in cultures of human PBMC and purified B lymphocytes. PBMC (containing both B and T lymphocytes) or purified B cells were infected with EBV, treated with DMDTC or phosphate buffered saline (PBS) and the outgrowth of colonies compared after 4 weeks. Pretreatment with DMDTC resulted in a significant dose-dependent increase in the transformation of B lymphoblasts in PBMC containing autologous T lymphocytes and in purified B lymphocytes at concentrations as low as 10 and 5nM, respectively (Fig. 1). To determine the effects of DMDTC on lymphocyte immortalization we assessed the cloning efficiency of these colonies by subculturing cells under conditions of limiting dilution for an additional 4 weeks and again scoring colony formation. Immortalization of EBV-transformed lymphocytes was enhanced 16 fold in PBMC and over 40 fold in purified B lymphocytes. These results demonstrated that DMDTC induced a marked increase in immunoblastic transformation in EBV-infected B cells and suggested that while treatment of EBV-infected cells with DMDTC increased lymphocyte proliferation it is arguably more potent in inducing immortalization.Fig. 1. Transformation and immortalization of EBV-infected B lymphocytes treated with DMDTC. Human Ficoll-purified PBMC (A) or isolated B lymphocytes (B) were infected with B95-8 EBV supernatants, treated with DMDTC or buffer, cultured for 4 weeks and colonies enumerated. Cells from resulting colonies were then subcultured under limiting dilution conditions for another 4 weeks and cloning efficiency expressed as the percent of immortalized colonies (%) for PBMC (C) or purified B lymphocytes (D). Results represent the average number of colonies per treatment group from triplicate cultures ±S.E.M. Asterisks indicate significant difference from buffer-treated cultures using Student t-test ($p<0.05$). Data is representative of 1 out of 6 independent experiments performed using PBMC, and 1 out of 8 independent experiments using isolated B lymphocytes. Cells for each experiment were obtained from different donors (n=14).

3.2 The kinetics of viral gene expression in primary EBV-infected B lymphocytes

To evaluate differences in early EBV gene expression that might correlate with DMDTC-induced lymphocyte transformation, we inoculated primary B lymphocytes with supernatants from EBV-producing B95-8 cells, treated the cultures 2h later with DMDTC or PBS alone and then measured viral gene- and protein-expression using flow cytometry and RT-PCR techniques (Fig. 2). Gene products associated with both lytic (BZLF1, vIL-10) and latent (EBNA2) EBV infection were present 12h post infection with a latent pattern of gene expression consistent with lymphocyte transformation (EBNA2, LMP-1) evident by 48h. Increases in viral capsid antigen (VCA) were not observed at any time point (Fig. 2: A1, B1), indicating that treatment-related differences in the number of viral-transformed cells were not due to an increased rate of virion production or lymphocyte infection. Biphasic expression of BCRF1 (24-72h) was consistently observed in untreated cells infected with EBV, and treatment with DMDTC always resulted in expression of vIL-10 at 48h post infection. However, DMDTC pretreatment did not result

in observable early changes in the kinetics of expression of other EBV genes typically associated with either transformation or lytic virion replication (Fig. 2: A2, B2). These findings suggest that pretreatment with DMDTC altered expression of vIL-10 in the absence of apparent changes in latent EBV gene expression or viral replication.

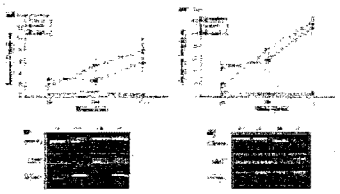

Fig. 2. Kinetics of gene expression in EBV-infected primary B lymphocytes. B cells were harvested at intervals as indicated (hours post-infection) and analyzed by flow cytometry or RT-PCR techniques. (A) Flow cytometric analysis of 2×10^6 EBV-infected B cells treated with PBS (A1) or 100nM DMDTC (A2) using antibodies against: EBNA2, BZLF-1 and VCA (viral capsid antigen p120). Values represent Mean+S.E.M. (n=4). (B) RT-PCR analysis of RNA extracts from 1×10^6 EBV-infected B cells treated with PBS (B1) or 100nM DMDTC (B2). Representative agarose gel (n=6) of PCR products for EBNA2, LMP1 and BCRF1 is shown. Primers for the specific transcripts and PCR conditions are indicated in Section 2.

3.3 The role of vIL-10 in DMDTC-induced transformation of EBV-infected B lymphocytes

We performed a series of experiments to evaluate the role of vIL-10 in DMDTC-induced transformation of EBV-infected cells. First, we determined the effects of DMDTC on vIL-10 producing cells using an ELISPOT assay (Fig. 3A). Treatment of EBV-infected B cells with DMDTC resulted in a significant increase in vIL-10 producing cells that was not observed following DMDTC treatment of uninfected B lymphocytes. We also ascertained that addition of neutralizing anti-IL-10 antibody functionally abrogated DMDTC-enhanced growth transformation (Fig. 3B). We then repeated the transformation experiments described in Fig. 1 using recombinant virus techniques. Freshly isolated purified B lymphocytes were infected with strains of EBV containing either a 3.3kb deletion between bp 9535 and 12870 that spans the entire BCRF1 open reading frame or a recombinant wild-type (WT) virus [28] (Fig. 3C). Cultures treated with and without DMDTC (1-100nM) were maintained for 4-6 weeks and scored for transformation. No differences in the rate of B lymphocyte transformation were observed in untreated cultures infected with either recombinant EBV strain. In contrast, a marked increase in B cell transformation was observed in DMDTC treated cultures infected with WT EBV but not the BCRF1-deletion mutant. These results demonstrated that the vIL-10 gene was essential for DMDTC-enhanced transformation of EBV-infected B lymphocytes.

Fig. 3. The role of vIL-10 in DMDTC enhanced B cell transformation. (A) The number of vIL-10 secreting cells was determined at 24, 48 and 72h in EBV-infected or uninfected B lymphocytes treated with PBS or 100nM DMDTC. Asterisks indicate significant difference between DMDTC-treated and untreated EBV-infected cultures using Student t-test (p-6 weeks and scored for transformation. No differences in the rate of B lymphocyte transformation were observed in untreated cultures infected with either recombinant EBV strain. In contrast, a marked increase in B cell transformation was observed in DMDTC treated cultures infected with WT EBV but not the BCRF1-deletion mutant. These results demonstrated that the vIL-10 gene was essential for DMDTC-enhanced transformation of EBV-infected B lymphocytes.Fig. 3. The role of vIL-10 in DMDTC enhanced B cell transformation. (A) The number of vIL-10

secreting cells was determined at 24, 48 and 72h in EBV-infected or uninfected B lymphocytes treated with PBS or 100nM DMDTC. Asterisks indicate significant difference between DMDTC-treated and untreated EBV-infected cultures using Student t-test ($p<0.05$). (B) Transformation of EBV-infected and/or DMDTC-pretreated B lymphocytes was evaluated in the presence and absence of anti-vIL10 antibodies. Colonies were scored after 4 weeks in culture. Asterisks indicate significant difference with and without addition of neutralizing anti-IL-10 antibodies using Student *t*-test ($p<0.05$). (C) Effect of DMDTC treatment on purified B lymphocytes co-cultivated with irradiated lymphoblastoid cell lines containing BCRF1-deleted or WT EBV recombinant strains. Colonies were scored after 6 weeks in culture. Error bars represent ±S.E.M. ($n=3$). Asterisks indicate significant difference from buffer-treated cultures using Student *t*-test ($p<0.05$).

3.4 DMDTC pretreatment of EBV-infected B cells enhanced their ability to inactivate autologous T cells

Our experiments indicated that DMDTC treatment directly enhanced transformation of EBV-infected B cells via a mechanism that was mediated via vIL-10. Treatment-related increases in B lymphocyte immortalization occur in the presence of T cells at concentrations of DMDTC that are as much as 500 times lower than those that directly inactivate human T lymphocytes [37]. However, vIL-10 alone is also known to abrogate the inhibitory capacity of T cells targeting EBV-induced B cell transformation [21]. Therefore we performed experiments to determine whether DMDTC pretreatment of B lymphocytes indirectly resulted in the inactivation of autologous T cells. EBV-infected purified human B lymphocytes were cultured under limiting dilution conditions with DMDTC for 7 days, washed, and then reconstituted with increasing numbers of autologous T lymphocytes previously unexposed to chemical. The addition of autologous T cells at any titer>1:1 effectively prevented the outgrowth of EBV-infected B cells in untreated cultures (Fig. 4). In contrast, pretreatment of B cells with DMDTC resulted in proliferation and outgrowth of B cell clones even in reconstituted cultures containing high ratios of T to B lymphocytes. Consistent with previous reports that different substituted DTC share a common chemistry and biological effects, we repeated these experiments using diethyldithiocarbamate (DEDTC) and obtained identical results (data not shown).

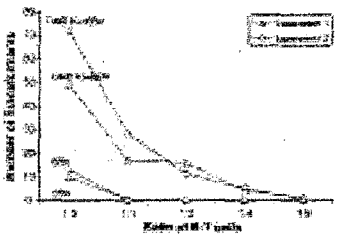

Fig. 4. Influence of DMDTC pretreatment on EBV-infected B lymphocyte immortalization in the presence of T cells. EBV-infected purified B cells were cultured for 1 week at a density of 500 cells per well in the presence of PBS or 100nM DMDTC. Cells were washed, varying numbers of autologous purified T cells added, cultured for an additional 4-6 weeks and scored for colonies. Symbols indicate the number of transformants per well from duplicate experiments. The ratio of B:T cells: 500:0 (1), 500:500 (1:1), 500:1000 (1:2), 500:2000 (1:4), 500:4000 (1:8).

