IL GENE DI FUSIONE FIP1L1-PDGFRA E LA MUTAZIONE V617F DI JAK2: LE NUOVE BASI MOLECOLARI DELLA TROMBOCITEMIA ESSENZIALE

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Riassunto

La Trombocitemia Essenziale (TE) è una delle malattie Mieloproliferative Croniche (SMP) ed è caratterizzata da un incremento del numero di piastrine senza altre cause di trombocitosi. L'assenza di caratteristiche specifiche rende difficile la diagnosi delle SMP. Per alcune di queste patologie, come la Leucemia Mieloide Cronica, esiste un *marker* molecolare; per le altre SMP sono stati introdotti criteri diagnostici clinici.

Spesso, però, l'assenza di uno specifico marcatore molecolare o di caratteristiche cliniche ben definite rende difficile discriminare tra i vari quadri clinici. In particolare la diagnosi clinica di TE è, in larga parte, basata su criteri d'esclusione. Numerosi sono gli studi volti ad individuare un *marker* molecolare anche per queste patologie. A livello molecolare, l'aberrante e costitutiva attivazione di specifiche chinasi, con conseguente attivazione inappropriata della cascata di trasmissione del segnale intracellulare, è progressivamente emersa come evento molecolare centrale e comune nella patogenesi delle SMP. Nel 2003 è stato individuato nei pazienti con ipereosinofila (HES) un gene di fusione (FIP1L1-PDGFRA) codificante per una tirosina chinasi aberrante. Nel 2005 una mutazione somatica in posizione 617 del gene che codifica per la tirosina chinasi JAK2 è stata trovata in pazienti con SMP. Queste due tirosine chinasi mutate in pazienti con SMP, si sono aggiunte alla crescente lista di tirosine chinasi coinvolte nella patogenesi delle neoplasie ematologiche.

Nel nostro lavoro, abbiamo studiato, mediante RT-PCR, quaranta pazienti con TE ed abbiamo trovato che sei dei quaranta pazienti studiati hanno il gene di fusione FIP1L1-PDGFRA. Come nella sindrome ipereosinofila, così anche nei nostri pazienti, il gene ibrido produce una forma attivata di tirosina chinasi PDGFRα.

L'analisi FISH del locus CHIC2 nei pazienti con PCR positiva per il gene di fusione ha mostrato che una percentuale di cellule midollari tra il 19 ed il 42% ha l'anomalia citogenetica caratteristica (una delezione interstiziale al cromosoma 4). Abbiamo, poi cercato la mutazione V617F di JAK2 nei 40 pazienti. Quindici di essi (40%) hanno tale mutazione. In un paziente coesistono entrambe le alterazioni.

I pazienti con TE, con gene di fusione FIP1L1-PDGFRA, sono candidati al trattamento con Imatinib, poiché come i pazienti con HES e LMC posseggono un *target* appropriato per il farmaco. Inoltre il marcatore molecolare del clone può essere l'oggetto del monitoraggio della risposta alla terapia.

Infine il nostro studio porta alla conclusione che il gene di fusione FIP1L1-PDGFRA è presente in una significativa percentuale di pazienti con TE e che può al momento essere considerato con la mutazione V617F di JAK2 uno dei marcatori molecolari della malattia.

Summary

FIP1L1-PDGFRA AND JAK2 V617F: MOLECULAR BASIS OF ESSENTIAL THROMBOCYTHEMIA

Essential thrombocythemia (ET), a disease characterized by primitive and pronounced increase of platelet count, belongs to the wide spectrum of chronic myeloproliferative diseases (MPD), which also comprise, as distinct entities, chronic myeloid leukemia (CML), polycythemia vera (PV), chronic idiopathic myelofibrosis (MF), and other Philadelphia (Ph) negative chronic myeloproliferative disorders including chronic eosinophilic leukemia (CEL) and the idiopathic hypereosinophilic syndrome (HES). For some of these disorders strict diagnostic criteria have been introduced into the clinical practice; however the lack of either categorized clinical features or specific molecular lesions makes difficult, in some MPD subtypes, a clear-cut discrimination among the different clinical pictures. Therefore, the clinical diagnosis is largely based upon exclusion criteria and the treatment is usually a nonspecific suppression of hematopoiesis. In particular, the diagnosis of ET, according to the Polycythemia Vera Study Group criteria, requires the exclusion of known causes of reactive increase of platelet counts, such as acute or chronic inflammatory conditions and the absence of features distinctive of other types of MPDs, such as marrow fibrosis, Philadelphia chromosome and increased red cell mass. At cellular level, several investigators have shown that various types of MPD hematopoietic progenitors belonging to the erythroid, myeloid or megakaryocytic lineage are hypersensitive to several growth factors. At molecular level, aberrant activation of specific kinases with the consequent inappropriate activation of signaling cascade has progressively emerged as the central and most common molecular event in the pathogenesis of these diseases. Indeed, the well studied model of CML indicates that the constitutive activation of a specific tyrosine kinase encoded by the BCR/ABL fusion gene primarily sustains the high proliferative potential of the myeloid clone and, hence, plays a central role for the neoplastic transformation and the clinical picture of the disease. The recent finding of the FIP1L1/PDGFRA fusion gene in one third of patients affected by HES adds to a growing list of activated kinases linked to the pathogenesis of MPDs. It has been shown that the FIP1L1/PDGFRα hybrid protein is able to transform hematopoietic cells and to sustain their growth factor-independent proliferation. Most importantly, in vitro data provided evidence that the

kinase activity of this fusion protein is inhibited by imatinib at a cellular IC_{50} much lower than the doses needed to inhibit BCR/ABL, and this observation led to the successful imatinib treatment of HES patients with the FIP1L1/PDGFRA fusion gene.
Very recently, four research groups independently showed that most patients with PV, and some with MF or ET, share an identical acquired point mutation of the Janus kinase 2 (JAK2) gene. This gene encodes a tyrosine kinase and the described mutation leads to a valine-to-phenylalanine substitution at position 617 (V617F) in the JH2 or autoinhibitory domain of JAK2, with the consequent constitutive activation of its kinase activity. This finding provided a novel clue for understanding the pathogenesis of these MPDs and for the development of a new, molecular-based, diagnostic approach of these disorders.

In the current study we provide evidence for the presence of the FIP1L1/PDGFRA gene in bone marrow precursors of a sizeable proportion of patients with ET. This gene, encoding an activated form of the PDGFR, represents, beyond the V617F JAK2 mutation, the second type of non-random molecular alteration found in ET patients.
In the period comprised between 2001 and 2006, 40 consecutive patients with diagnosis of ET were enrolled into the study. Baseline evaluation of all patients before the start of any therapy included, in addition to the general clinical and hematological evaluation, RT-PCR analysis for BCR/ABL transcripts to exclude a thrombocytemic presentation of CML, red cell mass assessment, marrow histology for marrow fibrosis, karyotypic analysis of marrow cells and ultrasound evaluation of spleen volume. Criteria for the diagnosis of ET were those established by the Polycythemia Vera Study Group (PVSG). Written informed consent was obtained from all the patients studied. Mean age of the patients, evenly distributed for gender, was 47 years. Eight of the 40 patients (20%) showed a mild leukocytosis; WBC counts never exceeded 20,000/μL. Conventional cytogenetic analysis failed in 6 cases for absence of mitoses and revealed the presence of clonal complex karyotype in four patients.
Mononuclear cells (MNC) were isolated using the Ficoll-Hypaque density gradient centrifugation method from bone marrow samples collected for the diagnostic work-up at presentation of the disease. Cells were washed twice in saline solution and resuspended in at least two aliquots for each patient.

Genomic DNA was extracted from a MNC aliquot of $2x10^6$ cells using the Nucleon BACC2 kit (Amersham Biosciences Europe GmbH, Freiburg Germany) following the manufacturer's directions. Total RNA

was purified from a MNC aliquots of $5x10^6$ cells cryopreserved at –80°C in 600μL of 4 M guanidinium isothiocyanate solution buffer. The "GenElute Total RNA" kit (SIGMA, S. Louis MO, USA) was used for the extraction following the manufacturer's directions. The quality of RNA was assessed on ethidium-bromide-stained 1% agarose gel containing 2.2mol/L formaldehyde.

All patient bone marrow samples were screened for the interstitial deletion at chromosome 4q12 by RT-PCR analysis of the FIP1L1/PDGFRA fusion gene. The RT-PCR analysis and Nested PCR for the presence of FIP1L1/PDGFRA fusion gene was performed as described by Cools et al.

Nested PCR revealed the presence of the FIP1L1/PDGFRA fusion gene in 6 of the 40 patients: five of them, showed a single amplification band, while in the remaining patient PCR yielded 4 distinct amplification bands. Sequence analysis confirmed in all cases that the amplified bands corresponded to an "*in frame*" junction between FIP1L1 and PDGFRA exons. The FIP1L1/PDGFRA junctions were highly variable.

The fusion between FIP1L1 and PDGFRA on chromosome 4 originates from an approximately 800-kb interstitial chromosomal deletion that comprises the cysteine-rich hydrophobic domain 2 (CHIC2) locus. To confirm the presence of the 4q12 interstitial deletion with the FIP1L1/PDGFRA fusion we applied the interphase FISH analysis using a single CHIC2 specific probe (RP11-367N1, IDs AF159423). Interphase FISH analysis was performed on whole bone marrow samples using standard procedures. The interstial 4q12 deletion results in the presence of a single hybridization signal (monosomy). A cut-off value of 6% for abnormal results (presence of 4q12 deletion) was calculated on a sample of 20 not-deleted bone marrow samples in which two independent operators scored 200 nuclei in a blinded fashion.

All patient BM samples were analyzed for the presence of the V617F JAK2 mutation recently described in patients with PV and ET at exon 12 of the JAK2 gene. The sequence analysis was performed by amplifying patient cDNA aliquots with a primer couple designed to flank the codon 617 at exon 12 of the JAK2 gene.

In 7 patients the presence of the V617F JAK2 mutation was confirmed using the amplification refractory mutation system (ARMS). Patient BM DNA was amplified using 1μM of a common reverse primer and 0.5 μM of two forward primers. The first forward primer is specific for the mutant allele and contains a mismatch at the third nucleotide of the 3' end to improve specificity, while the second primer

amplifies a sequence from both wild type and mutant alleles and is used as internal control of the assay.

Most importantly, our results showed that the presence of the FIP1L1/PDGFRA fusion gene and of the V617F JAK2 mutation are not mutually exclusive: in one patients, we found the FIP1L1/PDGFRA fusion gene at the RT-PCR analysis with a 34% of cells positive for CHIC2 monosomic deletion; in the same patient the V617F JAK2 mutation was evident on both strands at sequence analysis. Conversely, ARMS analysis excluded the presence of small subclones carrying the V617F JAK2 mutation below the sensitivity of sequence analysis in the remaining 5 patients.

Four subsets came out among our ET patients at molecular analysis: those with the somatic V617F JAK2 mutation (37,5% of the total); a second group with the FIP1L1/PDGFRA fusion gene, accounting for 15% of total cases; a larger group in which no genetic abnormality was detected, and, lastly, a single patient who showed the simultaneous presence of both mutations. No substantial differences were found concerning the main hematological features of these 4 subsets of patients. Those with the FIP1L1/PDGFRA fusion gene tended to be younger than those belonging to the other groups, but the low number of patients in each group could bias the comparison. Interestingly, eosinophil count was always in the normal range in the FIP1L1/PDGFRA positive patients, as well as in patients of the other groups; three of the 6 patients carrying this fusion gene were female. Lastly, additional chromosome abnormalities were detected in patients with the V617F JAK2 mutation as single genetic abnormality, but never in those with the FIP1L1/PDGFRA fusion gene.

In this study we confirm that JAK2 mutations may be frequently found in marrow precursors of patients affected by ET; most importantly, we provide a further insight into the molecular basis of ET, showing the presence of another acquired genetic alteration, the FIP1L1/PDGFRA fusion gene, that we have identified by both RT-PCR and FISH analysis in 15% of a series of consecutive ET patients. The structure of the FIP1L1/PDGFRA fusion gene is the same as that found in HES patients. Indeed, the PDGFRA breakpoints are invariably restricted to exon 12; hence, complete or truncated PDGFRA exon 12 is joined in frame to the 5' part of the FIP1L1, and, consequently, the derived fusion protein comprises the two PDGFRα tyrosine kinase domains. The PDGFRA exon 12 encodes a portion of the juxtamembrane domain that is known to serve as a negative regulator of PDGFRα kinase activity; this domain is disrupted in the fusion protein, with a consequent constitutive activation. This activated

form of tyrosine kinase proved to be able to transform hematopoietic cells both in vitro and in vivo. FIP1L1/PDGFRA transfection in the Ba/F3 hematopoietic cell line results in its interleukin-3-independent growth; in vivo, retroviral transduction of this fusion kinase into bone marrow cells of 5FU-treated donor mice, followed by transplantation into lethally irradiated recipients, produces a rapidly fatal myeloproliferative disease characterized by leukocytosis with myeloid predominance, splenomegaly, and extramedullary hematopoiesis. The peculiar mechanism, i.e. an interstitial chromosomal deletion, responsible for the formation of the FIP1L1/PDGFRA fusion gene, provides an optimal marker to assess clonality at bone marrow level through the use of interphase FISH analysis of the CHIC2 locus. In our ET cases bearing the FIP1L1/PDGFRA fusion gene, a percentage ranging from 19 to 42% of bone marrow cells showed a monosomic deletion of the CHIC2 locus, a finding indicative for a significant clonal expansion of marrow transformed cells bearing the 4q12 interstitial deletion.

The observation that a constitutive PDGFRα activation is shared by two different types of MPD, i.e. HES and ET, raises a question regarding the mechanisms by which the same mutation may produce to two different clinical entities. A series of experimental evidences suggest that the most plausible explanation is that the molecular lesion may occur in a multipotent progenitor, but an additional event is needed to drive the expansion of the eosinophilic or megakaryocytic compartment. This seems a general rule in MPD: in HES, the FIP1L1/PDGFRA fusion gene is detectable further than in the eosinophils, also in neutrophils, lymphocytes and monocytes; similarly, in the MPD patients the V617F JAK2 mutation is not restricted only to erythroid precursors or to megakaryocytes, and the Philadelphia chromosome is largely detectable in all hemopoietic lineages in CML patients. The remarkable finding that the FIP1L1/PDGFRA fusion gene requires constitutive expression of IL5 to induce a hypereosinophilic resembling syndrome in a recently published animal model, may corroborate the hypothesis of a multi*step* pathogenesis of ET, prompting to speculate that, after the acquisition of a first mutation, a second co-operating event is required to gain the full ET phenotype.

Beyond its relevant pathophysiologic interest, the identification of the FIP1L1/PDGFRA fusion gene in marrow hematopoietic progenitors of ET patients carries great potential for both clinical assessment and management of these patients.

A large number of studies have attempted to identify positive diagnostic criteria for ET, such as platelet size heterogeneity, platelet aggregation or spontaneous growth in cell cultures, but these abnormalities are neither sensitive nor specific. More promising appeared the assays looking for clonal hematopoiesis, at least at the level of the megakaryocyte fraction. However, these assays are particularly laborious, are applicable only on female subjects, and are not totally specific (healthy elderly women may show apparently clonal hematopoiesis). Therefore the diagnosis of ET, mainly based on exclusion criteria, is in some instances rather difficult. At present, in half of ET patients molecular data may provide useful markers that could be used for a rapid, unambiguous and reliable confirmation of the diagnosis of ET in patients with stable increased platelet count. Of the 40 ET patients who entered our study, 5 had the FIP1L1/PDGFRA fusion gene, 14 carried the V617F JAK2 mutation, and a single patient showed both genetic alterations; therefore, 20 of the 40 patients showed a recognizable molecular alteration.

It has been clearly shown that the kinase activity of the FIP1L1/PDGFRα fusion protein is a target for a new class of small molecules with tyrosine kinase inhibitory activity, among whom imatinib is a paradigmatic example. At cellular level, the effect of imatinib against PDGFRα has proved to be much higher than against ABL; indeed, HES patients carrying the FIP1L1/PDGFRA fusion gene may be induced into complete remission by imatinib at daily doses as low as 100 mg. Consequently, also ET patients harboring the FIP1L1/PDGFRA fusion gene are obvious candidate for imatinib treatment, since, like CML patient, they carry an appropriate target for the drug, which can be also used as a molecular marker for monitoring the response to the treatment.

In conclusion, our study shows that the FIP1L1/PDGFRA fusion gene is present in a significant proportion of ET patients and can be considered, along with the V617F JAK2 mutation, a molecular marker of this disease. This finding may have an immediate impact on the treatment of ET since the FIP1L1/PDGFRα fusion protein is a suitable target for imatinib. Further studies are necessary to understand how two different clinical entities may originate from a single molecular defect, to explain the male predilection of the FIP1L1/PDGFRA fusion gene in HES but not in ET and to evaluate in a larger cohort of ET patients whether the clinical picture might be correlated to the specific molecular lesion.

Introduzione

TROMBOCITEMIA ESSENZIALE

La Trombocitemia Essenziale (TE) è una malattia mieloproliferativa cronica, caratterizzata da un'intensa proliferazione megacariocitaria che determina un incremento del numero delle piastrine circolanti.[1-5] Questo disordine è caratterizzato da piastrinosi, splenomegalia, un'intensa iperplasia megacariocitica midollare una storia clinica priva di sintomi o caratterizzata da episodi emorragici o trombotici od entrambi.

La Trombocitemia, definita inizialmente primaria, fu descritta per la prima volta nel 1934 da Epstein e Goedel.[6] Gli autori descrissero il caso di un paziente con piastrinosi e ripetuti episodi emorragici. A lungo molti autori discussero se la trombocitemia primaria rappresentasse un'entità clinica distinta.[7]

Dameshek nel 1951 per la prima volta inserì la Trombocitemia primaria nel gruppo delle malattie mieloproliferative.[8] In seguito, studi di laboratorio hanno dimostrato che si tratta di un disordine clonale di cellule emopoietiche.[9-13] Queste subiscono una trasformazione che ne modifica il comportamento proliferativo, ma che consente loro di mantenere la capacità di differenziarsi; ciò porta alla produzione di una progenie matura, quantitativamente anomala, ma qualitativamente poco dissimile da quella normale.

La TE, dunque, caratterizzata da un primitivo e intenso incremento della quota piastrinica, appartiene al gruppo delle Sindromi Mieloproliferative (SMP) che comprende anche la Leucemia Mieloide Cronica (LMC), la Policitemia Vera (PV), Mielofibrosi Idiopatica (MF), Leucemia Cronica a Neutrofili (CNL), Leucemia Cronica ad eosinofili (CLE) e Sindrome Ipereosinofila Idiopatica (HES).

A livello cellulare, molti Autori hanno mostrato che, nelle SMP, i progenitori ematologici sono ipersensibili a diversi fattori di crescita.