A number of studies provide evidence that peripheral T cell tolerance or anergy can be overcome by addition of exogenous IL-2 which temporarily restores CD4 cell responsiveness [21,45]. We investigated the influence of exogenous IL-2 on the dynamics of CD4 cell activation and EBV-infected B cell transformation in DMDTC-treated and control PBMC. PBMC were infected with EBV, treated with 100nM DMDTC or PBS, cultured for 14 days and then supplemented with rhIL-2 or PBS. We confirmed in initial experiments that purified untreated T and B lymphocytes do not secrete IL-10 or IL-2 under the culture conditions used in these experiments (data not shown). Therefore, we used the number of IL-2-producing and vIL-10-producing cells, as determined by ELISPOT assays, as indicators of the numbers of activated CD4 cells and EBV-infected B cells, respectively. DMDTC pretreatment of EBV-infected cells resulted in a marked increase in vIL-10-producing cells relative to untreated cultures of EBV-infected cells (Fig. 5A). Addition of exogenous IL-2 on day 14 completely ablated vIL-10-producing cells in untreated cultures and also resulted in a delay and attenuation of vIL-10-producing cell proliferation in DMDTC-pretreated cultures. The addition of

exogenous IL-2 markedly increased the number of IL-2-producing cells in EBV-infected cultures. However, DMDTC-pretreatment did not affect the number or kinetics of these cells (Fig. 5B). Peripheral tolerance induced by vIL-10 has been shown to involve direct paralysis of B7-1-mediated co-stimulation and not down regulation of MHC or B7 expression [46]. Using flow cytometry, we determined that DMDTC pretreatment did not alter expression of HLA-DR, B-7.1 or B-7.2 in EBV-infected B cells over 7 days in culture prior to the addition of T cells (data not shown). These findings are consistent with the previously described mechanism for vIL-10 induction of local tolerance [25] and demonstrate that the continued presence of DMDTC is not necessary for EBV-transformed B lymphocyte evasion of cell mediated immune response.

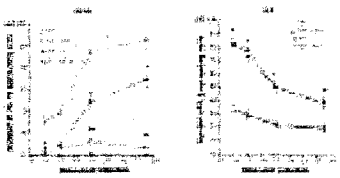

Fig. 5. Exogenous IL-2 partially restores T lymphocyte response against DMDTC-treated/EBV-infected B lymphocytes. Ficoll-purified PBMC were infected with EBV, treated with 100nM DMDTC, cultured for 14 days and then supplemented with PBS or 200 U rhIL-2. (A) vIL-10-producing B cells. Asterisks indicate significant difference compared with DMDTC pretreated cells without IL-2 supplementation using Student *t* test ($p<0.01$). Circle indicates significant difference compared with EBV-infected cultures without DMDTC pretreatment or IL-2 supplementation using Student *t* test ($p<0.01$); and (B) IL-2-producing T cells were enumerated by ELISPOT at 15, 17, 21 and 28 days post-infection. Data are expressed as the mean ±S.E.M. for secreting cells ($n=4$). Asterisks indicate significant difference compared with identical cultures without IL-2 supplementation using Student *t* test ($p<0.01$).

4 Discussion

Despite intensive investigation, a unified understanding of the biology of lymphocyte transformation by EBV remains elusive, and controversies persist with respect to the contribution of individual viral genes in mediating B lymphocyte transformation or immune evasion. vIL-10 has been shown to enhance B cell survival both by directly stimulating growth and differentiation as well as facilitating immune evasion by EBV-infected transformed B cells in vivo. Nevertheless, some previous studies have suggested that vIL-10 is not an absolute requirement for lymphocyte immortalization or survival [20]. Our experiments demonstrate that treatment of EBV-infected cells with DTC results in a marked increase in both B lymphocyte transformation and immortalization. Consistent with the previously demonstrated dual roles for IL-10 in enhancing B lymphocyte proliferation and promoting T cell anergy, our experiments indicate that vIL-10 plays an obligatory role in mediating DTC-induced increases in transformation as well as attenuation of T cell inhibition of EBV-infected B lymphocytes. These findings suggest that transient exposure of B lymphocytes to DTC early in the cycle of EBV infection can permanently alter the regulation of vIL-10 expression, suggesting that B lymphocyte transformation and survival may be subject to modulation by environmental or therapeutic agents.

However, the molecular basis for these effects remains unclear. Several laboratories including ours have reported that DTC directly suppresses cell-mediated immune response and T lymphocyte activation [33,35,37,47]. DTC are historically considered not to be mutagenic, although Soloneski et al. recently reported increases in chromosome aberrations in cells treated with relatively high concentrations of ethylene bis(dithiocarbamate) [48,49]. These effects occur at concentrations of DTC that are 200-500 times higher than those demonstrated to enhance transformation or immortalization of EBV-infected B lymphocytes in these experiments. Independently, we have not observed increases in structural chromosome aberrations in banded chromosome analysis of immortalized human B lymphocyte clones produced in our studies (data not shown).

The mechanism(s) whereby DTC specifically alter the regulation and expression of vIL-10 remain obscure, and very little is specifically known about the regulation of vIL-10 in infected B cells. DTC are generally regarded as

prototype inhibitors of transcriptional activation of NF-[kappa]B [50], and are implicated in both pro-oxidant radical scavenging and copper-dependent intracellular oxidation of protein thiols [35,51]. Transcription of hIL-10 is controlled by the constitutively expressed transcription factors, Sp1 and Sp3 [52], the induction of which have been demonstrated to be sensitive to oxidation of critical protein thiols [53,54]. Alternatively, the half-life of hIL-10 mRNA appears to be predominantly determined by post-transcriptional signals [55]. Homeostatic regulation of the subcellular distribution of copper is highly orchestrated [56], and alterations in intracellular copper concentration have been demonstrated to alter post-translational gene expression via multiple mechanisms [57,58]. Taken together it is attractive to hypothesize that DTC influence cell-specific gene expression of vIL-10 via copper-dependent direct oxidation of protein thiols.

IL-10 has been implicated in the development of immune tolerance for a variety of human tumors and LPD that are presumably mediated via cell-specific suppression of cell-mediated immune function, i.e. defective cytotoxic T-cell response, induction of antigen-specific anergy and T lymphocyte unresponsiveness [45,59-63]. Exploitation of cellular or viral IL-10 homologues by other intracellular pathogens appears to be a common mechanism of immune evasion [64], and a number of vIL-10 homologues have been identified including those encoded by EBV, equine herpes virus-type 2, and cytomegalovirus [64].

Over the past 20 years the incidence of sporadic B cell NHL steadily increased with almost a doubling of the incidence between 1980 and 2000 by about 3-4% per year worldwide [65]. In the past few years the incidence of NHL has apparently plateaued [31]. An etiological role for EBV in the pathogenesis of a subset of human cancers in non-immunosuppressed individuals, including cases of sporadic NHL and HD, has been repeatedly suggested [66-68], and it has been widely speculated that dietary, iatrogenic or environmental factors may influence viral carcinogenesis [9]. However, no direct biologically plausible mechanism has emerged to explain potential viral-chemical interactions in otherwise immunocompetent individuals in which EBV-infected B cells are normally held in check by T lymphocyte immunosurveillance [59]. The results of our studies reveal that exposure of human B lymphocytes to DTC at very low concentrations produces enhanced production of vIL-10 that is capable of inducing EBV-mediated B lymphocyte proliferation and immortalization in the presence of otherwise competent T cells. NHL is widely appreciated to be a complex and multifactorial disease. Numerous epidemiology studies have variously implicated a wide range of possible etiological associations between infectious agents, occupation, dietary factors, the environment and previous underlying diseases. However, consistent statistically significant results have been observed for only a relatively small subset of these [31]. Our results suggest the potential for collaboration between chemical and at least one Herpes virus, EBV, in the immortalization and immune evasion of human cells. These results provide a point of departure for further molecular epidemiology studies to better understand the biological basis of chemical-biological interactions and their potential influence on the development of NHL and perhaps other viral-associated malignancies.

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The World Health Organization (WHO) classification of tumors of the hematopoietic and lymphoid tissues. J Vardiman, University of Chicago, Chicago, IL, USA

The WHO classification of myeloid and lymphoid neoplasms utilizes morphology, immunophenotype, genetics and clinical features to define disease entities of clinical significance. It is a "consensus" classification for which a number of experts have agreed on the classification and the diagnostic criteria. In general, the classification stratifies neoplasms according to their lineage (myeloid, lymphoid, histiocytic/dendritic cell) and distinguishes neoplasms of precursor cells from those comprised of functionally mature cells. Further subclassification is based on biologic, phenotypic, genetic, and clinical features. Five major subgroups of myeloid tumors are recognized: myeloproliferative neoplasms (MPN, comprised mainly of mature cells with effective proliferation), myeloid (and lymphoid) neoplasms with eosinophilia and abnormalities of *PDGFRA*, *PDGFRB* and *FGFR1* (defined largely by the genetic abnormalities and significant eosinophilia), myelodysplastic/ myeloproliferative neoplasms (MDS/MPN, comprised mainly of mature cells with both effective and ineffective proliferative components), myelodysplastic syndromes (MDS, immature and mature cells with ineffective proliferation), and acute myeloid leukemia (AML, comprised of blasts with impaired maturation). Genetic abnormalities play an important role as diagnostic criteria for further subclassification of some myeloid subgroups, particularly AML, in which a number of clinicopathologic entities are recognized that are associated with specific genetic rearrangements or mutations. Although therapy-related AML and MDS often have genetic defects identical to those found in de novo AML and MDS, therapy-related myeloid neoplasms are classified separately within the AML category to emphasize their unique clinical and biologic properties. Lymphoid neoplasms appear to recapitulate stages of normal B-, T-, and NK-cell differentiation and to some extent are classified according to the corresponding normal stage. Thus, neoplasms of B cells can be divided into those of precursor, pre-germinal center, germinal center or post-germinal center B-cells. Because NK cells are closely related to T cells, neoplasms of these two cell types are often considered together, and broadly categorized as precursor T cell neoplasms or as peripheral T/NK cell neoplasms. Some lymphoid tumors, however, are recognized by unique clinical or biologic features, such as lymphomas associated with immunodeficiency states associated with immunosuppressive therapy or with aging.

Myelodysplastic syndrome (MDS): A case-case analysis of benzene exposure and clinical features using the WHO(2001)/(2008) criteria for diagnosis. RD Irons [a], SA Gross, XQ Wang, A Le, C Yan, AR Schnatter, H Fu. [a] Fudan-Cinpathogen Clinical and Molecular Research Center, Institutes of Biomedical Sciences, Fudan University, Shanghai, China

We diagnosed and characterized the prevalence of hematopoietic and lymphoid disease for 2923 consecutive patients presenting at 29 hospitals from Aug 2003 to Jun 2007. Diagnoses were made by a single laboratory using WHO criteria based on morphologic, immunophenotypic, cytogenetic, FISH and molecular data. A total of 611 subjects (322 males/289 females) were prospectively diagnosed with MDS using WHO (2001) criteria. Update and re-evaluation of cases using MDS (2008) criteria resulted in 649 MDS cases. Refractory cytopenia with multilineage dysplasia (RCMD) accounted for 68% of total cases, refractory anemia with excess blasts (RAEB), 16.3%, refractory anemia (RA), 6.5% and MDS-unclassifiable (MDS-U), 4.5%, based on 2008 criteria. Subjects were administered questionnaires and information on previous disease, work histories and exposures to potential etiologic agents such as benzene (BZ) was obtained. A total of 80/649 (13.2%) were determined to have some BZ exposure. The frequency of clonal cytogenetic abnormalities in the total MDS series was 30%, the most common being +8 >del(20)q > -7 >-5 , while the analogous frequency in BZ-exposed cases was only 22.5%. To further investigate the clinical features of MDS that may be associated with BZ exposure, we identified a subset of cases with high BZ exposure. These signal cases were matched by age and gender to cases with no known BZ exposure. When contrasting high versus no BZ exposure cases, we found a high odds ratio (OR) for WHO subtypes MDSU (OR = 6), followed by RAEB (OR = 2) and RCMD (OR = 0.5). Bone marrow morphology consistent with multilineage dysplasia with abnormal eosinophils was strongly associated with BZ exposure (OR = 42). Surprisingly, the relative risk of clonal cytogenetic abnormalities was reduced for high BZ exposed cases (OR = 0.56), reflecting the pattern in the total case series. (This work was supported by the Benzene Health Research Consortium).

A hospital-based case-control study of acute myeloid leukemia in Shanghai: Analysis of environmental and occupational risk factors by WHO subtypes. O Wong [a], F Harris, H Fu, et al. [a] Applied Health Sciences, San Mateo, California, USA

The objectives of the study were: (1) to investigate and identify potential environmental and occupational risk factors of acute myeloid leukemia (AML), and (2) to explore the relationships between risk factors and AML subtypes according to the World Health Organization (WHO) classification of myeloid neoplasms. The investigation was a hospital-based case-control study consisting of 722 confirmed AML cases and 1444 individually gender-age-matched patient controls at 29 hospitals in Shanghai. A 17-page questionnaire was used to obtain information on demographics, medical history, family history, lifestyle risk factors, employment history, residential history, and occupational and non-occupational exposures. Certain occupations of interest triggered a second questionnaire, which was occupation-specific and asked for more details about jobs, tasks, materials used and work environment. Exposure assessments were based on the questionnaires, on-site workplace investigations, data published in the Chinese literature, historical exposure measurements maintained by government health agencies, and expert opinions of a panel of local scientists who were familiar with workplaces in Shanghai. Risk estimates (odds ratios and 95% confidence intervals) of individual risk factors were calculated using conditional logistic regression models. A number of potential environmental and occupational risk factors were associated with an increased risk of AML (all subtypes combined) and/or individual subtypes; including home or workplace renovation, living on a farm, planting crops, raising livestock or animals, farm workers, metal workers, rubber and plastic workers, wood and furniture workers, printers, loading and unloading workers, automobile manufacturing, general construction, and food and drink industry (restaurants and other eateries). Exposures associated with an increased risk included benzene, metals, insecticides, herbicides, fertilizers, paints, glues and adhesives, and inks. Multivariate models were used to adjust for potential confounding exposures, and several potential risk factors were subsequently eliminated. The results of the investigation indicated that some risk factors applied to all or several subtypes (factors such as low-level education and living on a farm), while others were limited primarily to a single subtype (such as the association between raising livestock or animals and AML with multilineage dysplasia). Thus, some risk factors were subtype-specific. The difference in risk by subtype underscores the importance of the etiologic commonality and heterogeneity of AML.

A hospital-based case-control study of non-Hodgkin lymphoid neoplasms in Shanghai: Analysis of environmental and occupational risk factor by WHO subtypes O Wong [a], F Harris, H Fu, et al. [a] Applied Health Sciences, San Mateo, California, USA

The objectives of the study were: (1) to investigate and identify potential environmental and occupational risk factors of non-Hodgkin lymphoid neoplasms (NHLN), and (2) to explore the relationships between risk factors and NHLN subtypes according to the World Health Organization (WHO) classification of lymphoid neoplasms. The investigation was a hospital-based case-control study consisting of 649 confirmed NHLN cases and 1298 individually gender-age-matched patient controls at 25 hospitals in Shanghai. A 17-page questionnaire was used to obtain information on demographics, medical history, family history, lifestyle risk factors, employment history, residential history, and occupational and non-occupational exposures. Certain occupations of interest triggered a second questionnaire, which was occupation-specific and asked for more details about jobs, tasks, materials used and work environment. Exposure assessments were based on the questionnaires, on-site workplace investigations, data published in the Chinese literature, historical exposure measurements maintained by government health agencies, and expert opinions of a panel of local scientists who were familiar with workplaces in Shanghai. Risk estimates (odds ratios and 95% confidence intervals) of individual risk factors were calculated using conditional logistic regression models. A number of potential environmental and occupational risk factors were associated with an increased risk of NHLN (all subtypes combined) and/or individual subtypes; including home/workplace renovation, living on a farm, planting crops, raising livestock or animals, farm workers, fabric sewing and cutting workers, welders and sheet metal workers, masonry and plastering workers, product and chemical testing workers, toy manufacturing, agriculture industry, and beauty salon. Exposures associated with an increased risk included benzene, solvents, petroleum fuels, gasoline, metals, insecticides, herbicides, fertilizers, and glues and adhesives. Multivariate models were used to adjust for potential confounding exposures, and several potential risk factors were subsequently eliminated. The results of the investigation indicated that some risk factors applied to all or several subtypes (factors such as home/workplace renovation, and fabric sewing and cutting), while others were limited primarily to a single subtype (such as the association between solvents and precursor B-cell neoplasms). Thus, some risk factors were subtype-specific. The difference in risk by subtype underscores the importance of the etiologic commonality and heterogeneity of NHLN.

Influence of benzene exposure and other factors on metabolism and peripheral blood parameters in moderately exposed workers in Shanghai, China. AR Schnatter[a], P Kerzic, Y Zhou, M Chen, M Nicolich, K Lavelle, T Armstrong, M Bird, F Hua, R Irons. [a] ExxonMobil Biomedical Sciences, Inc., Annandale, NJ, USA

Benzene is an established leukemogen and bone marrow toxin yet the mechanism of these effects is still not known. The metabolism of benzene is thought to play a key role in these effects. Whether decrements in peripheral blood cells are also involved in marrow toxicity and leukemia is a matter of debate. Recent studies on benzene's metabolism have suggested that: (a) lifestyle habits such as smoking contribute to metabolite levels, (b) single nucleotide polymorphisms do not play a large role in levels of metabolites, (c) metabolism is more efficient at lower doses, (d) there may be important shifts in the proportion of metabolites over the range of exposures, and (e) toluene inhibits benzene metabolite formation. Recent findings on peripheral blood indices have variously suggested that lymphocytes, neutrophils or earlier, less differentiated cells may be more sensitive to benzene exposure. To test many of the findings which have resulted from recent studies, we studied 899 workers exposed to moderate levels of benzene in five factories near Shanghai China. Metabolite analyses focused on a subset of 208 workers in two factories for which five urinary benzene metabolites and same-day personal benzene exposure measurements were available. Benzene exposure was a strong predictor of metabolite levels. Segmented regression models suggest that benzene concentrations of 0.8 ppm are necessary to raise both hydroquinone and trans, trans muconic acid metabolites above background levels, while higher concentrations are necessary to affect background levels of phenol and catechol. Single nucleotide polymorphisms of key metabolizing and deactivating enzymes had little effect on metabolite levels. Despite previous data suggesting inhibition of benzene metabolism by toluene, in our data, toluene exposure showed a positive influence on metabolism, although the joint distribution of benzene and toluene exposure was compromised. When we examined metabolic efficiency across exposure, slightly increased metabolic efficiency was suggested for exposures below 1 ppm, although the magnitude of this effect was less than that observed in previous studies. In univariate analyses of peripheral blood counts, stronger effects for benzene exposure were noted for decreased erythrocyte and neutrophil counts and an increase in mean corpuscular volume. Further investigation of other blood parameters is ongoing.