A livello molecolare, l'aberrante attivazione di specifiche chinasi, con conseguente attivazione inappropriata della cascata di trasmissione del segnale intracellulare, è progressivamente emersa come evento molecolare centrale e comune nella patogenesi di queste malattie.[14-15]

In particolare il ben studiato modello molecolare della LMC, indica che l'attivazione costitutiva di una specifica chinasi codificata dal gene di fusione BCR/ABL, quale primo effetto sostiene l'attivazione del potenziale proliferativo della cellula staminale mieloide e gioca un

ruolo centrale nella trasformazione neoplastica e nel quadro clinico della malattia.[16]
La recente scoperta di un altro gene di fusione (FIP1L1/PDGFRA) in circa un terzo dei pazienti affetti da HES si aggiunge alla crescente lista di tirosine chinasi legate alla patogenesi delle SMP.[17] Molto importanti sono i dati in vitro che evidenziano come l'attività chinasica di questa proteina di fusione è inibita dall'Imatinib (Glivec®) ad una IC50 più bassa di BCR/ABL, e questa osservazione ha portato al successivo trattamento con Imatinib dei pazienti con HES con il gene di fusione FIP1L1/PDGFRA.[18]
L'eccessiva produzione di cellule mature, che caratterizza le SMP, frequentemente coinvolge due o tre linee emopoietiche contemporaneamente, anche se la definizione nosografica delle singole entità si basa sulla linea cellulare maggiormente espansa. Nella LMC, accanto alla caratteristica espansione di precursori della granulopoiesi, aumentano, quasi sempre, sia i granulociti che le piastrine; nella TE avviene lo stesso, anche se con rapporti reciproci invertiti tra granulociti e piastrine; nella PV l'espansione della mielopoiesi è trilineare, con maggiore rilievo per l'iperplasia eritroide. Nella MF, accanto alla patognomonica fibrosi del midollo osseo, aumentano di solito, nella fase florida, precursori della granulopoiesi, granulociti e piastrine.
Per alcune di queste patologie, come la LMC esiste un marker molecolare, per altre sono stati introdotti criteri diagnostici clinici, come per la PV ed in parte per la TE.[19-21]
Ma spesso l'assenza di un marcatore molecolare o di caratteristiche cliniche ben definite rende difficile discriminare tra i vari quadri clinici. In particolare la diagnosi clinica di TE è, in larga parte, basata su criteri d'esclusione ed il trattamento consiste in una soppressione non specifica dell'emopoiesi. Nella tabella 1 sono riportati i criteri diagnostici per la TE.[4]

Tabella 1: Criteri diagnostici di TE

Plt>600x10^9/L in due determinazioni, in un arco di tempo di sei mesi
nessuna causa di piastrinosi secondaria
nessuna evidenza di carenza di ferro
no evidenza di Policitemia Vera (massa eritrocitaria normale)
assenza del riarrangiamento BCR/ABL
assenza di fibrosi midollare
no evidenza di Sindrome Mielodisplastica

Nel 2005, quattro gruppi di ricerca in maniera indipendente, hanno mostrato che i pazienti con PV ed alcuni con MF e TE hanno un'identica mutazione puntiforme acquisita nel gene denominato *Janus kinases-2* (JAK2). Questo gene codifica per una tirosina chinasi; la descritta mutazione porta alla sostituzione di una valina con una fenilalanina in posizione 617 (V617F) nel dominio autoinibitorio di JAK2 (JAK2 *inhibitory domain 2* -JH2-) con la conseguente costitutiva attivazione della chinasi. Questa scoperta costituisce un nuovo punto per la conoscenza della patogenesi di queste sindromi mieloproliferative e per lo sviluppo di un nuovo approccio molecolare diagnostico di queste patologie.[22-25]

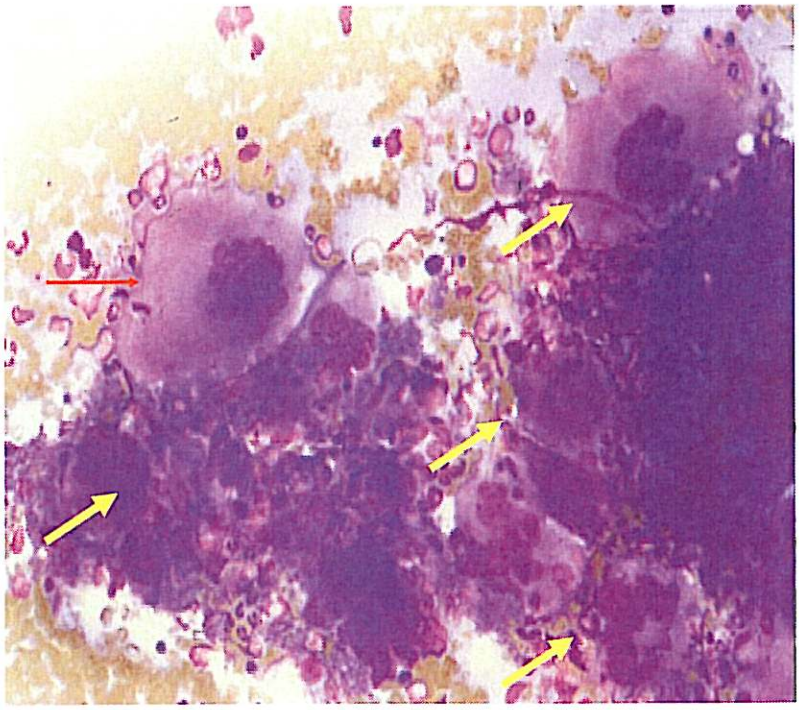


Figura1 **Aspirato midollare**. Donna di 60 anni seguita per TE dal 2000 presso la Sezione di Ematologia del Dipartimento di Biochimica e Biotecnologie Mediche, Università degli Studi di Napoli Federico II.
Microscopio ottico 40x. Colorazione May-Grünwald Giemsa: iperplasia megacariocitica (frecce gialle) e megacariociti displastici (freccia rossa)

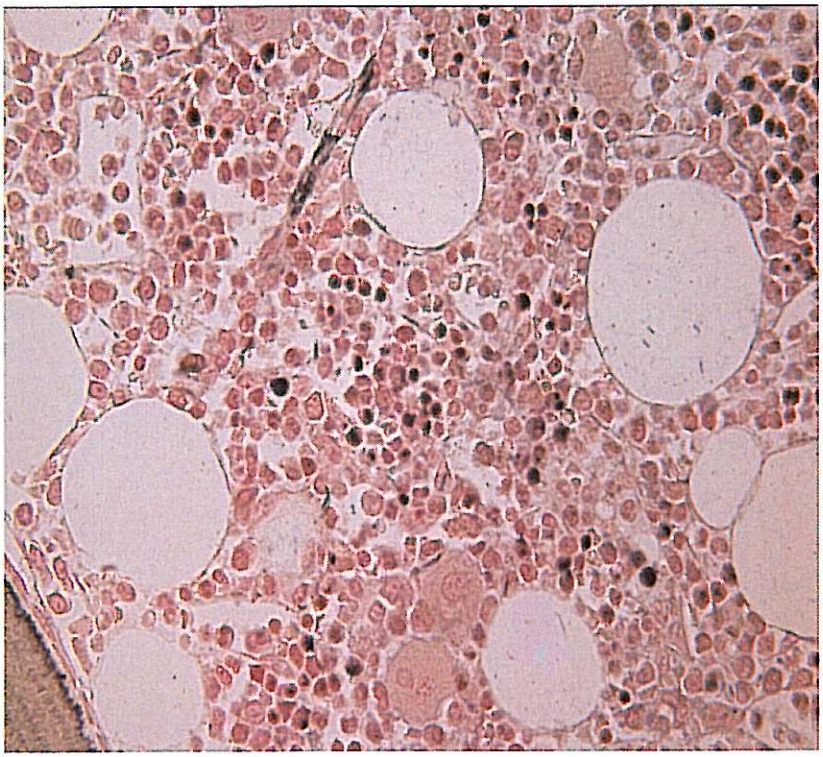


Figura 2 **Biopsia ossea**. Donna di 60 anni seguita per TE dal 2000 presso la Sezione di Ematologia del Dipartimento di Biochimica e Biotecnologie Mediche, Università degli Studi di Napoli Federico II
assenza di fibrosi in un pazienti con TE
Colorazione: impregnazione argentica sec. Gordon-Sweet
Per gentile concessione del prof Guido Pettinato e del dr Giuseppe Ciancia. Area di Anatomia Patologica, Dipartimento di Scienze Biomorfologiche e Funzionali.

TIROSINE CHINASI

Le tirosine chinasi (TK) sono enzimi capaci di trasferire gruppi fosfato dall'Adenosina Trifosfato (ATP) su residui di tirosina presenti su proteine bersaglio.
Esistono circa cento differenti tirosine chinasi nelle cellule umane e sono distinte in due famiglie: TK ad attività recettoriale e TK non recettoriali.
Le prime sono enzimi transmembrana altamente conservati che rispondono al legame con lo specifico ligando alla superficie cellulare e trasmettono il messaggio attraverso la fosforilazione di proteine *target*.
Le non recettoriali si possono trovare nel nucleo o nel citoplasma ed anch'esse sono coinvolte nella regolazione, crescita e morte cellulare.
Dal punto di vista strutturale le TK recettoriali sono glicoproteine di membrana di gruppo I, che attraversano la membrana una sola volta e sono formate da:

- una porzione extra-cellulare, costituita da un dominio proteico di legame N-terminale
- una porzione transmembrana, contenente residui di tirosina che vengono fosforilati quando il recettore è attivato
- un dominio ad attività tirosina-chinasica
- una porzone C-terminale disposta sul lato citoplasmatico della membrana.

Le TK non recettoriali mancano del segmento transmembrana e generalmente funzionano a valle dei recettori TK.
Dall'analisi di studi di cristallografia è emerso che queste proteine sono costituite da un lobo amino-terminale con una struttura a 6 foglietti β ed una α elica e da un lobo carbossi-terminale la cui struttura secondaria è costituita da α eliche; all'interno di questi lobi si trovano 11 domini conservati. Tra questi il dominio I rappresenta il punto di contatto della chinasi con l'ATP, mentre il dominio II è implicato nel trasferimento dei gruppi fosfato al peptide substrato contenuto nel lobo carbossiterminale. La porzione C-terminale contiene siti di autofosforilazione.
Nella conformazione inattiva, la posizione sterica del *loop* preclude l'accesso dell'ATP alla tasca di legame nel sito catalitico della chinasi; l'attivazione si associa ad un mutamento conformazionale del *loop* in modo da permettere il legame dell' ATP e, quindi, il funzionamento dell'attività enzimatica. La transizione dallo stato inattivo allo stato

attivo è reversibile, ed è mediata da numerose proteine intracellulari attraverso la fosforilazione di alcuni residui della proteina e le interazioni a livello dei siti regolatori SH2 ed SH3 (Src *homology*, così chiamati, perché descritti per la prima volta nella TK citosolica cSrc).
Il dominio SH2 è una regione di 100 amminoacidi che può interagire con il sito bersaglio di altre proteine; in condizioni fisiologiche tale sito è inattivo e non fosforilato.
Il legame del ligando al recettore determina la dimerizzazione del recettore e il conseguente cambiamento conformazionale del *loop* di attivazione e del dominio citoplasmatico, con successiva fosforilazione *in trans* dei residui di tirosina tra le due subunità recettoriali costituenti il dimero ed attivazione del dominio catalitico tirosin-chinasico del recettore stesso. [26]
Appartengono alla famiglia delle TK recettoriali molti recettori per fattori di crescita. La costituiva o disregolata attivazione delle tirosine chinasi, attraverso mutazioni od iperespressioni, gioca un ruolo fondamentale in numerose patologie neoplastiche.[26] Nella tabella 2 sono riportati le TK aberranti coinvolte di proteine chinasi in neoplasie umane.

Tabella 2. Tirosine chinasi mutate in neoplasie ematologiche.

Tirosina chinasi	Meccanismo attivazione	neoplasia
BCR/ABL	Traslocazione cromosomica t(9;22)	LMC[27] LAL[28] LAM[29]
TEL/ABL	Traslocazione cromosomica t(9;12)	LAL[30] LMA[31] LMC atipica[32]
TEL/JAK2	Traslocazione cromosomica t(9;12)	LMC atipica[33] LAL[34]
PDGFRβ	Traslocazione cromosomica t(5;10)	CMML[35] LAM[36]
FLT3	Duplicazioni interne in tandem	LAM[37] LAL[37] SMD[38]
PDGFRα	Delezione interstiziale 4q12	HES[17] Mastocitosi sistemica[39]
Alk-1	Traslocazione cromosomica t(2;5)	LNH anaplastico[40]
c-Kit	Mutazioni	LAM [39, 41-42]
FGFR	Traslocazione cromosomica t(8;13)	SMP/LAM[43]

LAL: leucemia acuta linfoide, LAM: leucemia mieloide acuta, CMML: leucemia mielomonocitica cronica, SMD: sindrome mielodisplastica;LNH: linfoma non Hodgkin.

FIP1L1-PDGFRA

Il gene di fusione FIP1L1-PDGFRA costituisce il primo esempio di gene di fusione che non è generato da una traslocazione cromosomica, ma è creato dalla delezione criptica interstiziale di 800-kb sul cromosoma 4q12 [del(4)(q12q12)].[17] La delezione non è visibile con le comuni tecniche di citogenetica.
Tale delezione rompe i gene FIP1L1 e PDGFRA e fonde la parte 5' del FIP1L1 alla parte 3' del gene PDGFRA. [17]

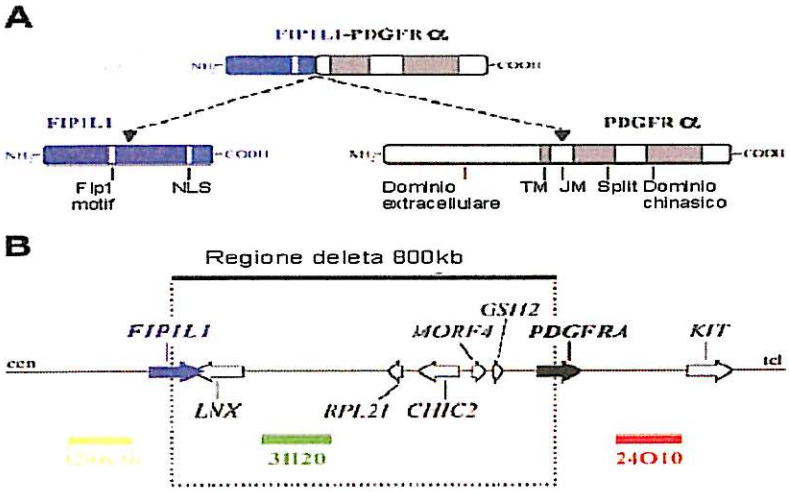


Figura 3 A rappresentazione schematica di FIP1L1, PDGFRA e della proteina di fusione.
NLS: segnale di localizzazione nucleare, TM:regione transmembrana, JM: regione juxtamembrana
Figura 3 B rappresentazione schematica di 4q12. Cen: centromero, Tel: telomero.

In ciascun paziente i *breakpoints* sono differenti, ma le giunzioni tra i geni FIP1L1 e PDGFRA sono sempre in *frame* e determinano la produzione di una proteina ibrida che contiene il sito catalitico del PDGFRα attivato costitutivamente.[17]
I *breakpoints* in FIP1L1 sono distribuiti su una regione di 40 kb (introne 7-10), mentre quelli in PDGFRA sono ristretti ad una regione molto piccola che interessa sempre l'esone 12.[17]
Nei *wild type* si ha lo *splicing* tra l'esone 11 e l'esone 12 di PDGFRA (Figura 4).

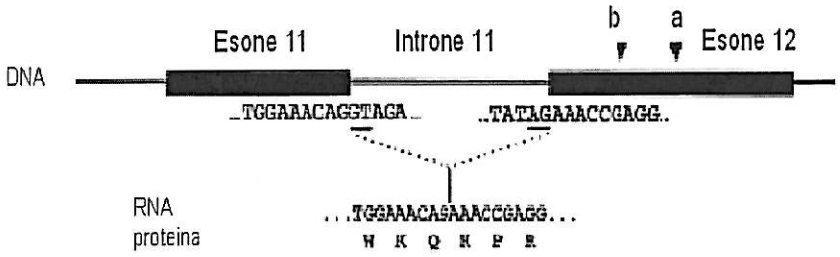
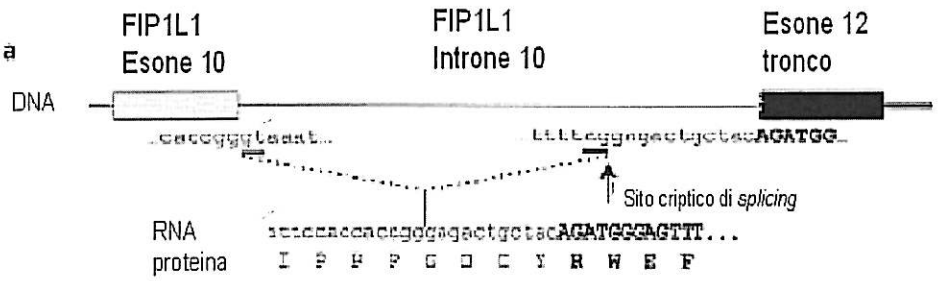


Figura 4 *Splicing* nei *wild type*: i siti sono sottolineati

Nei pazienti con del4q12, la parte 5' dell'esone 12 del PDGFRA è deleta, e pertanto lo *splicing* tra l'esone FIP1L1 e l'esone 12 tronco di PDGFRA può avvenire in due modi: [17,44]

- usando un sito criptico di *splicing* dell'introne di FIP1L1: si veda la figura 5a;



- usando siti di *splicing* criptici con l'esone 12 di PDGFRA: si veda la figura 5b.

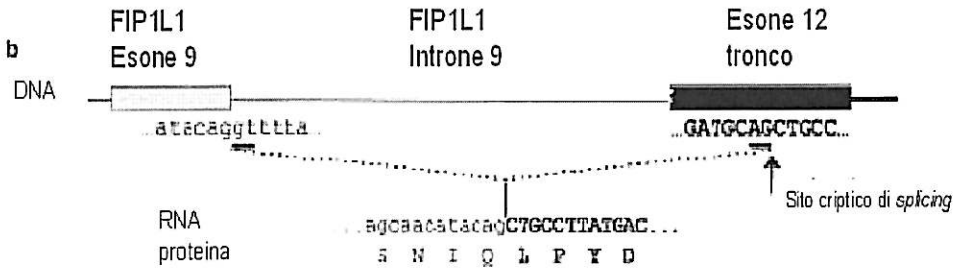


Figura 5 a-b Gli esoni sono segnati come rettangoli, i siti criptici sono sottolineati; la sequenza di FIP1L1 è riportata in minuscolo ed in grigio, quella di PDGFRA in maiuscolo e nero. RNA e la corrispondente proteina sono riportate sotto il DNA, in grigio per FIP1L1 ed in nero per PDGFRα

FIP1L1 è una proteina di 520 aminoacidi che contiene una regione di omologia a Fip1, una proteina implicata nella poliadenilazione.[45-46]
Proteine simili sono state trovate in altre specie animali ed in alcune specie vegetali. Tutte mostrano una regione ben conservata di 42 aminoacidi "Fip1", che è presente anche nella proteina di fusione FIP1L1-PDGFRα. La funzione precisa della proteina FIP1L1 è sconosciuta.

Il **PDGFR** appartiene al tipo III della famiglia dei recettori delle tirosine chinasi che include il c-KIT, FLT3 e CSFR1 ed è caratterizzato da un dominio transmembrana, uno juxtamembrana ed un dominio ad attività chinasica.

Recettori ad attività tirosina-chinasi tipoIII

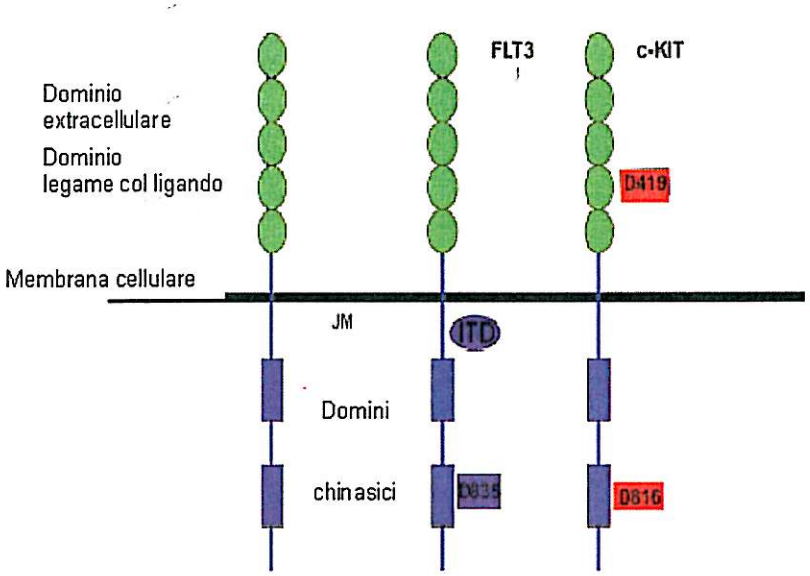


Figura 6 Recettori ad attività chinasica Tipo III.

PDGFR è il partner in molte traslocazioni cromosomiche. La traslocazione TEL/PDGFRβ, ad esempio, è la più frequente tra quelle che generano proteine di fusione non ABL, implicate con neoplasie ematologiche

Come le altre tirosine chinasi di fusione, FIP1L1-PDGFRα è una tirosina chinasi costitutivamente attiva, che trasforma le cellule ematopoietiche in vivo ed in vitro.[17,47] FIP1L1-PDGFRα fosforila se stessa e la proteina traduttrice del segnale ed attivatrice della trascrizione5 (STAT5) ma, in contrasto con la PDGFRα nativa non attiva il *pathway* di MAPK.[17,48-49] La differenza nel *signaling* di MAPK

può essere spiegata dalla differenza nella localizzazione cellulare, perché PDGFRα è una proteina transmembrana con accesso a RAS farnesilato, mentre FIP1L1-PDGFRα è citosolico. A supporto di questa ipotesi l'ancoraggio alla membrana di PDGFRβ è fondamentale per la sua capacità di attivare il *pathway* di MAPK.[50] Il meccanismo della costitutiva attivazione della tirosina chinasi FIP1L1-PDGFRα non è ben conosciuto. Si potrebbe dedurre, studiando il rapporto tra funzione e struttura di altre tirosine chinasi come BCR-ABL[51], TEL-PDGFRβ[52],TEL-ABL[31],H4-PDGFRbeta[35], HIP1-PDGFRβ[53], e TEL-JAK2[34], che FIP1L1 possa contribuire alla omodimerizzazione che serve ad attivare costitutivamente la chinasi PDGFRα.

L'esone 12 di PDGRFA codifica per un dominio juxtamembrana che ha una funzione autoinibitoria in altre tirosine chinasi. La rottura di questo dominio, con mutazioni *missense*, inserzioni o delezioni in *frame*, porta ad una costitutiva attivazione della tirosina chinasi interessata.

La regione juxtamembrana di PDGRFβ contiene un dominio WW che ha una funzione inibitoria per l'attività chinasica, e mutazioni puntiformi in *frame* del dominio WW risultano nella costitutiva attivazione della chinasi.[41] Tutti i geni di fusione FIP1L1-PDGFRA codificano per una proteina PDGFRα tronca nella regione WW, suggerendo che l'interruzione del dominio WW contribuisca all'attivazione di FIP1L1-PDGFRα. Allora, un'ipotesi plausibile è che l'interruzione nella regione WW dell'esone 12, in combinazione con l'iperespressione del gene di fusione che è controllato dal promotore della trascrizione di FIP1L1 siano gli eventi chiave nella trasformazione neoplastica delle cellule.

Il gene di fusione (FIP1L1/PDGFRA) è presente in circa un terzo dei pazienti affetti da HES e nei pazienti con mastocitosi ed eosinofilia. [17]

E' stato dimostrato che il gene di fusione FIP1L1/PDGFRA si trova in gran parte delle cellule della serie mieloide, tuttavia, i meccanismi molecolari che sono alla base dell'espansione preferenziale delle cellule eosinofili non sono ancora noti. Non vi sono, inoltre, dati riguardo alla espressione di FIP1L1 nei soggetti normali.

Il riarrangiamento FIP1L1-PDGFRA è stato decritto negli eosinofili, nei neutrofili e nelle cellule mononucleate con la FISH e la RT-PCR in pazienti con mastocitosi ed eosinofilia. [39] E' possibile che esso sia presente nelle linee mieloidi ma che solo gli eosinofili siano particolarmente sensibili ai segnali proliferativi di FIP1L1-PDGFRα.

JAK2

JAK2 è una tirosina chinasi che fu clonata nel 1989 e chiamata scherzosamente JAK per "*just another kinase*"
Fa parte di una superfamiglia di chinasi che nell'uomo è composta da altri tre elementi JAK1, JAK3 e TYK2.
JAK è legata alla porzione citoplasmatica del recettore dell'Eritropoietina (EPOr) ed è formata, dal punto di vista strutturale, da tre domini critici, JH1, il dominio ad attività chinasica, JH2 che ha attività inibitoria e il residuo amminoterminale FERM; quest'ultimo è responsabile del legame non covalente con la regione *box*1 presente nelle porzioni juxtamembrana citoplasmatiche, dei recettori tipo I delle citochine, come EPOr.
JAK, insieme ad EPOr ed all'eritropoietina (EPO) è importante nella maturazione delle cellule eritroidi.[54] Il *knock out* di JAK negli embrioni di topo è letale entro la 12ª giornata di vita embrionale per l' impossibilità di formare cellule eritroidi mature.
In assenza del ligando EPOr è in conformazione dimerica, con i domini transmembrana separati da 73 Å. Il legame di EPO al suo recettore provoca una modifica conformazionale nel recettore stesso, portando i due domini in posizione vicina con una distanza di 39 Å. (Figura7)

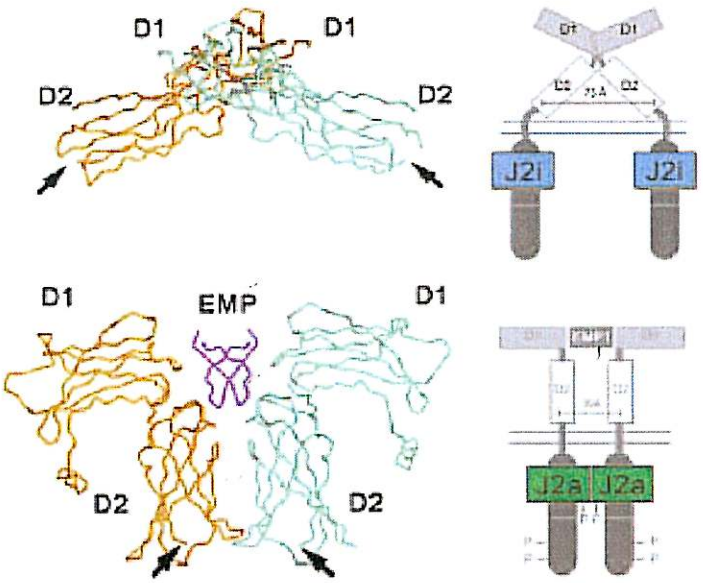


Figura 7 **Struttura di EPOr in assenza o presenza del ligando peptide Epo mimetico (EMP).** Rappresentazione degli eventi intracitoplasmatici che seguono al legame col ligando.
J2i: JAK2 inattivo; J2a: JAK2 attivo.

Numerosi sono i meccanismi molecolari che regolano l'attività chinasica di JAK2. La presenza di isolati domini JH1 di JAK2 portano alla costitutiva attivazione della chinasi. Invece, l'aggiunta di domini JH2 riduce molto il livello di autoattivazione [55]
Modelli molecolari di domini JH1 e JH2 di JAK2 basati sulla struttura dimerica dei recettori per fattori di crescita dei fibroblasti indicano che una regione JH2 interagisce con il *loop* di attivazione chinasica del dominio[56] (Figura 8).

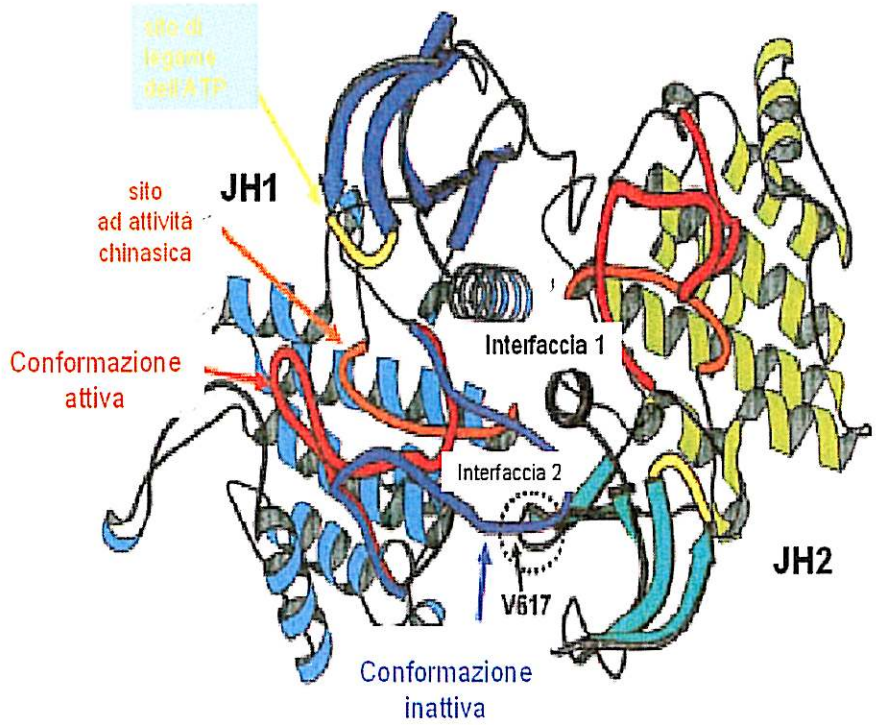


Figura 8 **Struttura di JAK2.**
Regioni JH1 (in blue) e JH2 (in verde). Il *loop* di attivazione di JH1 è mostrato nella possibile conformazione, attiva (rossa) ed inattiva (blue).
Il sito ad attività chinasica è mostrato in arancione ed il sito di legame dell'ATP in giallo. Il sito di interazione di JH2 ed il dominio attivo di JH1 è rappresentato nel cerchio, dove si trova la Val617

Numerosi esperimenti hanno dimostrato che la regione di aminoacidi da 619 a 670 è necessaria per l'azione inibitoria di JH2 sull'attività chinasica di JH1. [57]
Il meccanismo di trasmissione del segnale da EPOr inizia con il legame di EPO al recettore. L'omodimerizzazione del recettore provoca l'attivazione di JAK2.

Tre residui idrofobici di EPOr sono necessari per l'attivazione di JAK2.[58] Dopo l'attivazione del recettore, JAK viene fosforilato ai residui Y 1007 e 1008 e a molti altri residui Y, alcuni ad azione inibitoria (come 570) ed altri con attività stimolatoria del *signaling*.[59-60] JAK2 fosforila anche residui Y citoplasmatici di EPOr. Tutti i residui Y fosforilati di JAK2 ed EPOr diventano siti di legame ed attivazione di STAT5, STAT3, STAT1, SH-PTP1, CIS e shc-grb2.
Come conseguenza si ha un'attivazione del *pathway* di STAT, Map-chinasi, PI-3-chinasi ed AKT. Le conseguenze biologiche di questi eventi sono la sopravvivenza, la proliferazione e la differenziazione dei progenitori eritroide (Figura 9). In successione vengono attivate, proteine regolatrici come CIS e SOCS così come si attivano alcune fosfatasi come Shp2, che regolano in maniera negativa la trasduzione del segnale (Figura 10).

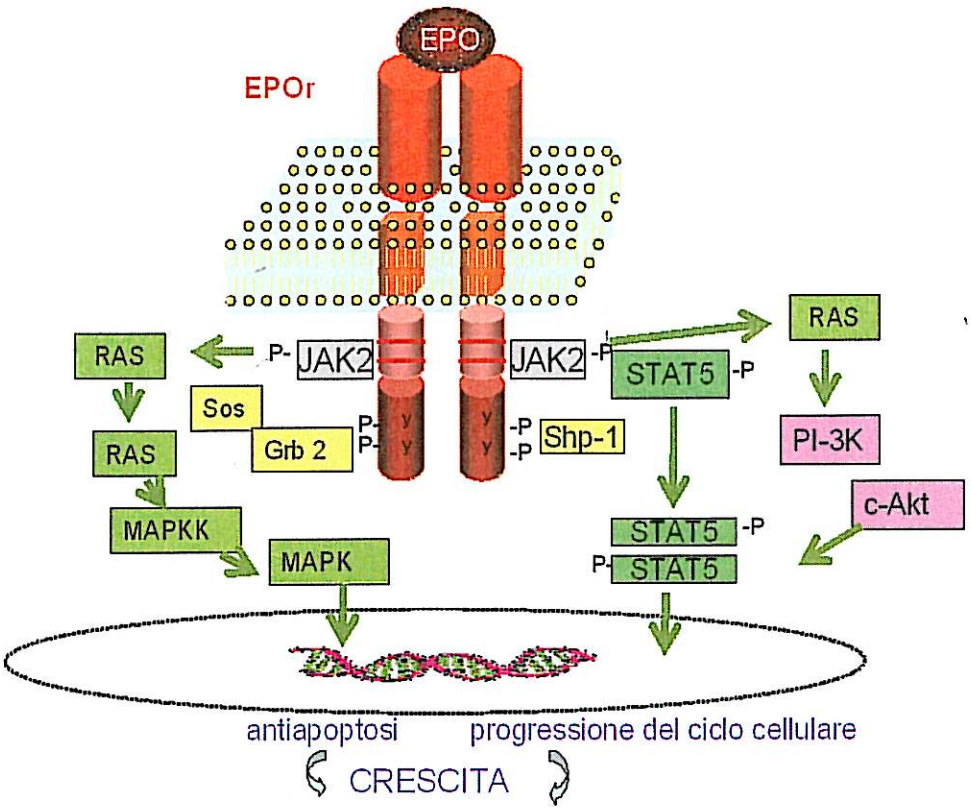


Figura 9 ***Signaling* di JAK2 nelle Sindromi mieloproliferative.** RAS, MAPKK, MAPK, STAT5, PI-3K, c-AKT proteine ad attività chinasica attivate dalla fosforilazione di JAK2

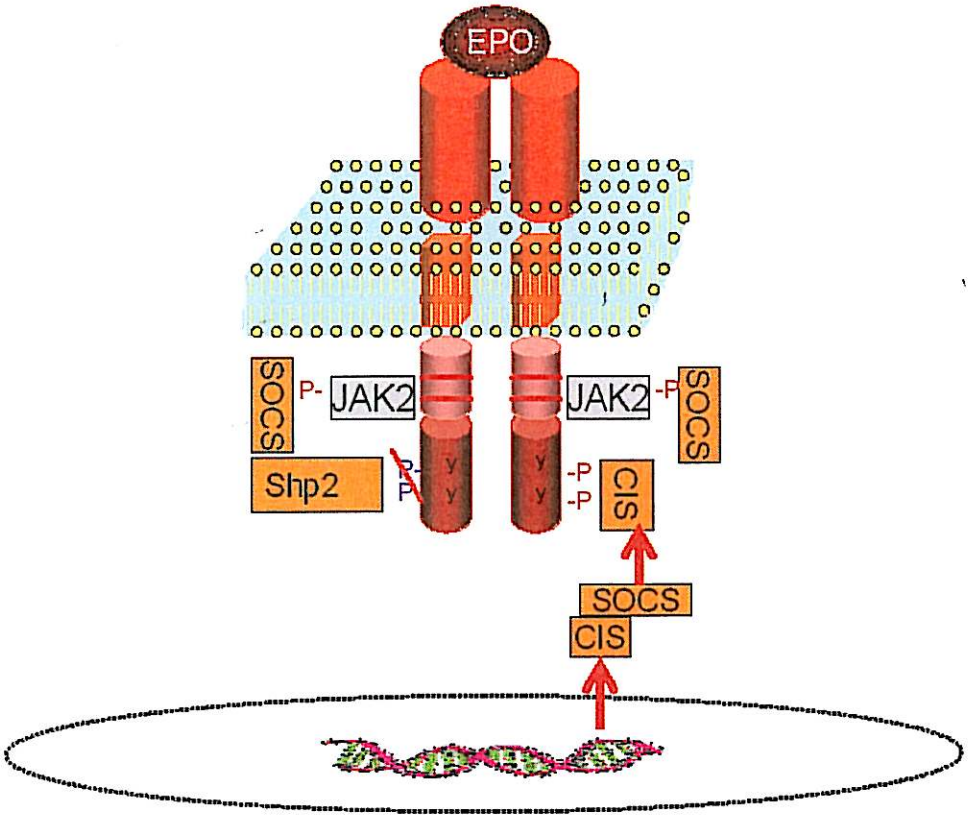


Figura 10 ***Signaling* di JAK2 nelle Sindromi Mieloproliferative.** Le proteine SOCS and CIS sono fosfatasi così come come Shp2, che modulano negativamente il *signaling.*

Nel 2005 è stata identificata una mutazione nel dominio JH2 di JAK2.[22-25] Tale mutazione è caratterizzata dalla sostituzione della valina con la fenilalanina in posizione 617 (V617). La sostituzione della piccola valina con la grossa fenilalanina provoca un'alterazione strutturale nel dominio JH2 modificando la capacità inibitoria (Figura 11).

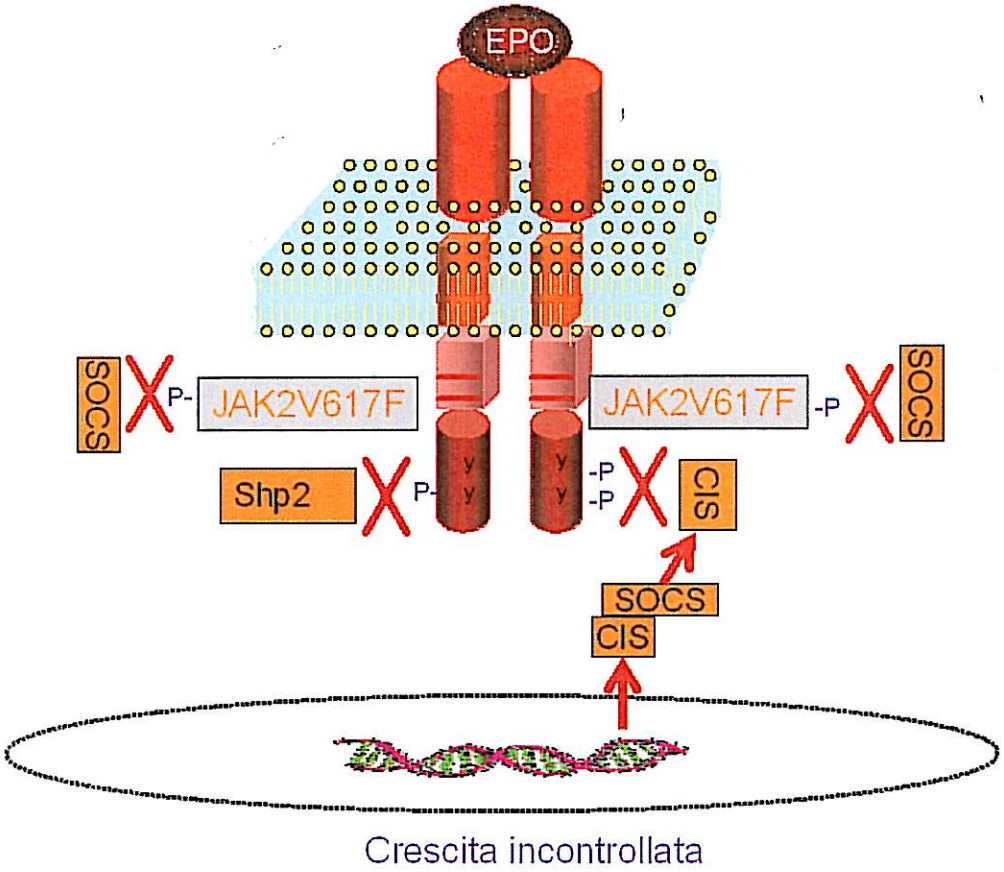


Figura 11 **Mutazione V617F di JAk2.**
La mutazione V617F di JAK2 modifica la struttura del dominio JH2 ed impedisce l'azione delle proteine inibitrici

La mutazione di JAK2 è somatica ed interviene nella cellula staminale ematopoietica.
Un modello in due *steps* per il ruolo di JAK2 spiega l'evoluzione clonale delle Sindromi Mieloproliferative. Il primo *step* consiste in una transversione G→T che sostituisce alla valina una fenilalanina alla posizione 617 in un allele JAK2 sul cromosoma 9p. Emerge, così, un clone che è eterozigote per JAK2 V617F e si espande e tenta di sostituire le cellule ematopoietiche senza la mutazione. Il secondo *step* consiste in una ricombinazione mitotica in una cellula ematopoietica che è eterozigote per JAK2 V617F. Questo genera una disomia uniparentérale e la perdita dell'eterozigosi del 9p (9pLOH) in una delle due cellule figlie. La cellula figlia che è omozigote per JAK2 V617F da origine ad un nuovo clone che si espande e reprime il precedente clone eterozigote (dominanza clonale). La transizione dalla normale emopoiesi all'emopoiesi clonale della cellula eterozigote ed il passaggio dall'eterozigosi all'omozigosi sono processi continui caratterizzati dalla presenza di una popolazione mista di cellule. [22]
In tutti gli studi la mutazione è stata trovata nelle cellule mieloidi, ma non nei linfociti T. Mutazioni di JAK2 sono state identificate nelle colonie eritroidi derivate da cellule progenitrici eritroidi.[23]
L'incidenza di questa mutazione nei pazienti con PV è molto alta (dal 65% al 97%). La mutazione V617F è stata trovata anche nei pazienti con MF (50%) e nei pazienti con TE (dal 25% al 57%). [22-25] In molti casi di TE la mutazione è stata trovata a titolo molto basso nel DNA ottenuto dalle cellule midollari, appena evidenziabile con le tecniche di sequenziamento. Questo può significare, come ci si aspetta, che l'emopoiesi nelle TE non è completamente clonale in molti pazienti e che la mutazione deve essere ricercata con tecniche più sensibili od in megacariociti isolati.