Benzene and childhood leukemia. DW Pyatt[a,b], S Hays, L Alyward. [a] Summit Toxicology, LLP, [b] University of Colorado Health Sciences Center, Schools of Public Health and Pharmacy, USA

Chronic exposure to high concentrations of benzene is an established cause of AML in occupationally exposed workers. Based on this well documented association, it is not unreasonable to assume that children could also get AML if they were exposed to comparable levels of benzene. Fortunately, reports of such exposures and subsequent AML development in children are non-existent. However, the question of whether children can develop leukemia at far lower, environmental levels of benzene remains. The existing scientific evidence relevant to this question will be addressed. Leukemias are the most common malignancy in children under 15 years of age. The majority (~85%) of childhood leukemia cases are acute lymphoblastic leukemia (ALL), with the remainder being almost exclusively acute myeloid leukemia (AML). Chronic forms of leukemia, either myeloid or lymphoid are exceedingly rare in children. Acute infant leukemia occurs before the age of 1 and shares phenotypic features with both ALL and AML. At this point, the risk factors for childhood leukemia remain essentially unknown as established etiological factors such as genetic conditions, ionization radiation and certain chemotherapeutic agents can explain only a small portion of cases. As a result, efforts to identify other potential etiologies remain an active research area. From a biological, clinical and epidemiological point of view, childhood ALL and AML are distinctly different diseases. Nonetheless, many older epidemiology studies on childhood leukemia have combined both types into one etiological group which is strongly influenced by the predominant ALL cases. In contrast, newer studies have begun to segregate out these two main subtypes and are reporting that childhood AML and ALL do not typically share the same risk factors. Combining them into a single group complicates the interpretation of this literature and may even obscure potential etiological relationships. Most epidemiology studies specific to children have included one or more of the following exposure pathways for benzene: 1) direct exposure to the child (via a variety of sources); 2) maternal exposures during pregnancy or while breastfeeding; and 3) exposure to either parent before conception. These pathways are not mutually exclusive and frequently occur together. The timing of exposure (e.g. age of the child) has also been an important consideration. Unfortunately, very few studies contain quantified exposure information and many have no meaningful exposure estimates at all. The use of various surrogates for exposure such as proximity to gas stations or traffic density makes interpretation very difficult, particularly with regard to chemical exposures. Parental exposures to benzene or other chemicals in the workplace and its potential effect in the offspring have also been assessed (with varying degrees of scientific rigor) in multiple studies. While there are a few positive findings, the collective literature does not indicate that exposure to environmental levels of benzene is related to an increased risk of childhood leukemia. Our understanding would be strengthened by additional studies that accurately characterize exposures as well as differentiate between the various forms of leukemias observed in children.

Benzene-initiated oxidative stress: Effects on embryonic signaling pathways.
HJ Badham [a], SJ Renaud, J Wan, LM Winn. [a] Department of Pharmacology and Toxicology, Queen's University, Kingston, Ontario, Canada

Approximately 90% of childhood cancers are of unknown etiology; however, it is hypothesized that *in utero* carcinogen exposure may contribute. Epidemiological studies have correlated parental exposure to benzene with an increased incidence of childhood leukemias. However, mechanisms of benzene-induced carcinogenesis following *in utero* exposure remain unknown. We hypothesize that *in utero* exposure to benzene causes alterations in the redox sensitive signaling pathways involving c-Myb, Pim-1, ERK, p38, and NF-kB via the production of reactive oxygen species (ROS) as a possible mechanism of *in utero* initiated carcinogenesis. Using a CD-1 mouse model we have shown increased oxidative stress in fetal tissue from embryos exposed *in utero* to benzene by measuring reduced to oxidized glutathione ratios, and increased levels of ROS in male fetuses using flow cytometry and the ROS sensitive fluorescent probe dichlorofluoroscein diacetate (DCFDA). In addition, using western blotting techniques we observed increased expression of fetal Pim-1, Pim-1 phosphorylation, c-Myb, and phosphorylated p38 (activated form) and lower protein levels of IKB-alpha, while phosphorylated ERK protein levels did not change. Interestingly, we found male fetuses more susceptible to benzene induced oxidative stress and alterations in embryonic signaling pathways, which is in agreement with the literature suggesting that males are more susceptible to benzene toxicity. Further studies evaluating the reason for this gender difference are ongoing.

Metabolic factors in susceptibility to benzene toxicity. D Ross, H Zhou, Department of Pharmaceutical Sciences, School of Pharmacy, University of Colorado, Denver, CO, USA.

Susceptibility to the toxic effects of benzene has been suggested to occur via variations in genes involved in benzene metabolism including cytochrome P450 2E1, epoxide hydrolases myeloperoxidase, glutathione-S-transferases and quinone reductases. However, genes involved in DNA repair, genomic stability and expression of cytokines and/or cell adhesion molecules have also been implicated in benzene susceptibility and an important role has also been suggested for p53 in influencing the response to benzene in animals. Consequently, individual susceptibility to benzene exposure in humans is likely to be multifactorial. Our work has focused on the quinone reductase NQO1 and the influence of the homozygous NQO1*2 polymorphism, which results in a null NQO1 phenotype, on benzene toxicity. The null NQO1 phenotype has been reported as a susceptibility factor for occupational benzene poisoning and NQO1 plays an important metabolic role in 1,4-benzoquinone detoxification. However, NQO1 may also impact other pathways in addition to metabolism of quinones due to protein-protein interactions or other mechanisms related to NQO1 activity. NQO1 has been implicated in stabilization of p53 and in maintaining microtubule integrity. NQO1 in bone marrow is primarily expressed in stromal cells and endothelial cells in stroma express high levels of NQO1. Since endothelial cells represent one of the major niches which influence bone marrow stem cell function, we have utilized transformed human bone marrow endothelial cells (HBMEC) as a model system to investigate the effects of benzene metabolites. HBMEC function was adversely affected by treatment with hydroquinone resulting in inhibition of endothelial cell tube formation, an effect modulated partly by upregulation of chondromodulin 1. In addition, inhibition of NQO1 activity in HBMEC using suicide inhibitors of NQO1 resulted in deficiencies of E-selectin, ICAM-1 and VCAM-1 adhesion molecule expression after TNFα stimulation. These results demonstrate how the metabolic susceptibility factor NQO1 may influence other potential susceptibility pathways for benzene toxicity such as p53 and adhesion molecule expression (Supported by NIH grant ES09554).

How much does benzene contribute to the overall burden of cancer due to occupation?
L Rushton [a], L Fortunato, S Hutchings. [a] Imperial College London, UK

Work-related cancers are largely preventable. We are estimating the burden of occupationally-related cancer in Great Britain to identify agents, industries and occupations for targeting risk prevention. We estimate attributable fractions (AF) and numbers (AN) for cancer mortality and incidence for all cancers, agents and occupations classified as IARC group 1 and 2A carcinogens. Benzene is classified as a group 1 carcinogen i.e. there is strong human evidence. The AF requires knowledge of the risk of the exposure and the proportion exposed. Relative risks (RR) estimates for acute non-lymphocytic leukemia (ANLL) were obtained from published literature: (i) RR = 2.47, 95% CI 1.38 - 4.07 (Rinsky *et al.* 2002)) for industry sectors with high exposures (ii) RR = 1.32 (95%CI 0.49 - 2.88) (Lewis *et al.* 2000) for land transport (iii) RR = 2.17 (95%CI 0.9 - 5.2) (Collins *et al.* 2003) for workers in industrial chemical manufacturing (iv) RR = 1.11 (95%CI 0.3 - 2.83) (Bloemen *et al.* 2004) for industry groups with low levels of benzene exposure. A latency of 0 - 20 years was assumed for leukemia and we defined the risk exposure period (REP) for deaths occurring in 2005 as 1986 - 2005. National data sources were used for estimating proportions exposed over the REP in different industry sectors, taking account of changing employment levels and employment turnover. Monte Carlo methods were used to obtain random error confidence intervals (CI). We estimate the AF for ANLL due to exposure to benzene as 0.26% (95%CI 0 - 4.9%) giving 5 attributable deaths (95% CI 0, 103) and 8 attributable cancer registrations (95% CI 0,158). The wide confidence intervals reflect small RRs. For leukemia from all IARC 1 and 2A occupational carcinogens (benzene, ionising radiation, ethylene oxide, formaldehyde, 1,3,butadiene, non-arsenical pesticides) estimates are: AF 0.7% (95%CI 0 - 5.7), AN (deaths) 30 (95% CI 0 - 239), AN (registrations) 47 (95% CI 0 - 363). The estimated overall occupational burden in GB due to all cancers (24 different cancers) is 5.5% (8.5% men and 2.3% women) giving over 8,000 deaths and 14,000 registrations attributable to carcinogens at work. Of the 41 different carcinogens for which separate estimates were made, benzene is ranked 31. Our results might thus be interpreted as indicating that benzene should not be an immediate priority for health and safety strategic planning and that reasons other than burden are required for prioritising research effort in this area.

Benzene is associated with the development of severe but not moderate aplastic anemia in Shanghai, China. SA Gross [a,b], RD Irons, AR Schnatter, AT Le, J Ryder, WX Qin, TW Armstrong, GB Copely. [a] Molecular Toxicology and Environmental Health Sciences Program, School of Pharmacy, University of Colorado Denver, CO; [b] Fudan-Cinpathogen Clinical and Molecular Research Center, Institutes of Biomedical Sciences, Fudan University, Shanghai, China.

Benzene is historically thought to be a cause of aplastic anemia (AA) in individuals exposed to high concentrations, although quantitative epidemiology studies are rare. Herein, we report the findings of a case control study of 137 consecutive patients diagnosed with AA in Shanghai, China over a 4 year period. Diagnoses were made by a single laboratory and subjects were age- and gender- matched to two hospital-based controls. Questionnaires were administered to all cases and controls, and information obtained on previous disease, work histories and exposures to potential etiologic agents such as benzene.. Subjects and their controls were further categorized into AA subtypes (moderate (MAA) and severe (SAA)) and odds ratios (OR) were calculated for individual risk factors. The strongest associations were observed for exposure to benzene and SAA (OR 3.12, 95% CI 1.12-8.65) and life on a farm and MAA (OR 3.08, 95% CI 1.44-6.56). Using a Conditional Logistic Regression model for analysis of multiple risk factors, consistent associations between the risk factors (i.e. benzene and life on a farm) and variation in subtypes of AA (i.e. SAA and MAA) emerged as the best explanations for the development of the disease (R^2 = 10.1% and 9.9%, respectively). These findings suggest that individual subtypes of AA may have distinct etiologies, and while exposure to benzene poses a significant risk of developing AA, the majority of cases of this disease remain unexplained. (This work was funded by the Benzene Health Consortium as part of the Shanghai Health Study.)

Analysis of hydroquinone and catechol in peripheral blood of benzene-exposed workers. PJ Kerzic [a], P Liu, P Pan, Y Zhou, AR Schnatter, RD Irons. [a] Fudan-Cinpathogen Clinical and Molecular Research Center, Institutes for Biomedical Sciences, Fudan University, Shanghai, China

We have developed a gas chromatography-mass spectrometry method for analysis of benzene (BZ) metabolites in human urine and blood. Here we describe peripheral blood concentrations of hydroquinone (HQ) and catechol (CAT) in total, protein bound, and unbound (free) forms obtained from BZ-exposed factory workers and controls. Total and unbound metabolites were directly measured in independent experiments, while bound forms were calculated as [total]-[unbound]. In this subset of a larger study, breathing zone benzene, toluene, and xylene were measured for the duration of a workshift, and end-shift blood samples taken from 143 individuals. Potential lifestyle and environmental influences were assessed by questionnaire and bioassay, and single nucleotide polymorphisms in xenobiotic metabolizing enzymes NQO1, MPO, Cyp2E1, and GSTT1 were also analyzed for potential contribution to differences in blood metabolite concentration. Positive correlations were observed between same-day BZ exposure and total CAT, bound CAT, total HQ, and bound HQ ($r>0.634$, $p<0.05$ for all), when compared to controls, while unbound CAT and HQ did not positively correlate with BZ exposure. Nearly all of the metabolites found in blood were bound to protein (CAT 96-99+%, HQ 78-92+%), and when the ratio of bound to unbound metabolites were compared in subsets of exposed workers, the increase in blood metabolite concentration was nearly all due to an increase in the protein-bound molecule. These findings suggest that a threshold for conjugation does not exist within the exposure spectrum studied. Correlations between exposure and blood metabolite concentration among subsets defined by genetic and lifestyle differences are also presented. This method demonstrates the feasibility of analyzing benzene metabolites in human blood, and should allow for further investigation of the health effects of benzene and its metabolites. [This work was supported by the Benzene Health Research Consortium].

Effect of hydroquinone on DNA double strand break and DNA-PKcs expression in HL-60 cells. MM Kong [a], WT Song, H You, JH Sun, Q Ma, YY Bi. [a] School of Public Health, Wuhan University, Wuhan, Hubei, China

Hydroquinone (HQ), a reactive metabolite of benzene, is myelotoxic and leukemogenic in humans. Carcinogenicity of HQ is dependent upon its conversion to reactive free radical species, such as semiquinone radical, in the bone marrow. Redox cycling of these radicals produces reactive oxygen species that damage DNA and induce DNA double strand breaks (DSBs). In mammalian cells, DSBs induce substantial phosphorylation of histone H2AX (γ-H2AX) at sites of DSBs, a marker of DSB formation. DSBs are mainly repaired through the error-prone, non-homologous end-joining pathway (NHEJ). NHEJ requires the DNA-dependent protein kinase (DNA-PK) that consists of two regulatory subunits (Ku70 and Ku80) and a catalytic subunit (DNA-PKcs). In this study, we investigated DSB formation in human promyelocytic leukemic HL-60 cells treated with HQ. Cells were treated with HQ of 10, 25, 50, or 100 μM for 6, 18, or 24 h. γ-H2AX foci formation was observed with fluorescent microscopy. Significantly higher amounts of γ-H2AX were found in cells treated with 50 and 100 μM HQ for 18 h and 24 h, respectively, indicating HQ induces DNA double strand breaks in concentration and time-dependent manners. To examine the DSB repair activity after HQ treatment, we analyzed the expression of DNA-PKcs in HL-60 cells at both transcription and translation levels. Cells were treated with HQ as above. The results showed a dose and time dependent induction of DNA-PKcs, indicating increased DSB repair activities after HQ treatment. Our findings reveal a role of HQ in the induction of DSB and DSB repair, both of which are relevant to HQ-induced myelotoxicity and leukemogenesis.

Exposure assessment for case-control (CC) epidemiology studies based in Shanghai, China: Summary of methods and results. T Armstrong[a], Y Zhou, C Zhang, S Bowes, Y Liang, O Wong, F Hua, A Schnatter. [a] TWA8HR Occupational Hygiene Consulting, NJ, USA

As part of a suite of epidemiology studies of leukemia, lymphoma and aplastic anemia conducted in Shanghai from 2001 to 2008, retrospective job and task focused exposure assessments (EA) of cases and matched controls were completed for benzene and selected other hazards of concern. Trained interviewers administered structured questionnaires (in Chinese) to study subjects recruited from 31 hospitals. Initial classifications (exposed, likely unexposed, or uncertain), done by team members not informed of case or control status, were made to focus subsequent investigations. Benzene EA resources beyond questionnaires included industry-specific extracts from the Shanghai Municipal Institute for Public Health Supervision database, Chinese literature on benzene exposures, on-site workplace investigations, summaries of key regulatory and technology changes, and task simulations. A Shanghai-based expert panel (EP) made preliminary exposure assignments, which were further refined via logic and consistency checks with source data, resulting in range estimates 0 to 4 (none, <1, 1 to 10, >10 to 100 and >100 mg/m^3) for benzene. For other hazards, sources included the EP's knowledge of relevant industries, and the Chinese and Western literature. A random 20% of the benzene EAs were checked by two independent approaches using job titles and key task data extracted from questionnaires. The CC database included over 21,000 work history events (WHE, or jobs). For study subjects, 754 WHE underwent further review for benzene exposure. The 754 WHE arose from diverse industries and trades including: shoe manufacturing, rubber production, printing, painters and machine repair. Nearly 40% of these WHs rated ≥ category 2 benzene exposure, with rank counts and percents of: 0 (150, 20%), 1 (317, 42%), 2 (144, 19%), 3 (101, 13%) and 4 (42, 6%). Review of initial differences between study ratings and the 20% sample ratings suggested that the more detailed data (beyond questionnaire extracts) available to the study team improved rating specificity. For other exposures, the EA covered 19,157 WH events with 15,231 in the final CC database. The most prevalent general category exposures included agricultural chemicals (2077), petroleum products (1339) and metals (908). The number, eras and diversity of WH events presented a large, challenging EA project, but the broad information base assembled supported the extensive, multiple substance EA. Information about the nature, extent and range of regulatory and technology changes in China, and the available Chinese exposure literature, were key sources on chemical uses that enhanced the EA approach.

Do we agree? : Comparison of semi-quantitative exposure assessments by three independent approaches. TW Armstrong[a], JW Cherrie, RF Herrick, M Chen, SM Bowes, AR Schnatter. [a] TWA8HR Occupational Hygiene Consulting, Branchburg NJ, USA (current affiliation)

As part of a review of exposure assessments (EA) for a population case-control study of acute myeloid leukemia and lymphoid neoplasm, we undertook a comparative evaluation of the study's semi-quantitative retrospective EA for benzene. The overall study goal was to rate the benzene exposure for each subject's described tasks. The study approach (TWA's) drew on broad information available, including historical monitoring data, Chinese medical/exposure literature, on-site workplace investigations, reports on technology changes for key industrial sectors, prior discussions with and written review notes from the Chinese experts. A stratified random process was used to select a 20% sample from the study database. Two raters (JWC, RFH) were provided partial data (from questionnaires only): start and end date for the work history, job title, type of industry, and a one to two sentence description of job duties. JWC's assessments were based on a structured subjective judgment approach developed for use in industry case-control studies, with results of then mapped to the study categories. RFH rated the exposures via the professional judgment, considering information on industry, job title and activities, materials used, processes, duration of work, and year. Following the ratings, TWA provided additional information from the study database that provided the rationale for study ratings that differed from the ratings of either JWC or RFH. Two of the raters (JWC, RFH) had somewhat less information than that drawn upon for the study EA. Due to these differences, a strict comparison of inter-rater comparability was not conducted. Weighted Kappas (generally in the range between 0.4 and 0.69) indicated reasonable agreement. Two categories represent no and very low (<1 mg/m^3) exposure, so comparisons were completed on "exposed" versus the two lowest categories as "unexposed". Those simple Kappas indicated moderate agreement. Another comparison showed scores were within plus/minus one category for approximately 85% of the subjects. JWC and RFH reviewed the additional information TWA provided and indicated this additional information would generally have altered their ratings in the direction of the study ratings. The three methods gave good agreement. The study ratings (TWA's) drew on additional information not available in the data extracts provided to RFH and JWC. Subsequent consideration of this additional information further improved the agreement of the raters. The comparison study suggests the additional information improved the EAs (TWA's) used in study epidemiologic analyses.