Scopi dello studio

Scopo dello studio è stato quello di valutare nei pazienti affetti da TE la presenza del gene di fusione FIP1L1/PDGFRA e quella della mutazione di JAK2.
Sono state quindi ricercate eventuali correlazioni tra il gene di fusione FIP1L1/PDGFRA o la mutazione di JAK2 e parametri clinici:

(i) parametri sierici della malattia quali LDH, beta2 microglobulina;
(ii) piastrinosi, leucocitosi ed eventuale eritrocitosi;
(iii) fenotipo clinico, per volumetria splenica ed eritromelalgia.

E' stata esaminata, inoltre, nei casi con duplice alterazione, la presenza di un fenotipo diverso, potenziale indicatore di sottogruppi di pazienti.

Materiali e metodi

Pazienti

Sono stati valutati tutti i pazienti con piastrinosi seguiti presso l'ambulatorio delle Sindromi Mieloproliferative Ph- della Sezione di Ematologia del Dipartimento di Biochimica e Biotecnologie Mediche dell'Università degli Studi di Napoli Federico II. Sono stati inseriti nello studio quaranta pazienti osservati tra il giugno del 2001 ed il giugno 2006.
La diagnosi di TE è stata formulata secondo i criteri del Polycythemia Vera Study Group, cioè piastrine >600x10^9/L, emoglobina o massa eritrocitaria normale, assenza del cromosoma Philadelphia, assenza di fibrosi midollare e nessuna causa di piastrinosi secondaria.[61]
Il consenso informato è stato ottenuto da tutti i pazienti arruolati.
Le caratteristiche ematologiche dei pazienti sono riassunte nella tabella 3.

Tabella 3. **Caratteristiche dei pazienti**

Pazienti (n)		40
M/F		18/22
Età (anni)	media *range*	47 19-84
Emoglobina (g/dL)		14,0 9,9-17,8
GB (x10^9/L)		9,6 4,4-19,1
Piastrine (x10^9/L)		816 545 -1936
Volume splenico (mL)		519 106 -1066
Cariotipo	normale	28
	alterato	4 cariotipi complessi
	non valutabile	8

Raccolta del sangue midollare, preparazione cellulare ed isolamento degli acidi nucleici.

Sangue midollare

In occasione della programmata procedura diagnostica in pazienti mai trattati in precedenza ed avendo ottenuto il consenso informato scritto, 8-10 mL di sangue midollare, previo anestesia locale, sono stati aspirati secondo le comuni metodiche, dalla spina iliaca postero superiore sinistra, in assoluta sterilità, e raccolti in *vacutainer* contenenti EDTA quale anticoagulante, conservati a temperatura ambiente ed inviati all'accettazione del settore dedicato.
L'analisi molecolare è stata effettuata presso il CEntro di INgegneria GEnetica (CEINGE) - Biotecnologie Avanzate.

Preparazione *pellet* cellulare

Le cellule mononucleate del sangue midollare (BMMNCs) sono state isolate mediante separazione con Ficoll-Hypaque al 20%; sono state lavate due volte in soluzione salina e risospese in 600 μL di una soluzione buffer di Guanidinaisotiocianato (GTC) e conservate a – 20°C, in almeno due aliquote per ciascun paziente.

Estrazione RNA e DNA

E' stato usato il kit "*GenEluate Total RNA*" (SIGMA, San Louis MO, USA) per l'estrazione, seguendo le indicazioni della casa produttrice con il seguente schema:
(a) le cellule sono state lisate in una specifica soluzione (*Sigma's GenEluteTM Mammalian Total* RNA Kit);
(b) il campione è stato più volte aspirato con siringa da 5 mL, attraverso un ago 22 G (frammentazione DNA genomico);
(c) 500 μL della sospensione ottenuta è stata caricata su una colonnina filtrante (*Sigma's GenEluteTM Mammalian Total* RNA Kit) e centrifugata in *microfuge* a 14000 x g per 2 minuti per la rimozione detriti cellulari;
(d) all'eluato ottenuto sono stati aggiunti 500 μL di etanolo al 70%;
(e) 700 μL della miscela sono stati caricati su colonnina legante l'RNA (*Sigma's GenEluteTM Mammalian Total* RNA Kit), il campione è stato centrifugato quindi per un minuto a 14000 x g ed è stato decantato l'eluato;
(f) 500 μL di soluzione di lavaggio 1 (*Sigma's GenEluteTM Mammalian Total* RNA Kit) sono stati caricati sulla colonnina per assicurare la rimozione del materiale legato alla resina ed il campione è stato di nuovo centrifugato a 14000 x g per un minuto;
(g) 500 μL di soluzione di lavaggio 2 (*Sigma's GenEluteTM Mammalian Total* RNA Kit) è stata caricata sulla colonnina ed è stata

di nuovo centrifugata a 14000 x g per un minuto. Tale fase è stata ripetuta due volte per rimuovere i residui di etanolo;
(h) la purificazione dell'RNA dalla colonnina è stata effettuata con 50 μL di soluzione di eluizione (*Sigma's GenEluteTM Mammalian Total* RNA Kit) precedentemente riscaldata a 65°C, centrifugando per due minuti a 14000 x g.
L'integrità dell'RNA totale è stata controllata elettroforeticamente su un gel d'agarosio all'1% con etidio bromuro contente 2.2 mol/L di formaldeide; la quantizzazione è stata effettuata con analisi spettrofotometrica a 260 e 280 nm (secondo le comuni procedure).
Il totale dell'RNA ottenuto (1 μg) è stato denaturato a 70 °C per 10 minuti e messo in ghiaccio.
Il DNA genomico è stato estratto da un'aliquota di $2x10^6$ cellule mononucleate usando il Nucleon BACC2 kit (Amersham Biosciences Europe GmbH, Freiburg Germany) seguendo le indicazioni della casa produttrice.

Amplificazione mediante RT-PCR di FIP1L1/PDGFRA

Reazione di retrotrascrizione

1 μg di RNA diluito in 7,2 μL di dd H_2O è stato denaturato a 70°C per 10 minuti, dopo l'aggiunta di 12,8 μL di RT mix contenente:
Buffer II (2 μL),
MgCl2 (4 μL),
DTT (2 μL),
Random esameri (2 μL),
dNTP (0,8 μL),
RNAsin (1 μL), RT (1 μL).
La reazione di retrotrascrizione è stata effettuata con il seguente profilo tempo/temperatura:
25 °C per 10 minuti
42 °C per 45 minuti
99 °C per 3 minuti.
Alla reazione finale di 20 μL (7,2 μL di RNA e 12,8 μL di H_2O) sono stati aggiunti 30 μL di H_2O (totale 50 μL).

Reazione PCR

L'analisi per la ricerca della presenza del gene di fusione FIP1L1/PDGFRA è stata eseguita con tecnica di PCR (a) e Nested-PCR (b) come descritto da Cools e collaboratori[17] con l' uso della coppia di primers FIP1L1-F4 e PDGFRA-R1 per la PCR e FIP1L1-F5 e PDGFRA-R2 per la Nested
La qualità del materiale è stata valutata con l'amplificazione di un gene di controllo quale il porfobilinogeno (c).

a) PCR

Primers utilizzati per la reazione di PCR[17]

Nome *primer*	Simbolo	Sequenza	Posizione
Forward PCR del 4q12	FIP1L1-F4	ACCTGGTGCTGATCTTTCTGAT	esone 7 (ultime 5 basi)- esone 8 del gene FIP1L1
Reverse PCR del 4q12	PDGFRA-R1	TGAGAGCTTGTTTTTCACTGGA	esone 14 del gene PDGFRA

Il cDNA ottenuto dalla reazione di trascrittasi inversa è stato amplificato dalla PCR in una miscela con volume finale di 50 μL contenente:
10mM Tris-HCL (pH 8.3)
50 mM KCl
2.5 mM $MgCl_2$
0.2 mM di ciascun deossiribonucleotide
1 U di Taq polimerasi,
0.4 μM dei *primers* FIP1L1-F4 e PDGFRA-R1.
La reazione di amplificazione è consistita di 35 cicli, in tre tempi, con il seguente profilo tempo/temperatura:
95°C per 30 secondi (denaturazione)
58°C per 1 minuto (*anniling*)
72°C per 1 minuto (polimerizzazione).

b) Nested PCR

4 μL della prima PCR sono stati usati per una seconda amplificazione, ottenuta con la coppia di primer *nested* FIP1L1-F5 e PDGFRA-R2.

Primers utilizzati per la reazione di *nested*-PCR [17]

Nome *primer*	Simbolo	Sequenza	Posizione
Forward Nested-PCR del 4q12	FIP1L1-F5	AAAGAGGATACGAATGGGACTTG	esone 8 (71-93bp di 131bp) del gene FIP1L1
Reverse Nested-PCR del 4q12	PDGFRA-R2	GGGACCGGCTTAATCCATAG	esone 13 del gene PDGFRA

La reazione *nested* è consistita di 35 cicli, con il seguente profilo tempo/temperatura:
95°C per 30 secondi (denaturazione)
60°C per 1 minuto (*anniling*)
72°C per 1 minuto (polimerizzazione)
I prodotti di PCR sono stati analizzati su gel di agarosio al 2x contenente etidio bromuro in TBE1x e le bande amplificate

sequenziate con apparecchiatura ABI3730 (Applera, Foster City, CA, USA) per stabilire la giunzione tra gli esoni FIP1L1 e PDGFA.

Amplificazione mediante PCR del gene di controllo (porfobilinogeno)

I *primers* usati in PCR per amplificare il porfobilinogeno (PBG-D) sono stati P-SE e P-AS. La sequenza di riferimento è il locus HSPBGDR2 con codice X04808: Human mRNA *for non erythropoietic porphobilinogen deaminase*:

Nome *primer*	Simbolo	Sequenza	posizione
Forward PBG-D	P-SE	CTGGTAACGGCAATGCGGCT	esone 1 (156-175 bp di 184 bp9 del gene PBG-D
Reverse PBG-D	P-AS	GCAGATGGCTCCGATGGTGA	esone 6 (76-57 bp di 78 bp9 del gene PBG-D

c) PCR Porfobilinogeno

Condizioni della PCR per il Porfobilinogeno .
Dopo la denaturazione iniziale a 95°C per 10 minuti la reazione di amplificazione si basata su 35 cicli, in tre tempi con il seguente profilo tempo/temperatura:
94°C per 30 secondi (denaturazione)
60°C per 1 minuto (*anniling*)
72°C per 1 minuto (polimerizzazione).

Sequenziamento degli amplificati FIP1L1-PDGFRA

La positività in Nested PCR è stata confermata mediante sequenziamento.
Le sequenze dei pazienti sono state allineate con:
1) NT_0022853: DNA *Homo sapiens chromosome 4 genomic coding*;
2) NM_006206: *Homo sapiens platelet-derived growth factor receptor, alpha polypeptide (PDGFRA), mRNA.*
3) NM_030917: Homo sapiens FIP1L1 (saccaromyces cerevisiae), mRNA.
L'analisi della sequenza dei prodotti amplificati rivela che il dominio chinasico del gene PDGFRA (*GenBank accession number NM_006206*) è unito al gene FIP1L1 (*GenBank accession number NM_030917)*.

FISH

La fusione tra FIP1L1 e PDGFRA sul cromosoma 4 si origina da una delezione interstiziale di circa 800kb che comprende il locus CHIC2 (*cysteine-rich hydrophobic domain2*).[17]

Dopo l'analisi mediante PCR, per confermare la presenza della delezione 4q12 abbiamo utilizzato la FISH in interfase ad un colore. Questa tecnica prevede l'uso di un probe diretto contro CHIC2 con la fluorescenza verde che si trova tra FIP1L1 e PDGFRA (Figura 12).

La perdita di uno dei due segnali di CHIC2 in un paziente con il gene di fusione FIP1L1-PDGFRA è stata descritta da Cools e collaboratori.[17]

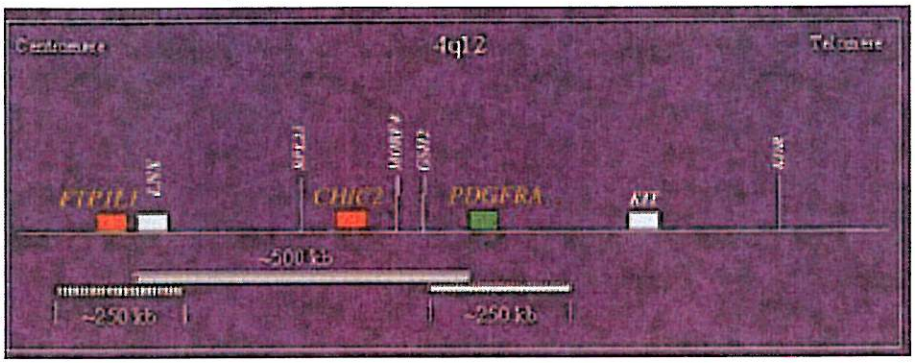


Figura 12 **Locus CHIC2 situato tra FIP1L1-PDGFRA**

Il *probe* è stato disegnato usando cromosomi artificiali di batteri BAC (*bacterial artificial chromosome*) clonati che coprivano l'aria di interesse sul 4q12.

I cloni BAC ricevuti dalla *ResGen Invitrogen*®, sono stati propagati subito dopo l'arrivo.

Per ciascun paziente il *pellet* di cellule midollari è stato conservato a -70°C con un fissativo formato da metanolo ed acido acetico (2:1). Tutti i campioni sono stati processati sia per la FISH che per la citogenetica convenzionale usando le procedure standard. [62]

Per la FISH sono stati usati vetrini di sangue midollare.

La sonda utilizzata è stata: RP11-367N1.

La delezione interstiziale 4q12 di uno dei due CHIC2 risulta da un solo segnale di ibridazione verde.
Due operatori, in maniera indipendente, hanno analizzato circa 70 nuclei (140 totali) tra il materiale di ciascuno dei sei pazienti con il gene di fusione FIP1L1-PDGFRA dello studio, di 20 individui sani e dei rimanenti 34 pazienti senza il gene di fusione. I *patterns* rappresentativi di nuclei in interfase normali ed anormali sono presentati in figura 13

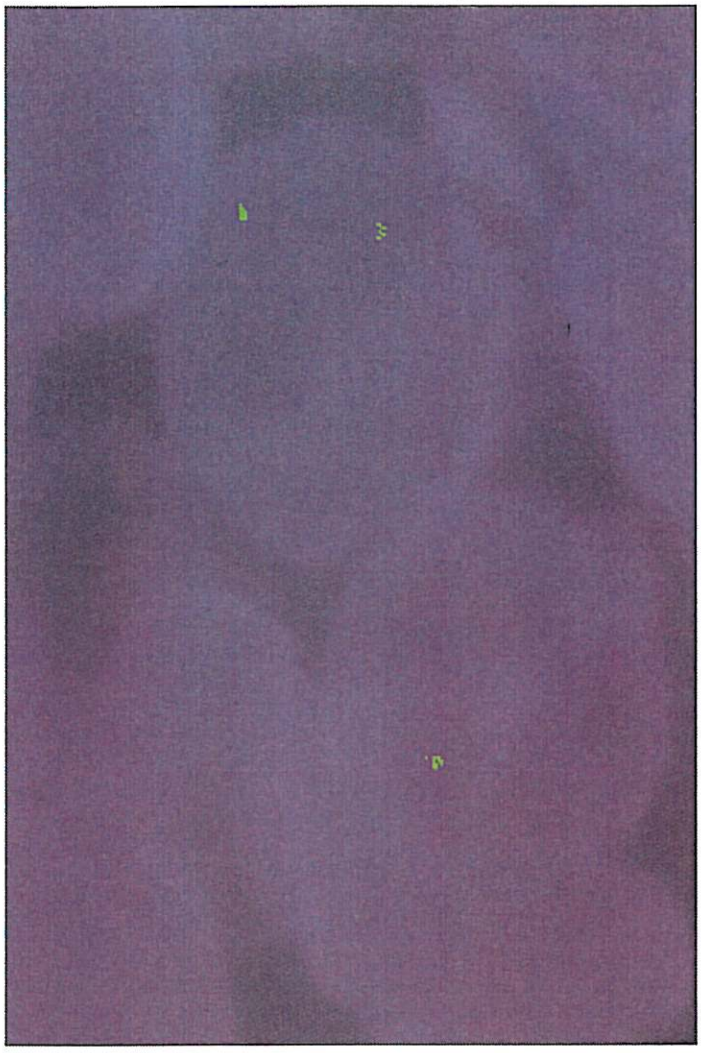


Figura 13 **FISH in due cellule in Interfase.**
Paziente 1: una cellula (in alto a sinistra) mostra due segnali CHIC2 (*pattern* normale) ed una cellula (in basso a destra) con un singolo segnale CHIC2 (*pattern* patologico).

Analisi della mutazione V617F JAK2

Tutti i campioni di sangue midollare sono stati analizzati per la presenza di V617F di JAK2, mediante sequenziamento diretto ed *amplification refractory mutation system* (ARMS) PCR.[23]
Sono stati disegnati dei primers che permettono di amplificare in RT-PCR una regione dell'esone 12 del gene JAK2, contente il codone GTC codificante per una valina nella sequenza *wild-type* o il codone TTC codificante per una fenilalanina nella sequenza mutata.

Nome *primer*	Simbolo	Sequenza	Posizione
Forward JAK2	JAK2-F	GCAGCAAGTATGATGAGCAAGC	esone 14 del gene JAK2
Reverse JAK2	JAK2-R	AAAATGCATGGCCCATGC	esone 15 del gene JAK2

La reazione di PCR è stata effettuata in una miscela con volume finale di 50 μL contenente:
10mM Tris-HCL (pH 8.3),
50 mM KCL,
2 mM $MgCl_2$,
0.2 mM di ciascun deossiribonucleotide,
2.5 U di Taq polimerasi,
0.5 μM di ciascun *primer*
Una denaturazione iniziale è stata ottenuta a 95°C per 10 minuti e poi la reazione è stata effettuata con 35 cicli con il seguente profilo tempo/ temperatura:
95°C per 30 secondi (denaturazione)
53 °C per 45 secondi (*anniling*)
72°C per 1 minuto (polimerizzazione)
I prodotti di PCR di 201 bp sono stati fatti migrare su gel di agarosio 2x in TBE1x.
La banda di 201bp amplificata è stata direttamente sequenziata in entrambe le direzioni con lo strumento per il sequenziamento ABI3730 (Applera, Foster City, CA, USA), e i campioni sono stati considerati mutati quando la sostituzione della base è stata trovata su entrambi i filamenti.

ARMS

In 7 pazienti la presenza della mutazione è stata confermata usando il sistema ARMS.[23]

Nome *primer*	Simbolo	Sequenza	Posizione
Forward AS1 *Forward* AS2	AS-F1 AS-F2	AGCATTTGGTTTTAAATTATG GAGTATATT ATCTATAGTCATGCTGAAAGT AGGAGAAAG	esone 12 del gene JAK2
Reverse AS	AS-R1	CTGAATAGTCCTACAGTGTTT TCAGTTTCA	esone 12 del gene JAK2

Il DNA dei pazienti è stato amplificato usando 1 μM di un *primer reverse* e 0.5 μM di due *primers forward*. Il primo *primer forward* è specifico per l'allele mutato e contiene un *mismatch* al terzo nucleotide del 3' terminale per aumentare la specificità, mentre il secondo *primer* amplifica una sequenza di entrambi gli alleli, sia il *wild-tipe* sia il mutato ed è usato come controllo interno del test.

Risultati

In un periodo compreso tra il 2001 ed il 2006, 40 pazienti con diagnosi clinica di TE sono stati arruolati nello studio. Tutti i pazienti hanno ricevuto diagnosi di TE secondo i criteri del PVSG.
Ciascun paziente è stato valutato con:

a) raccolta dei dati anamnestici e bioumorali secondo le linee guida comuni della *Good Medical Practice*;
b) analisi molecolare PCR per verificare l'assenza della traslocazione BCR/ABL, ed escludere una presentazione di LMC con trombocitosi;
c) analisi citogenetica;
d) studio della massa eritrocitaria con Cr_{51} nelle donne con Ht>45% e negli uomini con Ht>47%, per escludere una presentazione atipica della Policitemia Vera;
e) *grading* della fibrosi midollare per escludere una fase florida della mielofibrosi;
f) misurazione della volumetria splenica con tecnica ecografia.[63]

L'età media di questi pazienti, distribuiti in maniera uniforme per sesso, è risultata essere di 47 anni.
Otto di questi pazienti (20%) hanno mostrato a diagnosi una lieve leucocitosi, con una conta di globuli bianchi mai superiore a 20000/ μL. Nessuno dei pazienti ha presentato ipereosinofilia.
La citogenetica convenzionale ha fallito in 6 casi per l'assenza di metafasi ed ha rilevato in 4 pazienti un cariotipo complesso.
Le caratteristiche dei pazienti sono riassunte nella tabella 4

Tabella 4:caratteristiche molecolari dei pazienti.