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TITLE: An overview of published benzene exposure data by industry in China, 1960-2003

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BODY:

ABSTRACT

This article presents an overview of occupational benzene exposures in China based on data published in Chinese medical journals. The data were derived from 384 reports of benzene poisoning or industrial hygiene surveys published in Chinese medical journals between 1960 and 2003. The following information was extracted whenever available: industry, occupation, task, date, benzene levels, sampling location, workplace descriptions and, for case reports, medical diagnosis. Each paper provided one or more sets of benzene data, each set representing a sampling location or job title with one to several measurements including, mainly, breathing zone area concentration measurements, and much less frequently personal monitoring. Two criteria based on data quality were applied to select suitable data for analyses. The selected exposure data were analyzed by industry and time period. Nine hundred five sets of benzene measurements from 72 industries were reported in the 384 papers selected for this review, and 621 sets (68.6%) presented average benzene concentrations, which covered 55 industries. The distribution of the reported average benzene exposures was skewed with a median of 51.5mg/m^3. The average benzene concentrations were below 100mg/m^3 for 406 (65%) of the 621 reported average concentrations. The medians of the reported averages in mg/m^3 for the five industries with the

highest exposures were: 124.8 for leather products, 98.7 for electronic devices, 75.4 for machinery, 50.4 for shoes, and 50.3 for office supplies and sports equipment manufacturing. These data describe the concentrations and changing patterns of occupational benzene exposure by industry and time period in China.

FULL TEXT

1 Introduction

Benzene is one of the most widely used industrial chemicals in China. A wide variety of industries and occupations particularly the shoe and suitcase industries in China use benzene or benzene-containing solvents and adhesives [1]. The Chinese occupational medical literature is replete with reports of benzene over-exposure and benzene poisoning [2,3]. In 1979-1981 the Chinese Academy of Preventive Medicine carried out a national occupational health survey of more than 500,000 workers in China who were identified as having been exposed to benzene [4]. The reported geometric mean concentration of benzene was 18.1mg/m^3 and the 95% range at workplaces was 0.06-844.74mg/m^3 or 0.02-266ppm (1ppm=3.18mg/m^3), with some workplaces (1.3%) having benzene concentrations in excess of 1000mg/m^3. The data showed a skewed distribution with a second mode at approximately 200mg/m^3 and more than 10% of the data were at or above this value [4]. The nature of the distribution makes the median of these data difficult to interpret. Measurements of area breathing zone occupational benzene exposure from the 1950s to the late 1980s taken at factories in Shanghai were reported in a recent article [5]. This database of benzene measurements is maintained by the Shanghai Municipal Institute of Public Health Supervision. The arithmetic mean of benzene measurements for workplaces reported in the database was 132.7mg/m^3 and the median was 2.8mg/m^3.

In addition to ad hoc industrial hygiene surveys reported in the literature, there are incidents of acute poisoning and investigations of exposures associated with such events. For example, an investigation of 33 cases of serious benzene poisoning and nine deaths in 2002, associated with small-scale suitcase manufacturing, reported exposures as high as 2040mg/m^3[6].1

Industries in China have been regulated with occupational exposure limits (OELs) for benzene since the 1950s. These limits have gone through revisions over the years as shown in Table 1, including a shift from the prior area concentration basis to personal exposure measurements in 2002. The development of OELs in China has been discussed in a number of recent articles [1,7].

The current article presents an overview of occupational benzene exposures reported in Chinese medical journals between 1960 and 2003. The review includes 384 papers of benzene poisoning case investigations or industrial hygiene surveys, covering 72 industries in China classified according to the national industrial coding system [8]. Included in the overview is an analysis of benzene exposure data by industry and time period.

2 Material and methods

2.1 Information sources

Published literature was identified primarily through Internet searching of the China National Knowledge Infrastructure, which covers 1994-2003 and the Chinese Biomedical Literature Database, which covers 1978-1993. Manual searching was also carried out to supplement the online search, particularly for conference proceedings and articles published before 1978. Through this process, a total of 384 benzene poisoning case reports or industrial hygiene surveys published in Chinese journals were identified.

The benzene exposure data was systematically extracted into a database. From each paper, the following information was extracted whenever available: industry, occupation, task, year, benzene levels (minimum, average and/or maximum), workplace descriptions and, for case reports, medical diagnosis. Each paper provided one or more sets of benzene measurements, with each set representing one or more measurements for a sampling location or job title. Data for inclusion in the analyses were selected by applying standard acceptance criteria (as described below). Exposure

data were analyzed according to industry and time period. Table 2 provides an example of the data extracted for the database. The analyses do not distinguish between area concentration measurements and personal measurements because personal measurements were quite rare and the reports did not necessarily distinguish them, and the rare personal samples were unlikely to alter the general conclusions of the analyses in this report.

2.2 Selection criteria

Two criteria were applied to select the published data and information for inclusion in the analyses. The first criterion was used to select data for the analysis by industry type and the second criterion was used for selecting data for temporal analysis.

For the selection of data for the comparison of exposure level by industry type, the most important factors were the number of measurements in each set of measurements and the number of sets of measurements for the industry. A set of measurements consisted of: the sampling location or job title, the benzene measurements taken, and the year. Table 2 presents data from five published articles on benzene measurements. Article B in Table 2 provided three sets of measurements, covering three different jobs, while article E in Table 2 provided two sets of data, covering two different locations.

For the analyses in this report, we chose a weighted selection score (WSS) (described further below) of 18. This criterion considers the number of samples in a set and the number of measurement sets in a given industry. A score of 18 was the minimum for an industry to be included in the analyses. For example, using the leather industry data in Table 2: $WSS = 60 + 60 + 60 + 7 + 26 + 12 = 225$ Alternatively, if only report C had been available for the leather industry, the WSS=7, which is below our required score of 18 and would thus not be included in the trend analyses.

A key component of the WSS criterion for data sets in our review was the quantitative strength of the data. Our "rule of thumb" for this hinged on a commonly accepted sample size of six measurements in each set and six measurement sets in a given industry as a basis for a reliable estimate of an average concentration in typical industrial hygiene surveys [9]. It would have been optimal if each set of measurements had six samples as well to produce a score of 36 or above for calculating the median for each industry. However, this goal of six samples per set and six sets proved to be too stringent, so a compromise weighted score of "18" was chosen in order to include more data sets for the exposure level analysis according to industry type.

The criteria used to select information for the analysis of overall temporal trends of benzene exposure by industry required the published papers for the industry to include both average exposure concentration results and the year, and have at least six sets of measurements for each year.

2.3 Statistical analysis

Nonparametric tests followed by the Kruskal-Wallis *H*-test were applied to the statistical analysis by industry type. The Linear, Quadratic and Cubic Polynomial tests of one-way ANOVA contrasts were used for the analysis of overall temporal trends of benzene exposure from 1979 to 2001. $P<0.05$ was set to verify the statistical significance.

3 Results

3.1 General feature of occupational exposure to benzene

The articles included in the database were published between 1960 and 2003. Fig. 1 shows that few articles were published in the early 1960s and none between the mid 1960s and the late 1970s (the 10 years of the "Cultural Revolution" and its negative impact, which lasted for a few years afterwards). Starting in the 1980s, with the improvement of the national economy and health services, the frequency of inspections and air monitoring at workplaces greatly increased, resulting in a surge in the number of published papers.

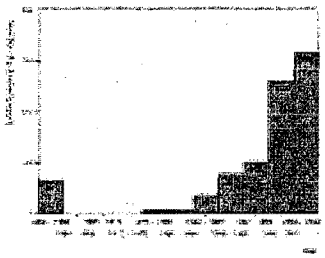

Fig. 1. Number of published reports included in the analyses by year. Numbers in each bar represent the number of reports included in the analyses for that time period.

Fig. 2 shows the distribution of average benzene exposure levels included in the present analysis of a total number of 621 sets data. It indicated that 264 data sets (42.4%) presented benzene concentration below 40mg/m^3 including nine data sets (1.4%) reported having not-detected levels of benzene that were expressed as "0". There were 357 sets (57.6%) that exceeded 40mg/m^3, the previous national benzene OEL adopted between 1962 and 2001 in China. Of those 621 sets, 142 sets (22.9%) presented concentrations between 40 and 100mg/m^3, and 147 sets (23.7%) presented levels between 100 and 500mg/m^3. There were 68 sets (11%) of measurements that far exceeded 500mg/m^3, including 15 sets that reported benzene exposure concentrations above 3000mg/m^3. The highest levels were found for the following occupations: paint coating/spraying and glue brushing (e.g., in shoes and suitcase industries). Some measurements were as high as several thousand mg/m^3. Levels of more than 10,000mg/m^3 were reported for work in confined spaces without adequate ventilation, which was usually associated with acute benzene poisoning. As further examples, acute benzene poisoning cases were reported among workers who painted the inner walls of oil storage tanks, worked in poorly ventilated basements, or in deep wells.