N	Sesso	età	FIP1L1-PDGFRA	JAK2	Citogenetica	del4q12 in FISH
1	M	22	Pos in N	W.T.	46,XY	si
2	F	24	Pos in N	W.T.	46,XX	si
3	M	28	Pos in N	W.T.	46,XY	si
4	M	52	Pos in N	Mutato	46,XY	si
5	F	54	Pos in PCR	W.T.	46,XX	si
6	F	56	Pos in N	W.T.	N.V.	si
7	M	71	neg	Mutato	45,X-Y[3] 46,XY[18]	
8	F	24	neg	Mutato	46,XX	
9	F	63	neg	Mutato	46,XX	
10	M	21	neg	Mutato	46,XY	
11	F	69	neg	Mutato	46,XX	
12	M	26	neg	Mutato	46,XY	
13	M	22	neg	Mutato	46,XY	
14	M	46	neg	Mutato	46,XY	
15	M	57	neg	Mutato	46,XY	
16	M	50	neg	Mutato	46,XY	
17	F	64	neg	Mutato	46,XX	
18	M	84	neg	Mutato	N.V.	
19	M	69	neg	Mutato	N.V.	
20	M	45	neg	Mutato	N.V.	
21	M	32	neg	W.T.	46,XY	
22	F	19	neg	W.T.	46,XX	
23	F	32	neg	W.T.	46,XX	
24	F	26	neg	W.T.	46,XX	
25	M	29	neg	W.T.	46,XY	
26	M	65	neg	W.T.	46,XY	
27	F	71	neg	W.T.	46,XY	
28	F	52	neg	W.T.	46,XX	
29	F	47	neg	W.T.	46,XX	
30	F	62	neg	W.T.	46,XX	
31	M	43	neg	W.T.	46,XY	
32	F	38	neg	W.T.	46,XX,del(11)(p15)[3] 46,XX [28]	
33	F	56	neg	W.T.	46,XX,inv(9)(p11q12)[19]	
34	F	38	neg	W.T.	N.V.	
35	F	59	neg	W.T.	N.V.	
36	F	53	neg	W.T.	N.V.	
37	F	49	neg	W.T.	N.V.	
38	F	22	neg	N.V.	46,XX	
39	M	64	neg	N.V.	46,XY	
40	F	38	neg	N.V.	46,XX,del(11)(p15)[3] 46,XX[14]	

N:Nested, WT: *wild type*, NV: non valutabile, mutato:mutazione V617F

Ricerca di FIP1L1
Tutti i campioni di sangue midollare sono stati *screenati* per la ricerca della delezione interstiziale del cromosoma 4q12, mediante analisi di RT-PCR e Nested PCR del gene di fusione FIP1L1-PDGFRA.
La Nested PCR ha rilevato la presenza del gene di fusione FIP1L1-PDGFRA in 6 dei quaranta pazienti: cinque di questi, hanno mostrato una singola banda di amplificazione, mentre in un paziente la PCR ha mostrato 4 distinte bande di amplificazione. Figura 14

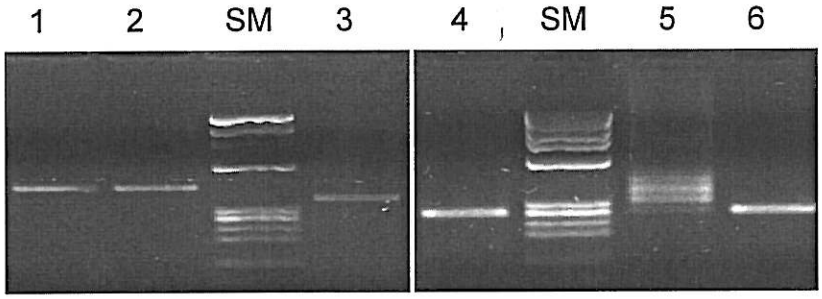


Figura 14 **I risultati della Nested-PCR.**
Presenza del gene di fusione FIP1L1-PDGFRA nei 6 pazienti. Le diverse bande del paziente 5 indicano la presenza di una variante di *splicing* del trascritto primario. SM: *size marker*.

L'analisi della sequenza ha confermato in tutti i casi che le bande amplificate corrispondono ad una giunzione in *frame* tra gli esoni FIP1L1 e PDGFRA.
La giunzione FIP1L1-PDGFRA è differente in ciascun paziente.
Le sequenze dei pazienti sono state allineate come descritto in precedenza;
i risultati degli allineamenti sono riportati in tabella 5.

Tabella 5: giunzioni in *frame* del gene di fusione

Paziente	
1	FIP1L1introne 9-ex10 (1-110 di 1-110) - PGDFRA ex12 (100-133 di 1-133nt)
2	FIP1L1 ex10 - PDGFRAex12 (100-133 di 1-133nt)
3	FIP1L1ex10 introne10(di30nt=3578435813) - PDGFRA ex12 (85-133nt)
4	FIP1L1ex10 - PDGFRA ex13
5variante1 5variante2	FIP1L1 ex10 - PDGFRA ex12 (100-133 di 1-133bp) FIP1L1 ex10 - PDGFRA ex13 (1-70 di1-105bp)
6	FIP1L1ex10 fuso FIP1L1 ex12 - PDGFRA ex12 (82-133 di 1-133bp)

Le sequenze del gene di fusione riscontrate nei pazienti sono le seguenti

Paziente1:

ATTACGGTCCAGGATCCTGTTGTGTTTCTATCAGCTTCTGG
TACATAATAGATATTCAGTTAAATGATGTTTACATTCTTCAC
CAAGATCCTGCAGTTTTACCTGTCAGGTACAGCAGGGAAG
AACTGGAAACTCAGAGAAAGAAACTGCCCTTCCATCTACCA
AAAGCTGAGTTTACTTCTCCTCCTTCTT][GTTCAAGACTGGG
CTTCCACCACCGAGCAGATGGGAGTTTCCAAGAGATGGAC
TAGTGCTTGTCGGGTCTTGGGGTCTGGAGCGTTTGGG AA

Paziente2:

CAATAAGATTACGGTACAGCAGGGAAGAACTGGAAACTCA
GAGAAAGAAACTGCCCTTCCATCTACAAAAGATGAGTTTA
CTTCTCCTCCTTCTTTGTTCAAGACTGGGCTTCCACCGAG
CAG][ATGGGAGTTTCCAAGAGATGGACTAGTGCTTGGTCG
GGTCTTGGGGTCTGGAGCGTTTGGGAAGGTGGTTGAAGG
AACAGCCTATGGATTAAGCCGGTCCC

Paziente3:

ACAATAAAATTACGGTACAGCAGGGAAGAACTGGAAACT
CAGAGAAAGAAACTGCCCTTCCATCTACAAAAGCTGAGTT
TACTTCTCCTCCTTCTTTGTTCAAGACTGGGCTTCCACCG
ACAGGTTCTTATTCCTATTTCTCTTTCCCTGCCTTATGACT
CAAGATGGGAGTTTCCAAGAG][ATGGACTAGTGCTTGGT
CGGGTCTTGGGGTCTGGAGCGTTTGGGAAGGTGGTTGA
AGGAACAGCCTATGGATTAAGCCGGTCCC

Paziente4:

CATTACGGTACAGCAGGGAAGAACTGGAAACTCAGAGA
AAGAAACTGCCCTTCCTCTACAAAAGCTGAGTTTACTTC
TCCTCCTTCTTTGTTCAAGACRGGGCTTCCACCGAGCA
G][GTCGGGTCTTGGGGTCTGGAGCGTTTGGGAAGGTG
GTTGAAGGAACAGCCTATGGATTAAGCCGGTCC

Paziente5:variante1

ATAAATTACGGCCGAAGGCTGTACTGGAAGTTACACCAG
GTGCAGAGAATCCAAGATGGCAGATTCAATCTTTTTAAG
GTACAGCAGGGAAGAACTGGAAACTCAGAGAAAGAAAC
TGCCCTTCCATCTACAAAAGCTGAGTTTACTTCTCCTCCT
TCTTTGTTCAAGACTGGGCTTCCACCGAGCAG[GTC]GGG
TCTTGGGGTCTGGAGCGTTTGGGAAGGTGGTTGAAGGA
ACAGCCTATTGGATTAAGCCGGTCCCA

Paziente5:variante2

ATAAGATTACGGTACAGCAGGGAAGAACTGGAAACTCA
GAGAAAGAAACTTGCCCTTCCATCTACAAAAGCTGAGTT
TACTTCTTCTCCTTCTTTGTTCAAGACTGGGCTTCCACC
GAG[CAG]ATGGGAGTTTCCAAGAGATGGACTCGTGCTT
GGTCGGGTCTTTGGGTCTGGAGCGTTTGGGAAGGTGG
TTGAAGGAACAGCCTATGGATTAAGCCGGTCCCA

Paziente6:

AGAGAAAGAAACTGCCCTTCCATCTACAAAAGCTGAG
TTTACTTCTCCTCCTTCTTTGTTCAAGACTGGGCTTTC
CACCGAGCAGGAGATTACCTGGGGCAATTGATGTTAT
CGGTCAAACTATAACTATCAGCCGAGTGAAGGCAGGC
GACGGGCAAATGAAAACAGCAACATACAGCTGCCTTA
TGAC TCAAGATGGG AGTTTCCAAG AGATGGACTA
GTGCTTGGTCGGGTCT][TGGGGTCTGGAGCGTTTGGG
AAGGTGGTCGAAGGAACAGCCTATGGATTAA

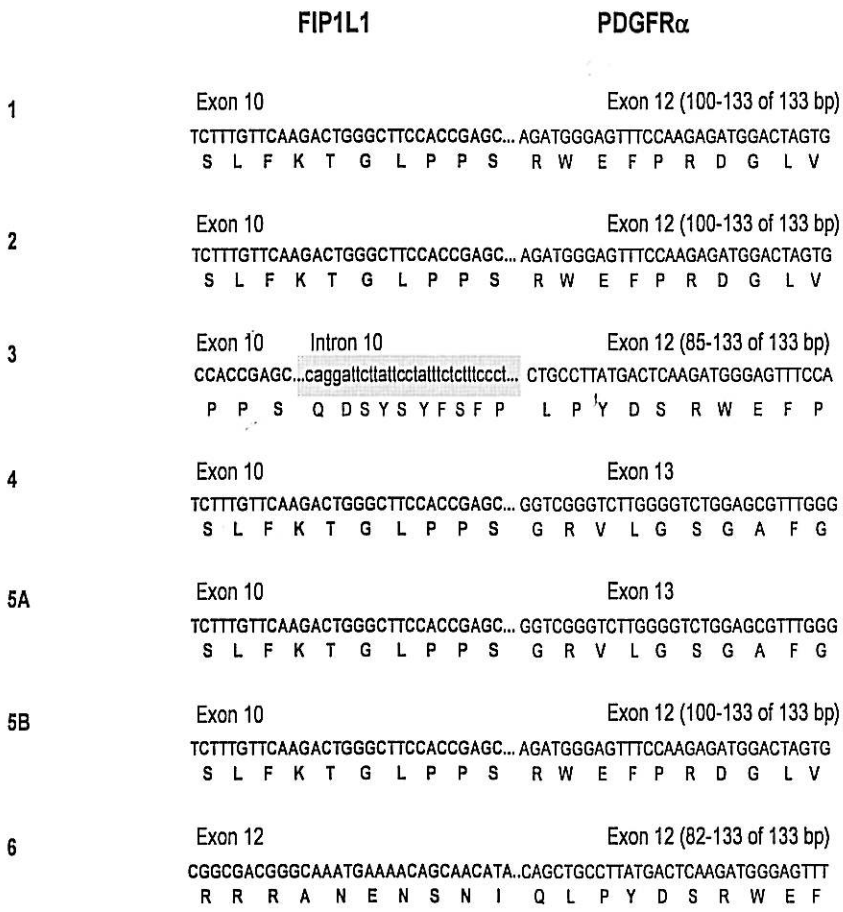


Figura15 **La sequenza delle giunzioni in *frame* del gene di fusione FIP1L1/PDGFRA** dei 6 pazienti. Nel caso di interessamento dell'esone 12 di PDGFRA è indicato il numero di basi nel trascritto di fusione. La sequenza segnata in grigio è derivata dell'introne 10 di FIP1L1.

In quattro pazienti l'esone 10 di FIP1L1 è unito alla sequenza di PDGRFA, mentre un piccolo frammento di trenta nucleotidi derivato dall'introne 10 di FIP1L1 è incluso alla giunzione alla giunzione FIP1L1-PDGFRA nel paziente 3; l'ultimo paziente mostra un *breakpoint* nell'introne 12 di FIP1L1 e l'intero esone12 di FIP1L1 è compreso nel trascritto ibrido.

I *breakpoints* del gene PDGFRA sono localizzati sulle regioni degli esoni 12 e 13. Inoltre una forma tronca di questo esone è stato trovato in quattro pazienti (1,2,3,6).
In un singolo caso il *breakpoint* di PDGFRA capita nell'introne 12 ed il corrispondente cDNA mostra una giunzione *in frame* tra l'esone 10 di FIP1L1 e l'esone 13 di PDGFRA (paziente 4).
Nel paziente 5 l'analisi della sequenza delle due bande amplificate indica la presenza di due varianti di *splicing* di FIP1L-1PDGFRA, una con una giunzione tra l'esone 10 e l'esone 12 e l'altra tra l'esone 10 e l'esone 13.

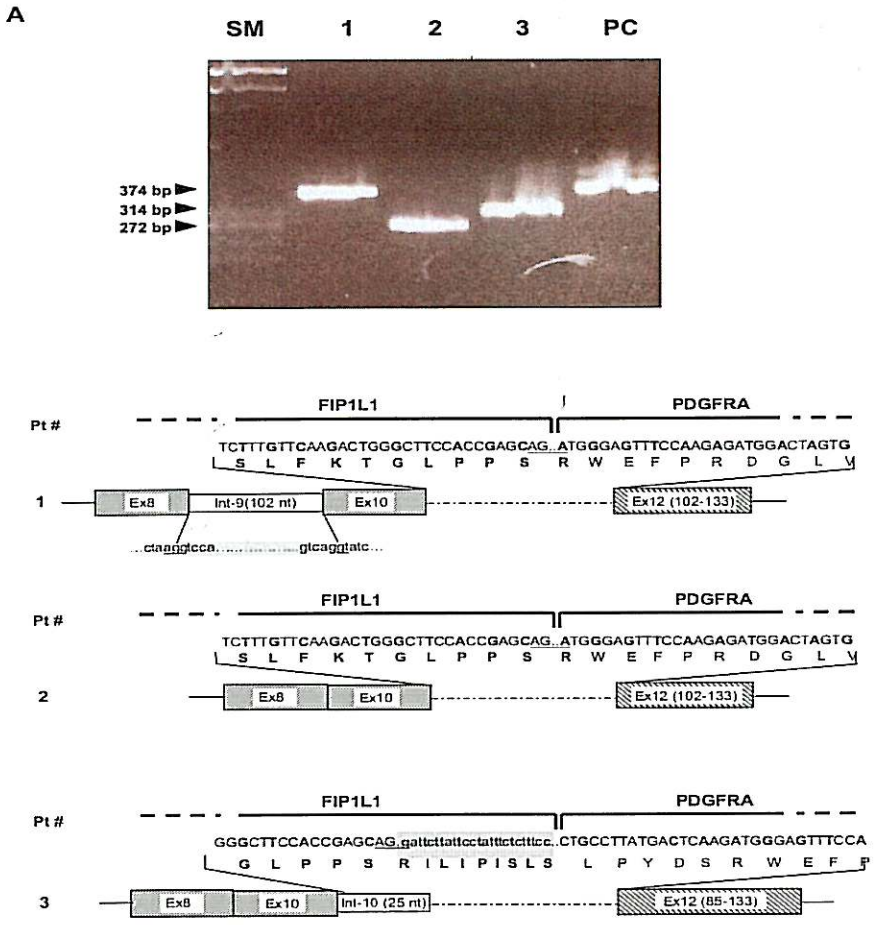


Figura16A in dettaglio i primi tre pazienti positivi. SM:*size marker*, PC:controllo positivo. Le frecce indicano l'altezza delle bande

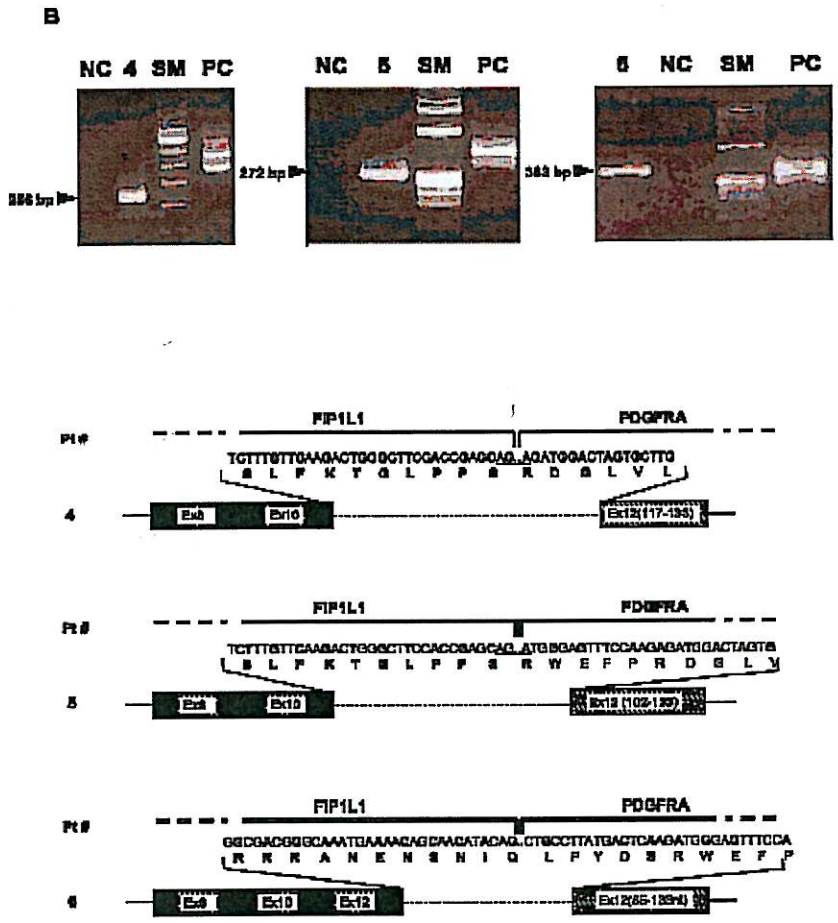


Figura16 B in dettaglio gli altri tre pazienti positivi. NC:controllo negativo,SM:*size marker*, PC:controllo positivo. Le frecce indicano l'altezza delle bande

FISH

I preparati citologici dei sei pazienti con analisi PCR positiva per il gene di fusione FIP1L1-PDGFRA sono stati analizzati con tecnica FISH in interfase per la ricerca di CHIC2 al locus 4q12 per confermare i dati molecolari e per valutare la percentuale di cellule clonali per ciascun paziente nel tessuto midollare.

Almeno 100 nuclei per ciascun paziente sono stati analizzati; la delezione di uno dei segnali CHIC2 è stato ritrovato in una percentuale variabile tra 19 e 42 in tutti i sei pazienti positivi (Tabella 6).

Tabella 6 Risultati dell'analisi FISH per CHIC2 nei sei pazienti con il gene di fusione FIP1L1-PDGFRA.

pazienti	Nuclei analizzati	Cellule con monosomia CHIC2
1	140	48 (34%)
2	120	51 (42%)
3	125	24 (19%)
4	135	39 (29%)
5	142	37 (26%)
6	120	43 (36%)
TE FIP1L1-PDGFRA negative (n=34)	100	8,1%
Midolli di controllo	200	3,5%

Da questi dati, è possibile ritenere che le cellule clonali nei pazienti con TE e delezione interstiziale del cromosoma 4q12 siano circa un terzo delle cellule midollari.

Mutazione V617 JAK2

Una singola mutazione somatica di JAK2 è stata recentemente descritta in pazienti con PV ed in una piccola percentuale di pazienti con TE. [22-25]

Partendo da questi dati, dopo aver valutato la presenza dell'alterazione molecolare FIP1L1-PDGFRA nelle TE, abbiamo analizzato i campioni di sangue midollare dei nostri 40 pazienti per ricercare la presenza della descritta mutazione di JAK2, prima in PCR, poi usando l'analisi delle sequenze che identificano la mutazione V617F di JAK2, derivata da una mutazione G→T in posizione 1849, quando essa interessa almeno il 20% delle cellule analizzate. [23] La mutazione V617F è stata ritrovata in 15 pazienti su 40 (40%); come atteso, il segnale della mutazione G→T è molto basso. (Figura 17).

Infatti l'emopoiesi clonale raramente mostra una larga espansione nei pazienti con TE come documentato anche dalla proporzione di cellule clonali stimata con l'analisi FISH nei sei pazienti con il gene di fusione FIP1L1-PDGRFA che non è mai stata superiore al 42%.

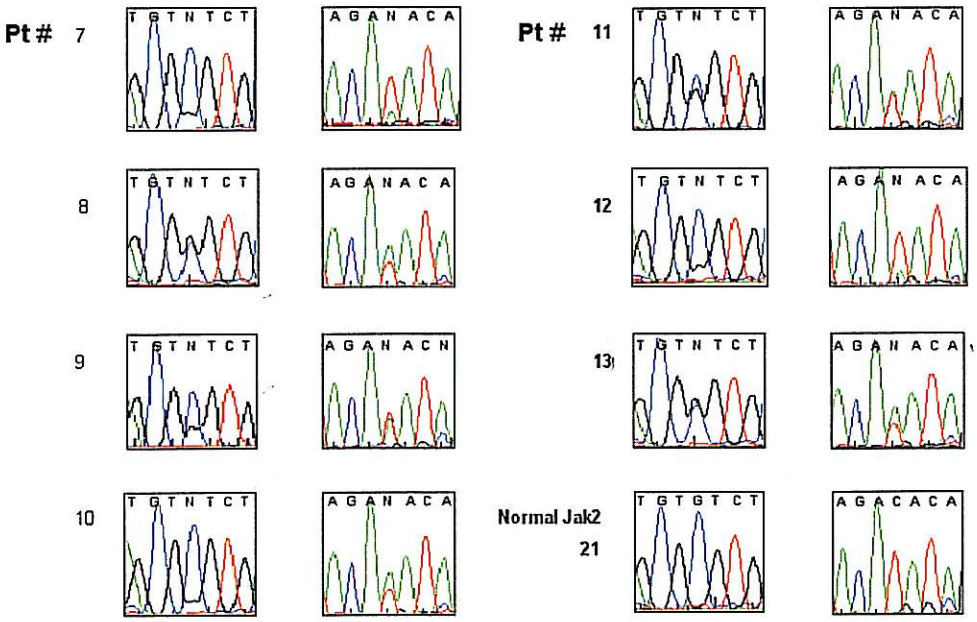


Figura17 **Ricerca della mutazione V617FJAK2**. Analisi della sequenza degli esoni 12 del gene JAK2 in 7 pazienti con TE. I risultati indicano un pattern misto di *wild type* e mutazioni G-T alla posizione 617. Il segnale corrispondente alla sequenza mutata è sempre più basso del *wild type*. I numeri fanno riferimento ai pazienti della tab4

I risultati dell'analisi delle sequenze sono state concordanti con quelle ottenute dall'ARMS in un numero ridotto di casi studiati (Figura 18). In tutti i casi le bande *wild type* hanno un segnale più forte rispetto alla banda V617F e questo conferma che l'emopoiesi clonale nei pazienti con TE è limitata.

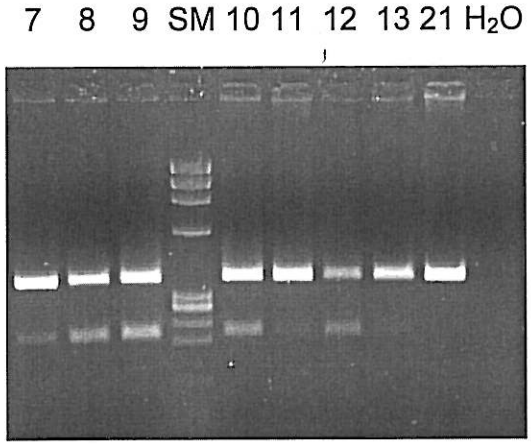


Figura 18 l'analisi ARMS ha confermato la presenza della mutazione in tutti e 7 i pazienti. SM: *size marker*, 21 controllo normale. I numeri si riferiscono ai pazienti della tabella4

Un aspetto molto importante è che i nostri risultati indicano che il gene di fusione FIP1L1-PDGFRA e la mutazione V617F di JAK2 non sono mutuamente esclusivi. In un paziente, infatti, abbiamo trovato dopo analisi con la PCR il gene di fusione FIP1L1-PDGFRA e il 34% di cellule positive per CHIC2 alla FISH; nello stesso paziente abbiamo trovato anche la mutazione V617F di JAK2. (paziente 4 tabella 4)
L'analisi ARMS ha escluso la presenza di un piccolo subclone con la mutazione V617F negli altri cinque pazienti positivi per il gene di fusione FIP1L1-PDGFRA.

Correlazione tra le caratteristiche dei pazienti e le alterazioni molecolari

Analizzati tutti i risultati ottenuti con le differenti tecniche è stato possibile identificare quattro tipologie di pazienti:

(i) con gene di fusione FIP1L1-PDGFRA (15%);
(ii) con la mutazione somatica di JAK2 V617F (40%),
(iii) senza nessuna delle due anomalie molecolari studiate (55%)
(iv) un solo paziente ha mostrato entrambe le anomalie. Tabella 7

Tabella 7 Caratteristiche dei 4 *subsets* molecolari differenti dei pazienti conTE

		FIP1L1/PDGFRA pos Jak2 –non mutato	FIP1L1/PDGFA neg V617F Jak2 mutato	FIP1L1/PDGFRA pos V617F Jak2 mutato	Nessuna anomalia
n		5	14	1	20
Età (anni)	media *range*	36,8 22-56	47 21-84	52	46 19-71
M/F		2/3	10/4	1/0	6/14
Hb(g/dL)	media *range*	15,2 13,5-17,8	13,9 13-15,7	14,3	13,4 9,9-15,4
GB ($x10^9$/L)		9,1 6,9-11,6	10,2 5,8-15,9	11,2	9,35 4,4-19,1
Plt ($x10^9$/L)		723 589-853	763 581-1218	838	868 548-1936
Eos ($x10^9$/L)		1,4 (0 – 5)	2,1 (0 – 6)	1	2,7 (0 – 5)
VS (mL)		568 140-1066	451 300-900	N.V.	543 106-960
Cariotipo anomalo		N.V.	4 cariotipi complessi	N.V.	

Hb:emoglobina, GB:globuli bianchi, Plt:piastrine, eos: eosinofili, VS: Volume Splenico, N.V.:non valutabile

Un aspetto interessante è che il numero di eosinofili nei sei pazienti positivi per la ricerca del gene di fusione FIP1L1-PDGFRA è sempre normale. Un altro aspetto peculiare è che la metà dei pazienti positivi sono donne (3/6) diversamente da quanto avviene nei pazienti con Sindrome Ipereosinofila con gene di fusione FIP1L1-PDGFRA (quasi tutti maschi).
Inoltre un'anomalia cromosomica aggiuntiva è stata individuata solo in un paziente con mutazione V617F di JAK2.
Il piccolo numero di pazienti di ciascun sottogruppo non permette alcuna valutazione statistica. Nessuna sostanziale differenza, comunque, sembra essere presente in questi quattro gruppi di pazienti.

L'unico aspetto è che i pazienti con il gene di fusione FIP1L1-PGDFRA tendono ad essere più giovani. Ma anche in questo caso non è possibile una valutazione statistica.

Discussione e conclusioni

La TE è inclusa nell'eterogeneo gruppo delle SMP Philadelphia negative, ed è caratterizzata dalla mancanza del patognomonico marcatore molecolare tipico della LMC.
La TE è poco frequente, con un'incidenza di circa 1-2 casi ogni 100.000 abitanti per anno. [64]
Sebbene le aspettative di vita di questi pazienti siano simili a quelle della popolazione normale, il decorso clinico dei pazienti con TE è complicato spesso da eventi trombotici ed emorragici. [65-66]
Le basi molecolari di questa patologia, cosi come quelle di altre SMP Ph- (PV,MF), sono in larga parte sconosciute.
Negli ultimi anni un crescente interesse del mondo scientifico si è manifestato nei confronti di queste patologie. La LMC ha rappresentato il modello molecolare con cui confrontarsi. In questa patologia l'attivazione costitutiva di una specifica chinasi codificata dal gene di fusione BCR/ABL, sostiene l'attivazione del potenziale proliferativo della cellula staminale mieloide mutata ed ha un ruolo chiave nella trasformazione neoplastica e nel quadro clinico della malattia. Dal 2000 la terapia di questa malattia è basata, in prima linea, sull'Imatinib, inibitore della tirosina chinasi, e capace di bloccare specificamente uno *step* della proliferazione neoplastica, senza interferire con la proliferazione delle altre linee cellulari normali.
Nel 2003 un altro gene di fusione (FIP1L1/PDGFRA) si è aggiunto alla crescente lista di tirosine chinasi legate alla patogenesi delle SMP. In circa un terzo dei pazienti affetti da HES è stata trovata la proteina aberrante codificata dal gene di fusione.[67] Oltre all'enorme importanza dal punto di vista molecolare, interessanti sono stati i dati in vitro che evidenziano come l'attività chinasica di questa proteina di fusione è inibita anch'essa dall'Imatinib. Per questo motivo i pazienti con HES con il gene di fusione FIP1L1/PDGFRA attualmente vengono trattati, anch'essi in prima linea, con l'Imatinib.[18]
Per le altre SMP senza un bersaglio molecolare l'uso di inibitori delle tirosine chinasi è stato valutato in maniera empirica, soprattutto nelle MF avanzate, con risultati molto diversi. Si è, dunque, speculato che a livello molecolare l'attivazione aberrante di specifiche chinasi con conseguente attivazione inappropriata della cascata di trasmissione del segnale intracellulare possa rappresentare, anche in queste patologie, l'evento molecolare centrale e comune.[14-15]
Ma nel 2005 lo scenario molecolare ha subito un inaspettato cambiamento: quattro gruppi di ricerca, in maniera indipendente l'uno dall'altro, hanno mostrato che la maggior parte dei pazienti con PV e

molti pazienti con TE e con MF mostrano un'identica mutazione puntiforme acquisita del gene JAK2 che codifica per una tirosina chinasi.[22-25] Questo porta ad una costitutiva attivazione dell'attività tirosinchinasica ed è responsabile dell'aumentata sensibilità alle citochine dei progenitori mieloidi osservata in questi pazienti.[24] Sembrerebbe, dunque, per le PV sicuramente, ed anche per alcune altre SMP che sia stato individuato un marcatore molecolare. L'importanza di questo nuovo dato molecolare è tale che è stato proposto di modificare i criteri diagnostici del PVSG, aggiungendo anche la ricerca della mutazione V617F tra i criteri maggiori da considerare per la diagnosi della PV e della TE.
Ancora una volta il marcatore molecolare coinvolge una tirosina chinasi.
Partendo da questi presupposti, abbiamo valutato nei pazienti con TE l'incidenza della mutazione V617F di JAK2, ed abbiamo cercato di individuare un'altra anomalia molecolare: la presenza del gene di fusione FIP1L1-PDGFRA.
La scoperta che questa anomalia molecolare è presente, seppure in un numero ridotto di questi pazienti, ha dato un ulteriore impulso alla conoscenza delle basi molecolari della TE. Il gene di fusione FIP1L1-PDGFRA è stato identificato mediante analisi PCR e FISH nel 15% dei pazienti. Tale percentuale esclude la casualità e può essere considerata di riferimento per la conoscenza delle basi molecolari di questa malattia in un sottogruppo di pazienti.
La struttura del gene di fusione FIP1L1-PDGFRA è la stessa individuata nei pazienti con HES.[18] Infatti, anche nei nostri pazienti il *breakpoint* del gene PDGFRA è ristretto all'esone 12.
L'esone 12 completo o tronco è unito "*in frame*" alla parte 5' del FIP1L1; e di conseguenza, la proteina di fusione comprende i due domini ad attività tirosina-chinasi del PDGFRA.
L'esone 12 di PDGFRA codifica una porzione del dominio juxtamembrana che serve come regolatore negativo dell'attività chinasica del PDGFRA stesso, e la sua rottura nella proteina di fusione provoca una attivazione costitutiva. Questa forma attiva di tirosina chinasi è capace di trasformare le cellule emopoietiche sia in vitro che in vivo. La trasfezione di FIP1L1-PDGFRA in linee cellulari emopoietiche Ba/F3 le rende indipendenti per la crescita allo stimolo dell'Interleuchina3 (IL3).[17]
In vivo, invece, la transduzione retrovirale di questa chinasi di fusione nel midollo di topi donatori trattati con 5FU, seguito da trapianto in topi irradiati, produce una malattia mieloproliferativa, rapidamente fatale,

caratterizzata da leucocitosi neutrofila, splenomegalia ed emopoiesi extramidollare. [47]
Il meccanismo peculiare, cioè una delezione cromosomica interstiziale, responsabile per la formazione del gene di fusione FIP1L1-PDGFRA, fornisce un ottimo marcatore per valutare la clonalità a livello midollare attraverso l'uso della analisi FISH in interfase del locus CHIC2. [14]
Nei nostri pazienti con TE che hanno presentato il gene di fusione FIP1L1-PDGFRA, una percentuale di cellule midollari tra il 19 ed il 42% ha mostrato la delezione monosomica del locus CHIC2, una scoperta indicativa di una espansione clonale significativa di cellule midollari trasformate con la delezione interstiziale 4q12.
Questi dati confermano l'eterogeneo e non completo interessamento del tessuto emopoietico midollare nei pazienti con TE, peraltro già mostrato dall' analisi della clonalità basata sull'inattivazione del cromosoma X. [13,67]
A questo proposito è significativo sottolineare che il test di PCR per la ricerca del gene di fusione FIP1L1-PDGFRA può essere poco sensibile; eccetto che per il paziente con il 42% di cellule midollari con CHIC2 deleto, solo la Nested PCR è stata in grado di dimostrare la presenza di questa anomalia.
L'espansione clonale delle cellule con del4q12 è la più evidente prova che il gene di fusione FIP1L1-PDGFRA sostiene il vantaggio di crescita di queste cellule.
Nell'insieme questi dati sono indicativi di un ruolo diretto della proteina di fusione FIP1L1-PDGFRα nella patogenesi delle TE e che la forma attiva di PDGFR α è responsabile dell'ipersensibilità dei progenitori eritroidi a specifiche e differenti citochine e probabilmente alla base dell' iperplasia megacariocitaria osservata in questa patologia.
Se due distinte alterazioni molecolari, per esempio la fusione FIP1L1-PDGFRA e JAK2 mutato sono presenti nella stessa malattia, si può comprendere il *pathway* molecolare che media il loro effetto patologico.
JAK2 mutato incrementa i livelli intracellulari di STAT5 fosforilato nei progenitori midollari, sia nei pazienti con PV, sia in quelli con TE. [22-23]
Le linee cellulari ematologiche Ba/F3 transfettate con FIP1L1-PDGFRA mostrano alti livelli di STAT5; [17] l'attivazione di questi fattori trascrizionali potrebbe indicare un forte legame fra i meccanismi molecolari delle cellule emopoietiche alterate da queste due lesioni molecolari.
Al contrario, l'osservazione che l'attivazione di una comune tirosina chinasi quale PDGFRα sia presente in due differenti sindromi

mieloproliferative, l'HES e la TE, pone la questione di come una singola mutazione possa essere responsabile di due entità cliniche differenti. E' innanzitutto possibile ipotizzare che questo difetto genetico sorga sempre in progenitori emopoietici *committed*, ma già *subsets* differenti di cellule midollari, od, in alternativa, che le lesioni molecolari occorrano in progenitori multipotenti ed una lesione aggiuntiva porti all'espansione del compartimento eosinofilo o megacariocitario.
Una serie di evidenze sperimentali indicano che la seconda è la spiegazione più plausibile. Sia la TE che l'HES sono disordini che originano dalla cellula staminale ed una serie di evidenze sperimentali e cliniche indicano che l'espansione clonale non è ristretta ad una singola linea emopoietica.[64] Nelle HES il gene di fusione FIP1L1-PDGFRA è rintracciabile oltre che negli eosinofili anche nei neutrofili, linfociti e monociti.[68] E' stato, anche, segnalato il caso di un paziente con mastocitosi sistemica ed eosinofilia, in cui il gene di fusione FIP1L1-PDGFRA è stato identificato non solo negli eosinofili ma anche in altri precursori del compartimento mieloide;[39] ugualmente, nei pazienti con SMP la mutazione V617F non è ristretta ai precursori eritroide o megacaiocitari[22] ed ancora, il cromosoma Philadelphia è largamente ritrovabile in tutte le cellule emopoietiche nei pazienti con LMC.[69]
La scoperta interessante che il gene di fusione FIP1L1-PDGFRA richieda una costitutiva espressione di IL-5 per indurre una sindrome simile all'ipereosinofila in modelli animali, può rafforzare l'ipotesi di un patogenesi *multisteps* delle SMP, e della TE in particolare: dopo l' acquisizione della prima mutazione, un secondo evento co-operante è verosimilmente richiesto per ottenere il fenotipo della TE. [70] Così come interessante è la scoperta della associazione, in uno dei nostri pazienti, di entrambe le anomalie molecolari; questo dato ulteriormente rafforza l'ipotesi di una patogenesi complessa.
Oltre il suo interesse molecolare, l'identificazione del gene di fusione FIP1L1-PDGFRA nei progenitori emopoietici midollari nei pazienti con TE porta con se un grande potenziale sia per la valutazione clinica, sia per il *management* di questi pazienti. Un numero aumentato di piastrine può essere osservato come evento reattivo in pazienti con condizioni infiammatorie acute o croniche cosi come nelle carenze di ferro.[71] Esso può essere presente anche in altre SMP, oltre che nelle TE.[64] Numerosi studi hanno cercato di identificare criteri diagnostici per la TE, come l'eterogeneità delle dimensioni delle piastrine, anomalie dell'aggregazione delle piastrine o crescita spontanea nelle colture cellulari, ma queste anomalie si sono rilevate poco sensibili e

specifiche.[72] Più promettenti appaiono le tecniche per la ricerca delle dell'emopoiesi clonale a livello dei megacariociti;[68] ma queste tecniche appaiono particolarmente laboriose, sono applicabili solo alle donne, e non totalmente specifiche. Allora la diagnosi di TE, basata per la maggior parte su criteri di esclusione, può essere difficile.
Attualmente, nella metà dei pazienti con TE l'analisi molecolare può fornire un marcatore a conferma della diagnosi clinica.
Dei quaranta pazienti arruolati nello studio, cinque hanno il gene di fusione FIP1L1-PDGFRA, 14 hanno la mutazione V617F di JAK2 ed un singolo paziente mostra entrambe le alterazioni; dunque venti su quaranta hanno un marcatore molecolare diagnostico.
Infine, la scoperta del gene di fusione FIP1L1-PDGFRA nei pazienti con TE apre uno scenario nuovo nel trattamento dei pazienti con questo tipo di Sindrome Mieloproliferativa. E' stato chiaramente mostrato come l'attività chinasica di questa proteina di fusione FIP1L1-PDGFRalfa sia il *target* per una nuova classe di piccole molecole con effetto inibitorio sull' attività delle tirosine chinasi. L'Imatinib è l'esempio paradigmatico.[26]
A livello cellulare, l'effetto dell'Imatinib contro il PDGFRα è maggiore rispetto a quello contro l'ABL e, quindi, i pazienti con HES con gene di fusione FIP1L1-PDGFRA possono raggiungere la remissione molecolare con una bassa dose giornaliera dell'ordine di 100 mg.[18] I pazienti con TE, con gene di fusione FIP1L1-PDGFRA, sono candidati al trattamento con Imatinib, poiché come i pazienti con HES e LMC posseggono un *target* appropriato per il farmaco. Inoltre il marcatore molecolare del clone può essere l'oggetto del monitoraggio della risposta alla terapia.
Infine il nostro studio porta alla conclusione che il gene di fusione FIP1L1-PDGFRA è presente in una significativa percentuale di pazienti con TE e che può al momento essere considerato con la mutazione V617F di JAK2 uno dei marcatori molecolari della malattia. Ulteriori studi sono necessari per capire come due differenti entità cliniche possano originare da un singolo difetto molecolare, per spiegare la predilezione per il sesso maschile del gene di fusione nelle HES ma non nelle TE, e per valutare in una larga coorte di pazienti con TE se le caratteristiche cliniche possano essere correlate alle differenti lesioni molecolari.