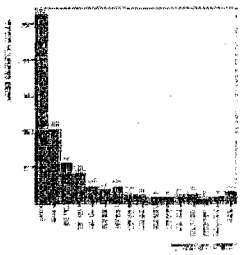

Fig. 2. Distribution of the average benzene exposure levels. Numbers in the bars denote the number of reports in the analyses in the given concentration range.

3.2 Comparison of benzene exposure by industry

Reports for 39 industries included both average benzene concentration data and the number of samples. According to the selection criteria, 27 (69.2%) industries (1972-2002) of the 39 met the required WSS of 18 and were included in the analysis according to industry. The results, given in Table 3, show that the median benzene exposures in the nine industries with the highest reported concentrations exceeded the previous OEL standard of 40mg/m^3, and 59% (16 of 27) of the industries had median exposure levels above the new standards of either 10mg/m^3 (permissible concentration-short term exposure limit; PC-STEL) or 6mg/m^3 (permissible concentration-time weighted average; PC-TWA).

The previous OELs for chemical substances and dusts were expressed as maximum allowable concentrations that were mainly based on stationary area breathing zone monitoring samples until 2001. The newly developed OELs in terms of TWA and STEL have been adopted since 2002 while the area breathing zone monitoring samples at workplace are still used because personal samplers have not been commonly available. Therefore, the data remain comparable.

Table 3 compares benzene measurements by industry, sorted by the median of the reported average exposures. Twenty-seven of the most related industries were ranked by median benzene concentrations in the table and further analyses were conducted to verify the statistical differences of exposure levels by nonparametric tests. Table 3 shows the five industries with the highest exposures (that met the criteria of six sets or more of measurements) were: leather products, electronic devices, machinery, shoes, and office suppliers and sports equipment manufacturing.

3.3 Historical trends of the benzene exposure data

According to the selection criteria described above, the reports of medians of the exposure levels for the 22 years between 1979 and 2001 were adequate for temporal trend analysis. The benzene exposure levels for the 22 years were overall significantly different ($P<0.05$). Fig. 3 shows the trend by year for the median exposure levels.

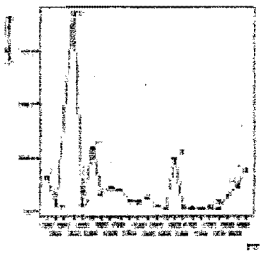

Fig. 3. Overall trend in median benzene exposure in Chinese industry, 1979-2001. (*) Indicates the number of measurement sets in the database.

These trends were most likely influenced by several historical events. First, the national economy started growing in the late 1970s, which resulted in increased numbers of small-scale industries (SSIs) in rural areas. Second, the improvement of occupational health services after the mid 1990s brought about more stringent health inspections and workplace monitoring and resulted in more published data. Third, the new Occupational Disease Prevention and Control Act was adopted by the Standing Committee of the National People's Congress in 2001, which required the reporting of benzene over-exposures in industries, including in the SSIs in rural areas [10].

As shown by Figs. 4-7, average benzene exposure levels in several major industries showed a similar but variable decline over the years. More significant declines were evident for the earlier periods. Most of these industries show slightly increased exposure concentrations at the beginning of the 21st century. The industries (Figs. 4-7) were chosen for the analysis according to industry type mainly because of the amount of data available for them. This may in part be due to more frequent investigations of these particular industries.

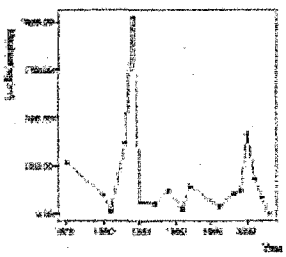

Fig. 4. Change in average benzene exposure levels in leather manufacturing over time (1975-2003).

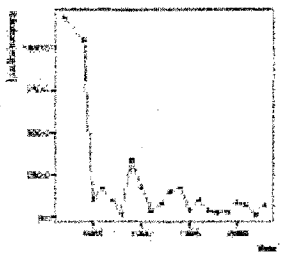

Fig. 5. Change in average benzene exposure levels in leather shoes manufacturing over time (1982-2003).

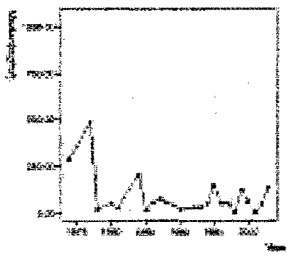

Fig. 6. Change in average benzene exposure levels in spray painting over time (1974-2003).

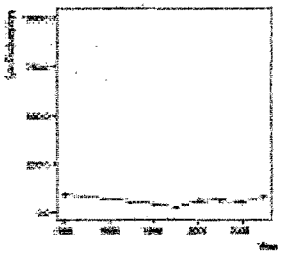

Fig. 7. Change in average benzene exposure levels in furniture manufacturing over time (1994-2003).

4 Discussion and conclusions

This review article describes the Chinese data for occupational benzene exposure and its changing patterns according to industries and time period based on the data extracted from the published Chinese medical literatures between 1960s and 2003. The results provide important information for prioritizing the prevention strategy of benzene poisoning. The median values of the reported average concentrations and the ranges of the averages for the five highest industries (with six or more sets of measurements) are: leather products, 124.8 (3.7-267.8mg/m^3); electronic devices, 98.7 (4.5-254.9mg/m^3); machinery, 75.4 (4.2-152.7mg/m^3); shoes, 50.4 (1.3-1488.6mg/m^3); and office supplies and sports equipment manufacturing, 50.3 (10.7-256.0mg/m^3). These coincide with previously reported industries with higher prevalence of benzene-related hematopoietic diseases [4]. The percentage of industries with reported medians exceeding the prior OEL of 40mg/m^3 was 33% (9 of 27). For 59% (16 of 27) of the industries, medians of the exposure levels were below the prior OEL (40mg/m^3) but above the new standards of either 10mg/m^3 (PC-STEL) or 6mg/m^3 (PC-TWA).

With innovations in production technology, which included the use of toluene and xylene instead of pure benzene and improved working practices, and the improvement of occupational health services, overall average benzene exposure levels appeared to decline over the years. In particular, industries traditionally with high exposures such as leather products manufacturing, shoes, paint spraying and furniture industries showed declines. Relatively high levels (270.38mg/m^3) were observed during the period between 1981 and 1985, but dropped to 73.85mg/m^3 in the second half

of 1980s. The exposure levels declined further to 48.42mg/m^3 during the period between 1996 and 2000. However, the exposure levels fluctuated due to the influence of the national economic development and newly implemented legislations. For example, the average of the reported exposures rose to 121.25mg/m^3 at the beginning of the 21st century, which might partially be due to the wider disclosure of over-exposures in small workshops commonly found in rural areas. Additionally, the current public awareness and the frequency of health inspection might bias the results to a certain extent. The declining tendencies of occupational benzene exposures were also shown by a Job-Exposure Matrix investigation in Shanghai based on a database maintained in Shanghai Municipal Institute of Public Health Supervision (1953-1989) [4].

There are a number of limitations associated with the published information and the analyses currently presented. These include:

1) Different sampling and analytical methods were employed over the decades covered. Although this may introduce some bias, we believe that the differences are minor and will not significantly alter the findings of this review.
2) Our analysis did not distinguish between benzene poisoning case reports and industrial hygiene surveys. Benzene poisoning case reports refer to investigation of the Chinese regulation-defined occupational benzene poisoning. Either acute or chronic benzene poisoning cases may thus represent high-end exposures. On the other hand, industrial hygiene surveys were commissioned for a variety of reasons, and are not necessarily targeted at facilities with benzene poisoning cases or high benzene exposures. The industrial hygiene survey reports thus may or may not better represent typical conditions.
3) Our selection criteria may have excluded some informative but smaller data sets. We believe the criteria gave us the more reliable data to use in our analysis, resulting in more robust summaries.
4) The original investigators who reported the data may have had a range of investigative needs and approaches that introduced indeterminate biases into their reported data. For example, what motivated or initiated the authors to write up benzene poisoning reports or industrial hygiene surveys and submit them for publication?
5) The data in the original reports are area samples that represent concentrations in the air at the location and time measured. However, these concentrations might not directly reflect worker exposures, particularly for full-shift exposures, because they did not account for various potential exposure zones.
6) The data for trend analyses in this report are averages given in the reports. These averages should give a good estimate of the typical concentrations. However, the averages do not give insights into the range and variability of the concentrations in the industries covered.

Acknowledgements

We are indebted to the Benzene Health Research Consortium for sponsoring the Shanghai Health Study, as well as Dr. Jerry Rice, Chair, Scientific Review Panel, Dr. Patrick Beatty, Chair, Technical Committee, Prof. Richard D. Irons, Dr. A. Robert Schnatter, and Gail Jorgensen for their continuing support and encouragement in conducting the investigation of benzene exposures. The authors are also grateful for colleagues and medical students working at the University of Colorado Health Sciences Center and Fudan University Medical Center Joint Clinical and Molecular Laboratory, who contributed to the literature searching and database development.