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1) "Measurement of spleen volume by ultrasound scanning in patients with thrombocytosis: a prospective study"
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Picardi M, De Rosa G, Selleri S, Scarpato N, Soscia E, Martinelli V, **Ciancia R**, Rotoli B
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Camera A, Andretta C, Villa MR, Volpicelli M, Picardi M, Rossi M, Rinaldi C, Della Coppa P, **Ciancia R**, Selleri C, Rotoli B
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8) "α Interferon treatment for pregnant women affected by Essential Thrombocythemia: case reports and a review"
Martinelli P, Martinelli V, Aganti A, Maruotti GM, Paladini D, **Ciancia R**, Rotoli B
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9) "High number of circulating CD 34+ cells in patients with myelophthisis"
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10) Estimation of bulky lymph nodes by power Doppler ultrasound scanning in patients with Hodgkin's lymphoma: a prospective study.
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11) "Severe reactivation of hepatitis B virus infection in a patient with Hairy Cell Leukemia: should Lamivudine prophilaxis be recommended to HbaAg-negative, Anti-HBc-positive patients?"
Orlando R, Tosone G, Tiseo D, Piazza M, Portella G, **Ciancia R**, Martinelli V, Montante B, Rotoli B
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2) "Peg-Intron alfa 2b in Essential Thrombocythemia: phase II study for determination of minimum effective, safe and tolerated dose. Preliminary data"
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16) "Spleen volume assessment in hematologic diseases: CT versus US"
Soscia E, Picardi M, Mainenti P, **Ciancia R**, Martinelli V, De Rosa G, Sodano A, Montante B, Rotoli B.
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Haematologica, (abstract) vol 88 (suppl) n.ro 15, pag 416, Ottobre 2003

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Picardi M, De Rosa G, Selleri C, Scarpato N, Soscia E, Martinelli V, **Ciancia R**, Rotoli B
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18) "A novel strategy for cell therapy of chronic myeloid leukemia using ex vivo treatment of Ph positive cells with decitabine and with the by stander transfer of CD40L"
Quintarelli C, Izzo B, Muccioli Casadei G, Bartiromo M, Sparacino A, **Ciancia R**, Biagi E, Intrieri M, Luciano L, Amabile M, Rosti GA, Martinelli G, Brenner M, Pane F.
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Ciancia R, Martinelli V, Cosentini E, Picardi M, Petruzziello F, Matano E, Rotoli B.
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20) Diagnosis of NHL by the combined use of histology and flow cytometry on cell suspensions from lymph nodes, gastric mucosa, cutis and lachrymal gland
Montante B, Morabito P, Scalia G, Lo Pardo C, Ciancia R, Ciancia G, Pettinato G, Martinelli V, De Renzo A, Napolano F, Del Vecchio L, Rotoli B.

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Ciancia R, Martinelli V, Zatterale A, Gentile G, Picardi M, Petruzziello F, Montante B, Rotoli B.
Haematologica, (abstract) vol 90 (suppl) n.ro 3, pag 388, Luglio 2005

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Haematologica, (abstract) vol 90 (suppl) n.ro 3, pag 241, Luglio 2005

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26) Lymphoma cells microdissected from primary splenic low grade Non Hodgkin Lymphoma but not normal splenic cells contain HCV genome
De Renzo A, De Angelis B, Perna F, Ciancia G, Quintarelli C, Picardi M, **Ciancia R**, Persico M, Rotoli B, Pane F
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27) Essential Thrombocythemia and pregnancy: preliminary report of the pregnancy committed of the Registro Italiano Trombocitemie (RIT)
Melillo L, Tieghi A, Candoni A, **Ciancia R**, Martinelli V, Latagliata R, Specchia G, Scalzulli PR, Fanci R, Comitini G, Cascavilla N, Gugliotta L
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Rossi A, Bonatti S, Mallardo M, Martinelli V, **Ciancia R**, Gravetti A, Rotoli B
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30) Occurrence and distribution of VHL-dependent polycythemia in south Italy
Perrotta S, Martinelli V, Ferraro M, **Ciancia R**, Petruzziello F, Rossi F, Picardi M, Ladogana S, Nobili B, Rotoli B, Della Ragione F
Haematologica, (abstract) vol 91 (suppl) n.ro 3, pag 55, Settembre 2006

31) Deformability based in vitro analysis of red blood flowing in microcapillaries
Tomaiuolo G, Simeone M, Guido S, Marrucci G, **Ciancia R**, Rinaldi C, Martinelli V, Rotoli B
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32) Circulating CD34+ cells in Philadelphia negative myeloproliferative diseases: a steady state parameter or a spy of progression?
Petruzziello F, Cosentini E, **Ciancia R**, Picardi M, Bruno P, Aufiero D, Montante B, Napolano F, Martorelli MC, Martinelli V, Rotoli B
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33) Clinical relevance of intrahepatic HBV DNA in HbsAg negative HBcAb positive patients undergoing stem cell trasplantation for hematological malignancies
Picardi M, Muretto P, Montante B, **Ciancia R**, De Rosa G, Selleri C, Pane F, Rotoli B
Haematologica, (abstract) vol 91 (suppl) n.ro 3, pag 124 Settembre 2006

APPENDICE

Elenco pubblicazioni allegate

1) "Measurement of spleen volume by ultrasound scanning in patients with thrombocytosis: a prospective study"
Picardi M, Martinelli V, **Ciancia R**, Soscia E, Morante R, Sodano A, Fortunato G, Rotoli B.
Blood, 2002; 99: 4228-30

2) "Lymphnode localization of extramedullary myeloid cell tumor in myelodysplastic syndrome: report of one case diagnosed by fine needle cytology."
Fulciniti F, Zeppa P, Marino G, Martinelli V, **Ciancia R**, Del Vecchio L, Rotoli B, Palombini L.
Diagnostic Cytopathology 2003; 28:136-139

3) "Intravenous itraconazole for treating invasive pulmonary aspergillosis neutropenic patients with acute lymphoblastic leukemia"
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5) Intestinal toxicity during induction chemotherapy with cytarabine-based regimens in adult acute myeloid leukemia.
Camera A, Andretta C, Villa MR, Volpicelli M, Picardi M, Rossi M, Rinaldi C, Della Coppa P, **Ciancia R**, Selleri C, Rotoli B
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7) "A randomized comparition of power doppler ultrasound directed excisional biopsy with standard excisional biopsy for the caracterision of lymphadenopathies in patients with suspectal lymphoma"
Picardi M, Gennarelli N, **Ciancia R**, De Renzo A, Gargiulo G, Ciancia G, Sparano L, Zeppa P, Martinelli V, Pettinato G, LoBello R, Pane F, Rotoli B
Journal Clinical Oncology 2004; 22 (18): 3733-3740

8) "α Interferon treatment for pregnant women affected by Essential Thrombocythemia: case reports and a review"
Martinelli P, Martinelli V, Aganti A, Maruotti GM, Paladini D, **Ciancia R**, Rotoli B
American Journal of Obstretics and Gynecology, 2004; 191(6): 2016-20

9) "High number of circulating CD 34+ cells in patients with myelophthisis"
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Haematologica, July 2005;90:976-77

10) Estimation of bulky lymph nodes by power Doppler ultrasound scanning in patients with Hodgkin's lymphoma: a prospective study.
Picardi M, **Ciancia R**, De Renzo A, Montante B, Ciancia G, Zeppa P, Lobello R, Pane F, D'Agostino D, Nicolai E, Sirignano C, Salvatore M, Rotoli B.

Haematologica. 2006 Jul;91(7):960-3.
11) "Severe reactivation of hepatitis B virus infection in a patient with Hairy Cell Leukemia: should Lamivudine prophilaxis be recommended to HbaAg-negative, Anti-HBc-positive patients?"
Orlando R, Tosone G, Tiseo D, Piazza M, Portella G, **Ciancia R**, Martinelli V, Montante B, Rotoli B
Infection, 2006; 34:282-284

Measurement of spleen volume by ultrasound scanning in patients with thrombocytosis: a prospective study

Marco Picardi, Vincenzo Martinelli, Rosanna Ciancia, Ernesto Soscia, Roberto Morante, Antonio Sodano, Giuliana Fortunato, and Bruno Rotoli

Spleen size was assessed in 73 patients with thrombocytosis and in 15 healthy subjects, comparing palpation with ultrasonography (US) measurement of longitudinal diameter and volume. Intraobserver and interobserver variability for volume on US, checked in 12 patients, was very low. Correlation between spleen volume measured by US and that measured by computed tomography was excellent. Splenomegaly was detected by palpation in 25% of patients, by US assessment of longitudinal diameter in 33%, and by US assessment of volume in 52%. After diagnostic work-up, 54 patients had a diagnosis of essential thrombocythemia (ET), 4 of idiopathic myelofibrosis (IMF), and 15 of secondary thrombocytosis (ST). Spleen volume in patients with ST was in the normal range (138 ± 47 mL) and was significantly lower than that in patients with ET or IMF (370 ± 210 mL; $P < .001$). Thus, US-measured volume was the most sensitive method for identifying nonpalpable splenomegaly in patients with primary myeloproliferative diseases, and it may help in distinguishing these diseases from reactive disorders. (Blood. 2002;99:4228-4230)

Introduction

Enlargement of the spleen is an important criterion in diagnosing primary myeloproliferative diseases.[1-3] Because splenomegaly may occur in patients in whom the spleen is not palpable, the most sensitive imaging procedure for measuring splenic size has been sought. Nuclear medicine techniques, which are relatively complex and not routinely available, expose patients to radiation.[4-6] One-dimensional ultrasonography (US) is often inaccurate.[7] Computed tomography (CT) and magnetic resonance imaging are relatively expensive. Thus, stringent criteria for defining splenomegaly by using imaging methods are still controversial.[1-7] We tested the accuracy of US-measured spleen volume compared with palpation and US-measured longitudinal diameter in detecting splenomegaly in patients with thrombocytosis subsequently classified as having myeloproliferative diseases or reactive conditions. We used a group of healthy subjects to establish reference ranges for US volume and a patient subgroup to assess variability in US measurements and examine the correlation between US-measured and CT-measured spleen volume.

Study design

In the past 3 years, we prospectively studied 73 consecutive patients (43 women and 30 men; median age, 50 years [range, 13-75 years]; median body-surface area, 1.7 m^2 [range 1.4-2.0 m^2]) who presented with thrombocytosis (median platelet count, 720×10^9/L; range, 600-1898 $\times 10^9$/L) of unknown origin. Patients with overt spleen enlargement (ie, 3 cm from the costal border at palpation) or with a disease associated with splenomegaly (eg, portal hypertension or thalassemia) were excluded from the study. Informed consent to participation was obtained from all subjects studied.

All spleen US scans were performed by the same operator, who used an EUB 525 Hitachi (Tokyo, Japan) instrument with a 2.5/3.5-MHz broadband curvilinear probe. The spleen was scanned in patients who were fasting, in the longitudinal and transverse planes by using an intercostal approach, a subcostal approach, or both. The patient was placed in a supine or right-sided position until complete organ visualization was achieved.[8-10] Perimeter, longitudinal diameter, and area, defined as the maximum measurements with splenic borders and angles clearly defined, were measured, and volume (in milliliters) was calculated automatically. Reference values for volume were obtained from measurements in 15 healthy subjects matched with the patients for sex, age, and body-surface area. For each subject, the mean value of 3 measurements repeated on the same occasion was calculated and recorded for final analysis.

Twelve unselected patients were studied by repeated US measurements on 2 occasions at a 1-week interval by the same operator (intraobserver reproducibility) and by another operator unaware of the previous results, with both operators using the same US machine (interobserver reproducibility).[11,12] After informed consent to participation was obtained again, the same 12 patients underwent a CT examination. Spleen axial images were obtained by using a multirow helical instrument (Mx 8000; Marconi Medical Systems, Cleveland, OH), to produce a 3-dimensional model used to calculate volumes automatically.[13] Technical parameters included 6.5-mm slice width with identical reconstruction index, pitch 1, 200 mA, 120 kilovolt potential, and a rotation time of 0.75 seconds.

All patients underwent physical examination, full blood counts, assessment of blood smears and serum ferritin levels, and a search for possible causes of reactive thrombocytosis. Morphologic, cytogenetic, and molecular studies of bone marrow aspirates and a trephine biopsy with use of the Perls reaction for iron deposits and the silver-impregnation method for grading reticulin fibrosis were performed in all nonsecondary cases. In accordance with the updated criteria of the Polycythemia Vera Study Group,[1,2] 54 patients were found to be affected by essential thrombocythemia (ET), 4 by idiopathic myelofibrosis (IMF), and 15 by secondary thrombocytosis (ST) due to inflammatory

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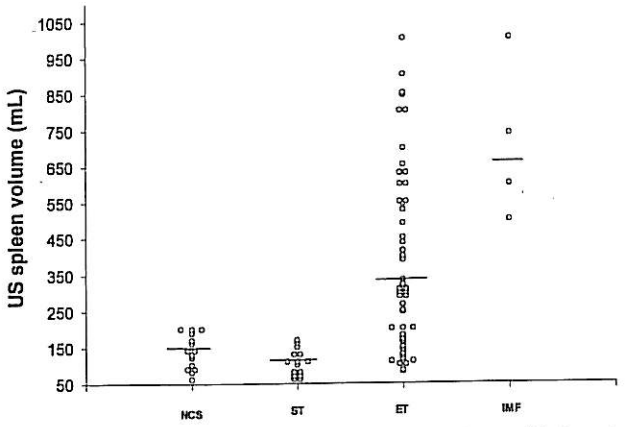

Figure 1. Spleen volume in healthy subjects and in patients with thrombocytosis. Spleen volume was measured by US in healthy control subjects (NCS) and in patients with secondary thrombocytosis (ST), essential thrombocythemia (ET), or idiopathic myelofibrosis (IMF).

conditions, iron-deficiency anemia, amyloidosis, or metastasizing malignant disease.

Statistical evaluations, including χ^2 testing, analysis of variance with Bonferroni correction, and Pearson correlation, were performed with SPSS for Windows software (version 9.0; SPSS, Chicago, IL).

Results and discussion

Intraobserver reproducibility and interobserver reproducibility of measurements of spleen volume by US were excellent, with Pearson values of 0.999 and 0.997, respectively. Spleen-volume measurements by US and by CT scanning were strongly correlated, with a Pearson value of 0.945 ($P < .001$).

In the healthy subjects, spleen longitudinal diameter ranged from 8 to 11 cm (median, 9.5 cm) and spleen volume from 60 to 200 mL (median, 140 mL). Among the 73 patients studied, spleen enlargement was detected by palpation (between 0.5 and 2 cm below the costal margin) in 18, by US assessment of longitudinal diameter in 24, and by US assessment of volume in 38. A Pearson value of 0.407 indicated a clear disagreement between the volume and longitudinal-diameter measurements. Measurement of volume had significantly higher sensitivity in detecting splenomegaly compared with measurement of longitudinal diameter and palpation (52% versus 33% or 25%; $P = .003$). Of the 54 patients with ET, 16 (29%) had palpable splenomegaly, 22 (40%) had increased longitudinal diameter on US, and 34 (63%) had volume enlargement on US (overall median, 320 mL; range, 81-1000 mL). The difference in median spleen volume between patients with ET and healthy subjects was significant ($P < .001$). All 4 patients with IMF had marked spleen-volume enlargement (median, 670 mL; range 510-1000 mL), whereas only 2 of them had splenomegaly on palpation or US assessment of longitudinal diameter. In the group with ST, spleen-volume estimation by US was in the normal range (median, 110 mL).

In a preliminary analysis, we stratified the patients in the ET cohort according to US-measured spleen volume. Twenty patients with a normal volume (≤ 200 mL) had a median serum lactic dehydrogenase (LDH) level of 350 U/L (range, 200-509 U/L; normal value, 227-450 U/L) and no or mild bone marrow fibrosis; 18 patients with a spleen volume between 246 and 490 mL had a median LDH level of 452 U/L (range, 360-774 U/L), with mild to moderate bone marrow fibrosis in 11; and 16 patients with a spleen volume at least 500 mL had a median LDH level of 462 U/L (range, 200-748 U/L), with mild to moderate bone marrow fibrosis in 14. In 2 patients in the latter group, IMF developed after a median follow-up time of 12 months, whereas none of the patients in the other groups had onset of IMF after a median follow-up time of 18 months. Moreover, patients with ET and a spleen volume at least 500 mL had less response to a first-line interferon treatment; only one of the 5 patients in this group had normalization of platelet counts, compared with 11 of 14 patients in the groups with no to moderate (< 500 mL) spleen-volume enlargement.

In conclusion, in contrast to other studies employing different methods,[5-7] we found a high rate of splenomegaly in patients with ET by using US measurements of volume (Figure 1). These findings are consistent with those of another series, in which spleen volume was measured by a radionuclide technique (single-photon emission CT).[4] Because the spleen is an irregularly shaped organ that may enlarge at different rates in its various dimensions, physical examination[1-3] and imaging using one-dimensional measurements[5-7] may underestimate the true organ size, thus suggesting

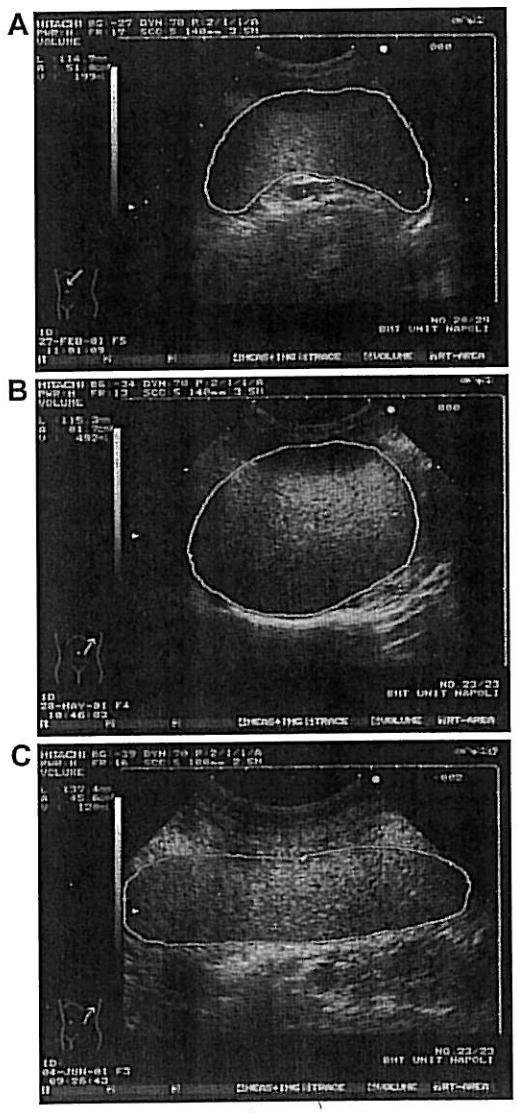

Figure 2. Various spleen shapes detected by US. (A) Spleen in a control subject having normal longitudinal diameter and volume. (B) Spleen in a thrombocythemic patient having longitudinal diameter like that in Figure 2A but clearly enlarged volume. (C) Spleen in a control subject showing enlarged longitudinal diameter but normal volume (rodlike spleen).

an erroneously low incidence of splenomegaly in patients with ET. Multidimensional US spleen-volume measurement (defined as the integration of longitudinal diameter, perimeter, and area) is a rapid, reliable, and low-cost method for detecting the true spleen size (Figure 2). In addition, our data support the concept of clinical heterogeneity in patients with ET[3,14,15]; a role for spleen-volume measurement in subgrouping these patients will be better evaluated by studies in progress.

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Lymphnode Localization of Extramedullary Myeloid Cell Tumor in Myelodysplastic Syndrome:

Report of One Case Diagnosed by Fine-Needle Cytology

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Trilineage extramedullary myeloid tumor (EMT) is an uncommon medical condition mostly diagnosed in patients affected by acute or chronic myeloid leukemia or, more rarely, by a myelodysplastic syndrome, among which the most frequent is refractory anemia with excess of blasts in transformation (RAEB-t). The prognostic significance of EMT is still unclear, although the appearance of trilineage EMT is often considered to affect the outcome adversely. A 70-year-old lady with previous history of intestinal resection for colonic adenocarcinoma in 1995 and subsequently treated with 5-fuorouracyl developed a refractory anemia with excess of blasts (RAEB) in 1998. During the follow-up, a progression to RAEB-t was recorded. During chemotherapy for this condition, slight enlargement of left supraclavicular and right submandibular nodes was noticed. Fine-needle biopsy was performed with ancillary studies. A diagnosis of trilineage extramedullary myeloid tumor was reached. The patient was treated with low doses of chemotherapy with a good response lasting 12 months. The peculiar cytologic picture of this condition when corroborated by ancillary studies (immunocytochemistry and flow cytometry) is diagnostic of this rare condition. Furthermore, the extramedullary myeloid tumor in this case did not significantly affect the response to the chemotherapy of RAEB-t. Diagn. Cytopathol. 2003;28: 136–139.