TABLES

Table 1

Years	Standard	Reference
1956-1979	50mg/m^3, Area breathing zone concentration	[11,12]

1979-2002	40mg/m^3, Area breathing zone concentration	[13]
2002-Present	10mg/m^3, Permissible concentration-short term exposure limit; 6mg/m^3, permissible concentration-time weighted average	[14]

Past and current benzene occupational exposure limits in China

Table 2

Article	No. of cases and diagnosis	Industry	No. of sets	Year	Task/area description	Min. conc. (mg/m^3)	Aver. conc. (mg/m^3)	Max. conc. (mg/m^3)	No. of samples
A	5 (atypical anemia)	Leather manufacturing (shoes)	1	1983		0	370	1530	NA
B	77 (chronic poisoning)	Leather manufacturing (shoes)	3	1985	Soles adhesive brushing	NA	1128.39	NA	60
Underlay adhesive brushing	NA	440.39	NA	60					
Assembly finishing	NA	48.43	NA	60					
C		Leather manufacturing (watch bands)	1	1989		45	122.5	300	7
D	17 (chronic poisoning)	Leather manufacturing (shoes)	1	NA		4.3	NA	308	26
E		Leather manufacturing (suitcases)	2	2001	Garment I	NA	191.71	NA	NA
Garment II	NA	126.44	NA	12					

Examples of benzene exposure data extracted from the published Chinese medical literature for leather manufacturing

NA: not available.

Table 3

Code	Type of industry	No. of sets	No. of samples	Median	Average (range)
252	Leather products[a]	18	1487	124.8	124.1(3.7-267.8)
449	Electronic devices manufacturing[a]	6	1930	98.7	120.2(4.5-254.9)

42, 43	Machinery manufacturing[a]	6	6815	75.4	75.6(4.2-152.7)
243	Shoes manufacturing, leather[a]	70	12197	50.4	149.9(1.3-1488.6)
291	Office supplies and sports equipment[a]	6	106	50.3	79.4(10.7-256.0)
321	Spray painting	29	1186	39.8	53.4(0-226.8)
270	Furniture manufacturing	8	618	39.3	36.6(2.0-72.0)
444	Msc. electronic parts manufacturing	7	197	33.6	50.5(3.0-105.6)
472	Automobile manufacturing	6	3478	32.8	56.8(0-196.1)
314	Organic chemical industry	19	650	23.8	39.3(12.8-130.5)
341	Rubber products manufacturing	15	182	22.9	114.6(0.1-633.6)
998	Other industries	10	6799	18.5	23.8(2.2-85.5)
320	Paint manufacturing	37	525	13.2	23.9(1.0-127.5)
31	Chemical industry	18	859	7.6	19.3(0-123.9)
294	Printing industry	8	6416	6.5	7.2(0-23.6)
401	Metal-based products processing	10	77	1.4	7.5(0-38.0)
296	Toy manufacturing	2	2531	132.9	132.9(1.5-264.3)
363	Coal products manufacturing	3	23	96.0	79.8(12.8-130.5)
361	Crude oil processing	3	992	62.6	54.4(7.4-93.2)
512	-7) were chosen for the analysis according to industry type mainly because of the amount of data available for them. This may in part be due to more frequent investigations of these particular industries.Fig. 4. Change in average benzene exposure levels in leather manufacturing over time (1975-2003).Fig. 5. Change in average benzene exposure levels in leather shoes manufacturing over time (1982-2003).Fig. 6. Change in average benzene exposure levels in spray painting over time (1974-2003).Fig. 7. Change in average benzene exposure levels in furniture manufacturing over time (1994-2003).4 Discussion and conclusionsThis review article describes the Chinese data for occupational benzene exposure and its changing patterns according to industries and time period based on the data extracted from the published Chinese medical literatures between 1960s and 2003. The results provide important information for prioritizing the prevention strategy of benzene poisoning. The median values of the reported average concentrations and the ranges of the averages for the five highest industries (with six or	3	22	57.2	41.9(5.8-62.6)

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analyses currently presented. These include:1) Different sampling and analytical methods were employed over the decades covered. Although this may introduce some bias, we believe that the differences are minor and will not significantly alter the findings of this review.2) Our analysis did not distinguish between benzene poisoning case reports and industrial hygiene surveys. Benzene poisoning case reports refer to investigation of the Chinese regulation-defined occupational benzene poisoning. Either acute or chronic benzene poisoning cases may thus represent high-end exposures. On the other hand, industrial hygiene surveys were commissioned for a variety of reasons, and are not necessarily targeted at facilities with benzene poisoning cases or high benzene exposures. The industrial hygiene survey reports thus may or may not better represent typical conditions.3) Our selection criteria may have excluded some informative but smaller data sets. We believe the criteria gave us the more reliable data to use in our analysis, resulting in more robust summaries.4) The original investigators who reported the data may have had a range of investigative needs and approaches that introduced indeterminate biases into their reported data. For example, what motivated or initiated the authors to write up benzene poisoning reports or industrial hygiene surveys and submit them for publication?5) The data in the original reports are area samples that represent concentrations in the air at the location and time measured. However, these concentrations might not directly reflect worker exposures, particularly for full-shift exposures, because they did not account for various potential exposure zones.6) The data for trend analyses in this report are averages given in the reports. These averages should give a good estimate of the typical concentrations. However, the averages do not give insights into the range and variability of the concentrations in the industries covered.AcknowledgementsWe are indebted to the Benzene Health Research Consortium for sponsoring the Shanghai Health Study, as well as Dr. Jerry Rice, Chair, Scientific Review Panel, Dr. Patrick Beatty, Chair, Technical Committee, Prof. Richard D.

Irons, Dr. A. Robert Schnatter, and Gail Jorgensen for their continuing support and encouragement in conducting the investigation of benzene exposures. The authors are also grateful for colleagues and medical students working at the University of Colorado Health Sciences Center and Fudan University Medical Center Joint Clinical and Molecular Laboratory, who contributed to the literature searching and database development.TABLESTable 1YearsStandardReference1956-197950mg/m3, Area breathing zone concentration[11,12]1979-200240mg/m3, Area breathing zone concentration[13]2002-Present10mg/m3, Permissible concentration-short term exposure limit; 6mg/m3, permissible concentration-time weighted average[14]Past and current benzene occupational exposure limits in ChinaTable 2ArticleNo. of cases and diagnosisIndustryNo. of setsYearTask/area descriptionMin. conc. (mg/m3)Aver. conc. (mg/m3)Max. conc. (mg/m3)No. of samplesA5 (atypical anemia)Leather manufacturing (shoes)1198303701530NAB77 (chronic poisoning)Leather manufacturing (shoes)31985Soles adhesive brushingNA1128.39NA60Underlay adhesive brushingNA440.39NA60Assembly finishingNA48.43NA60CLeather manufacturing (watch bands)1198945122.53007D17 (chronic poisoning)Leather manufacturing (shoes)1NA4.3NA30826ELeather manufacturing (suitcases)22001Garment INA191.71NANAGarment IINA126.44NA12Examples of benzene exposure data extracted from the published Chinese medical literature for leather manufacturingNA: not available.Table 3CodeType of industryNo. of setsNo. of samplesMedianAverage (range)252Leather productsa181487124.8124.1(3.7-267.8)449Electronic devices manufacturinga6193098.7120.2(4.5-254.9)42, 43Machinery manufacturinga6681575.475.6(4.2-152.7)243Shoes manufacturing, leathera701219750.4149.9(1.3-1488.6)291Office supplies and sports equipmenta610650.379.4(10.7-256.0)321Spray

painting29118639.853.4(0-226.8)270Furniture manufactur-ing861839.336.6(2.0-72.0)444Msc. electronic parts manufactur-ing719733.650.5(3.0-105.6)472Automobile manufactur-ing6347832.856.8(0-196.1)314Organic chem-ical in-dustry1965023.839.3(12.8-130.5)341Rubber products manufactur-ing1518222.9114.6(0.1-633.6)998Other in-dustries10679918.523.8(2.2-85.5)320Paint manufactur-ing3752513.223.9(1.0-127.5)31Chemical in-dustry188597.619.3(0-123.9)294Printing in-dustry864166.57.2(0-23.6)401Metal-based products pro-cessing10771.47.5(0-38.0)296Toy manufac-turing22531132.9132.9(1.5-264.3)363Coal products manufactur-ing32396.079.8(12.8-130.5)361Crude oil pro-cessing399262.654.4(7.4-93.2)512Petroleum & geological prospecting

238	Other textile industries/printing & dyeing	1	178	26.2	26.2
520	Civil engineering & construction	3	137	20.3	122.2(1.2-345.2)
374	Pottery & porcelain products manufacturing	3	26	20.2	22.4(7.1-40.0)
450	Electronic circuit manufacturing	3	26	20.2	22.4(7.1-40.0)
342	Plastic products manufacturing	2	1216	15.2	15.2(2.3-28.2)
499	Other precision instruments manufacturing	2	44	14.3	14.3(8.7-19.9)
407	Household metal hardware manufacturing	1	1139	2.3	2.3

Comparison of the average benzene concentrations (mg/m^3) by industry

[a]Denotes the top five industries with more than six measurement sets in an individual industry. Industries following the blank space (after 401, metal-based processing) are those for which fewer than six data sets were available.

FOOTNOTES

1. No systematic monitoring data with the report were available as the governmental occupational health inspection has not always effectively accessed the informal work sectors in rural areas.

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