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The collective term *extramedullary myeloid cell tumor* (EMT), introduced in the medical literature by Davey et al.,[1] now encompasses all the extramedullary complications of leukemia, as granulocytic sarcoma[2] and leukemia cutis,[3,4] also including the non-mass-forming leukemic infiltrates. While this grouping may be considered arbitrary, some common molecular risk factors, as translocation 8–21 and the expression of neural-cell adhesion molecule (NCAM; Leu19, CD56) on blast cells coming from these lesions,[5] probably warrant this collective designation, although the cellular composition of the lesions should be always studied by descriptive morphology and immunocytochemistry.[6] While most EMTs follow acute or chronic myeloid leukemia (AML or CML), a significant proportion can be observed during the course of myelodysplastic syndrome (MDS).

To our knowledge, only three cases of trilineage EMT have undergone fine-needle biopsy.[7] All these patients were suffering from refractory anemia with excess of blasts in transformation (RAEB-t); the lesions were located in the soft tissues and were studied by the same authors. In this study, we report a new case with multiple lymph node localizations in a patient with RAEB-t.

Case Report

The patient was a 66-year-old white female who was initially found to be affected by hypochromic anemia. The diagnostic work-up included a positive stool blood test; endoscopy disclosed an infiltrating adenocarcinoma of the descending colon, which was treated with anterior resection in 1995. The patient was then treated with 24 cycles of

Table I. Prospect of the Disease Course*

	Physical examination						*Blasts %*			
Time	*Spleen*	*Leukem*	*Lymph*	*Hb*	*WBC*	*Platelets*	*PB*	*BM*	*PRBC (%)*	*Therapy*
November 1998 to June 2000	0	No	No	9.0/11.8	2.6/18.9	19/77	0/13	11	No	Observation, danazol
July 2000 to November 2000	0	No	No	8.5/11.3	30.1/59.4	28/113	3/23	n.s.	Yes (1)	Etoposide (6 courses), EPO
November 2000 to January 2001	5	?	Yes/no	7.5/9.0	33.7/102.4	33/75	7/26	n.s.	Yes (4)	ARA-c (3 courses)
January 2001 to September 2001	5	No	No	8.4/11.5	5.7/99.9	20/177	2/49	n.s.	Yes (1)	6-MP (9 courses)
September 2001 to December 2001	6/25	Yes	Yes/no	5.4/10.0	2.0/82.2	2/173	2/44	n.s.	Yes (12)	6-MP + etoposide (3 courses)

*Spleen, centimeters from the left costal margin; Leukem = leukemids; Lymph = lymphnode enlargement; PB = peripheral blood; PRBC = peripheral blast cells (%); BM = bone marrow; 6-MP = thyoguanine (6-mercaptopurine).

5-fluorouracyl; a slight leucopenia was noticed during treatment. A new endoscopy performed in February 1997 disclosed an adenomatous polyp that was treated endoscopically; rectal hemorrhage of moderate to severe degree complicated the polipectomy.

In that occasion, a slight systemic hypertension, chronic infection by hepatitis C virus, and diabetes mellitus were diagnosed. The patient was hospitalized again in August 1998; her blood tests showed thrombocytopenia and neutropenia. Splenomegaly was also found.

In October 1998, she was found affected by refractory anemia with excess of blasts (RAEB). A cytofluorimetric study performed on medullary blood disclosed 11.3% of blasts, of which 71% were $CD34^+$. Cytogenetic study on medullary blood showed the following karyotype: 46,XX,del(16)(q21)[20]/46,XX[11]. Peripheral blood indexes were as follows: Hb 11.8 g/dl, WBC 2,600 (of which 28% were neutrophils, 70% were lymphocytes, 2% were monocytes), platelets 77,000/μl.

In February 1999, a small monoclonal IgG κ-band was detected. The hematologic picture worsened in July 1999, with symptomatic thrombocytopenia and increase of medullary blasts; a diagnosis of RAEB-t was established and therapy with danazol and glucocorticosteroids was initiated. The hyperglycemic state was corrected by insulin and the only symptoms recorded by the patient were profuse sweating and diffuse petechial hemorrhages.

In May 2000, she reported the appearance of subcutaneous nodules in her left antibrachium and right poplyteal region. Fine-needle cytology was performed in our institution, with a cytologic diagnosis of chronic inflammatory lesion of the subcutaneous fat.

In June 2000, the WBC count rose (18,900/μl) and circulating blast cells were detected; a cytogenetic and cytofluorimetric study on peripheral blood showed the following blast phenotype: DR^+, $CD13^+$, $CD33^+$, $CD56^+$, $CD11b^+$, $CD11c^+$, $CD117^+$, $CD34^+$; the blast karyotype was 46,XX,del(16)(q21)[8];46,XX[10]. In July 2000, progression of anemia required blood transfusions; a trial with erythropoietin was started. Further increase of leukocytosis required oral etoposide therapy (six cycles).

In November 2000, a further increase of leukocytosis (WBC 75,000, of which 26% were blasts) granted chemotherapy with low-dose arabincytoside-C (ARA-c). There was only minimal response to this therapy. In January 2001, there was a worsening of the patient's general condition; she was transfused again and started therapy with oral thyoguanine. The presence of small left supraclavicular and submandibular lymph nodes were detected. Fine-needle cytology was performed on both lymph nodes by a 23 G needle; a lymph node extension of the trilineage myeloproliferative disease was diagnosed.

Following this diagnosis, six further cycles of thyoguanine were given with disappearance of the enlarged lymph nodes, good hematological response, and diminished need for transfusions. Her hematological values in March 2001 were as follows: Hb 10.5 g/dl, WBC 31,5000/μl, with 49% neutrophils, 6% eosinophils, 4% monocytes, 10% promonocytes, 9% myelocytes, 1% metamyelocytes, 21% blasts.

In September 2001, clinical progression of the disease with marked splenomegaly (to the left iliac fossa), anemia, and leukocytosis was noticed. Moreover, skin leukemids, accompanied by laterocervical, bilateral axillary lymphadenopathy and gingival hypertrophy, appeared. The patient was classified as WHO stage 5 and was treated with chemotherapy with two drugs: thyoguanine 80 mg/day for 6 days and etoposide 100 mg. i.v. for 3 days. A partial response to the therapy was obtained; the patient died on December 2001 by a sepsis. The course of the disease is summarized in Table I.

Cytopathologic Picture

Several slides were obtained from the lymph node aspirates, which were air-dried or fixed in 95% ethanol for May-Grünwald-Giemsa (MGG) and Papanicolaou (Pap) stain, respectively. Part of the aspirated material was injected into a vial containing 2 mLs of phosphate-buffered saline solution and sent for flow cytometric analysis while a separate aspiration was injected into another vial containing 2 mLs of Hank's balanced saline and used to make cytospin preparations for immunocytochemistry

The cytopathologic picture was similar in both of the aspirated lymph nodes and showed a moderate to high cellularity. The most striking feature at low magnification was the evident scarceness of lymphoid cells as opposed to the abundance of cells of myeloid lineage in various degrees

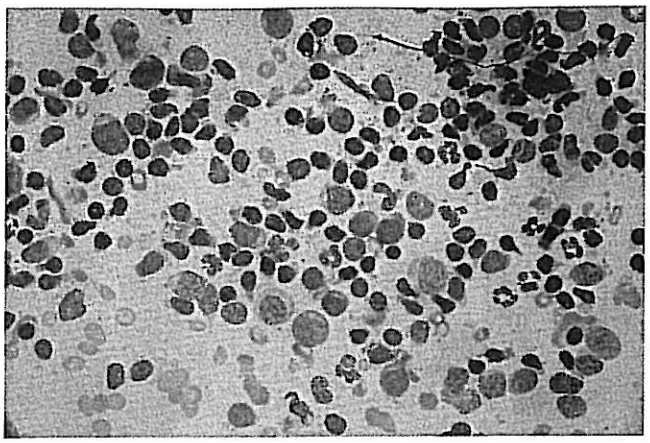

Fig. C-1A

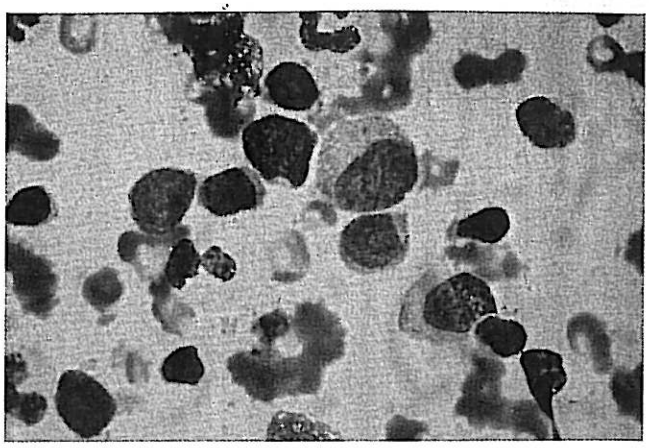

Fig. C-1B

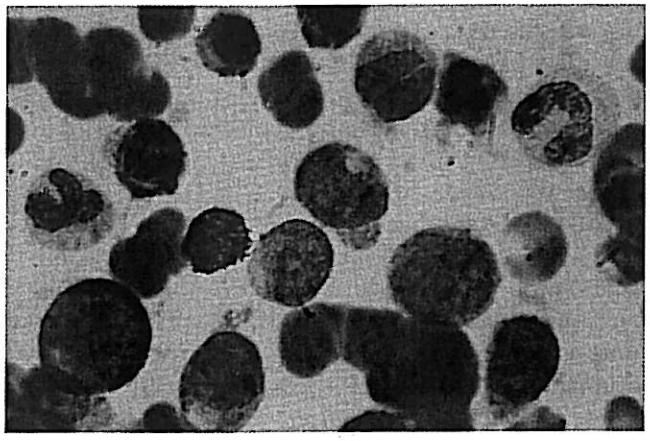

Fig. C-1C

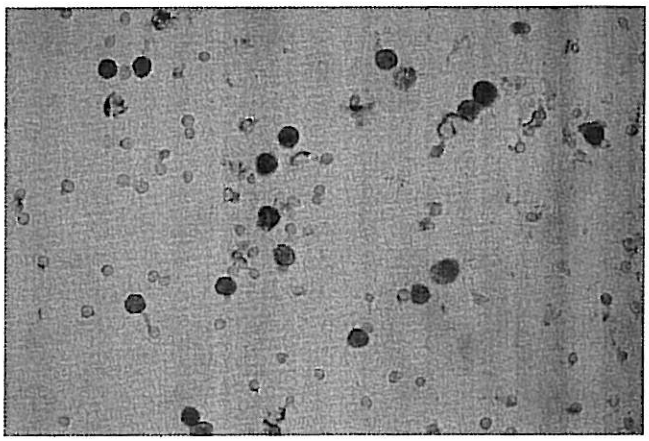

Fig. C-1D

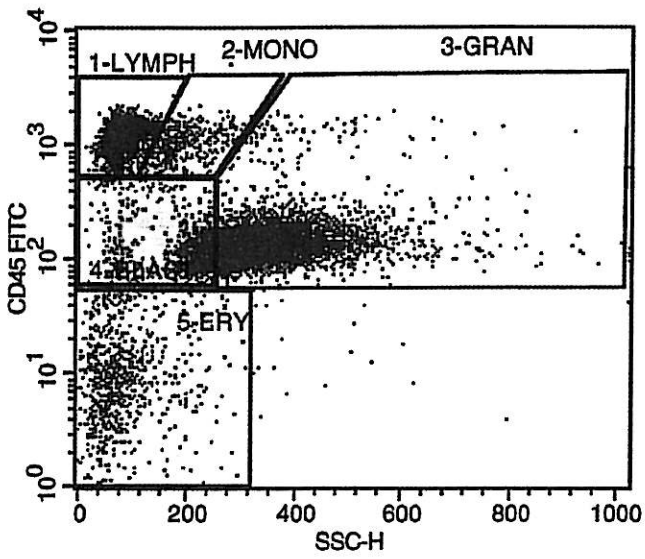

Fig. C-2A

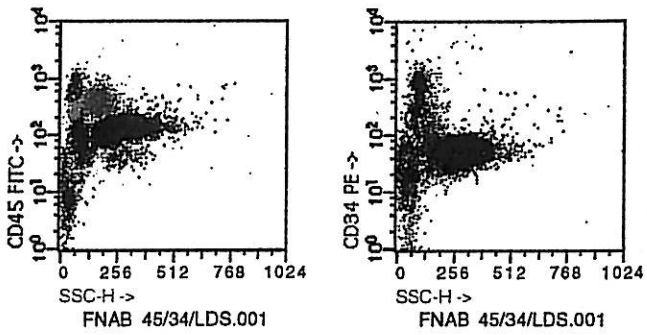

Fig. C-2B

Fig. C-1. A: Fine-needle biopsy of supraclavicular lymph node. Notice the massive substitution of the lymphoid cells with a polymorphic infiltrate of myeloid cells with numerous undifferentiated blasts. MGG, ×250, original magnification. **B:** Bone marrow smear. Notice the close similarity of the cellular picture with that of the lymphnode fine-needle biopsy. MGG, ×400, original magnification. **C:** Bone marrow smear: higher magnification showing a well-formed Auer rod (lower right) and several immature myeloid cells in the background. Myeloperoxidase cytochemistry, ×1,060, oil. **D:** Fine-needle biopsy of the supraclavicular lymphnode: myeloperoxidase immunocytochemical stain. Diffuse cytoplasmatic positivity of the cell population. Immunoperoxidase, ×125, original magnification.

Fig. C-2. A: flow cytometric analysis of bone marrow performed by using the CELLQUEST software (Beckton Dickinson). X-axis, side scatter; Y-axis, CD45FITC. Bone marrow cells are separated into five cell populations (lymphocytes, monocytes, granulocytes, blasts, and erythroblasts). $CD34^+$ cells are depicted in yellow. **B:** Flow cytometric analysis of lymphnode aspirate performed by using the PAINT-A-GATE software (Beckton Dickinson). X-axis, side scatter; Y-axis, CD45 FITC (left) and CD34-PE (right). Cells are separated into six cell populations and depicted with different colors: green (lymphocytes), orange (monocytes), blue (granulocytes), red (blast cells, $CD36^+$), cyan (immature lymphocytes), and violet (erythroblasts).

of maturation (Fig. C-1A). A number (about 10%) of these cells were undifferentiated type 1 myeloid blasts, with large, round, hyperchromatic, nucleolated nuclei surrounded by a narrow rim of slightly azurophilic cytoplasm in MGG-stained smears (Fig. C-1C); some of these showed small-sized, multiple, well-formed Auer rods. Granular, type 2 myeloblasts constituted about 55% of the blasts. A higher percentage of cells was represented by promyelocytes and differentiating myelocytes. Among the more mature cell types, hypogranular metamyelocytes, eosinophils, and segmented neutrophils prevailed. Rare dysplastic megakaryocytes and more than occasional erythroblasts were also observed.

Immunocytochemistry performed on cytospins obtained from lymph node aspirates showed diffuse positivity of the undifferentiated blasts and promyelocytes for myeloperoxidase (Fig. C-1D) and CD34, while the more mature cell compartment resulted positive to CD13 and CD15.

Flow cytometric study of the lymph node aspirations gave the following results: mature lymphocytes, 3%; monocytes, 12%; granulocytes, 64%; myeloblasts, 10%; erythroblasts, 7% (Fig. C-2A). Cytopathologic findings in the lymph node aspirations were compared to routine bone marrow smears, which had been taken before the onset of lymphadenopathy. A remarkably similar cellular composition of the marrow smears was found as compared to the FNAB findings. This similarity was maintained when the flow cytometric charts pertaining to the lymphnode aspirate and the bone marrow aspirate were compared; the two populations had substantially the same $CD45/CD34^{+}$ phenotype (Fig. C-2B). Moreover, smears of peripheral blood, though more diluted, showed the same qualitative cell composition of the lymph node and bone marrow aspirate. The karyotype of the lymph node aspirate showed a normal female pattern.

Discussion

EMT may precede or complicate the course of acute or chronic leukemias or various types of MDS, including RAEB-t.[3–5] Clinical and histopathological criteria for the diagnosis of EMT have been set in the last decade and EMTs have been found in various anatomical sites, including lymph nodes.[6] Three subsets of EMT have been considered in this classification: well differentiated (WD), showing evidence of trilineage myeloid maturation; poorly differentiated (PD), more monomorphic with rare evidence of myeloid differentiation; and a blastic-cell type, which corresponds to the so-called granulocytic sarcoma (GS), in which evidence of myeloid cell derivation is based only on immunocytochemistry, flow cytometry, and other ancillary studies.

A recent study has reported the fine-needle aspiration biopsy cytology findings in three new cases of WD EMT diagnosed in the course of RAEB-t, as in the current report.[7] Cytopathologic criteria for the diagnosis of WD EMT in this subset of MDS consist, as in histopathology, in finding a polymorphic population of myeloid cells in which immature myeloid blasts generally do not exceed 20%–29%. These latter show most of the times Auer rods. Among the more mature cell types, hypogranular metamyelocytes, erythroblasts, and dysplastic megakaryocytes may also be found.[2,5,6]

In aspirates of WD EMT, whatever the site might be, the cytopathologist's impression is that of a polymorphic cell picture as opposed to a clonal monomorphic cell proliferation. In cases with a high percentage of undifferentiated blasts, differential diagnosis has to be made with chronic myeloid leukemia and with chronic myelomonocytic leukemia; the finding of Auer rods and of a maturation hiatus may help identify EMT. This differential is generally resolved by keeping into proper account the clinical and hematological data, differential cell counts, and, more importantly at present, flow cytometric studies.

While there is a reasonable agreement on the cytohistopathological classification of EMT, the basic nature of the process is still a controversial matter. The pathologist's view tends to unify WD, PD EMT, and GS, considering them as different faces of a soft-tissue sarcoma made up by myeloid elements and identifying in these processes two of the features of malignancy, the invasion of distant tissues/sites and mass formation.[7] The hematologist's view is that EMT should be considered as a malignant process only in case of GS.

In the subset of MDS, the presence of EMT in the absence of an accompanying acute myeloid leukemia is considered not necessarily a potential forerunner of malignancy, as demonstrated by the present case.

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Intravenous itraconazole for treating invasive pulmonary aspergillosis in neutropenic patients with acute lymphoblastic leukemia

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***Aspergillus* infection is associated with high mortality rate in immunocompromised hosts; more effective drugs for this infection are needed. Oral itraconazole has been studied in neutropenic fungus infected patients. Using a novel formulation (intravenous) of itraconazole, we successfully treated severe necrotizing pneumonias due to *Aspergillus* species occurring during a post-chemotherapy prolonged aplastic phase in two patients with acute lymphoblastic leukemia.**

Invasive pulmonary aspergillosis is an emerging complication in patients with acute leukemia. Its increasing incidence has been dramatically documented in the last decade, in both ante- and post-mortem studies (1,2). The overall response rates to conventional amphotericin B (cAMB) is unsatisfactory, ranging between 35% and 45%; long-term therapy is badly tolerated owing to nephro- and infusion-related toxicity (3). More effective and less toxic drugs for this infection are needed. We report the use of i.v. itraconazole for the treatment of invasive pulmonary aspergillosis in two patients who had undergone intensive chemotherapy for acute lymphoblastic leukemia (ALL) and were subsequently included in an international phase IV study (Protocol ITR-INT-92; Sporanox IV, Janssen Pharmaceutica, Beerse, Belgium).

Case report 1

Ph-positive ALL-L2 was diagnosed in a 39-year-old woman in November 1999. She proved to be resistant to 2 induction courses, and no bone marrow donor was available. In June 2000, she received additional chemotherapy (cytarabine 11 gr daily for 4 days and idarubicin 22 mg daily for 3 days). Antimicrobial prophylaxis with ciprofloxacin and fluconazole was given. After 10 days of severe neutropenia, she started having high fever followed by chest pain, cough, dyspnoea, and moderate hypoxia, and received broad-spectrum antibiotics. A week later the persistence of symptoms and the appearance of pulmonary infiltrates (figure 1A) led to antifungal treatment with i.v. cAMB 1.5 mg/Kg/day and G-CSF 5 _g/Kg/day. After 10 days, cAMB was stopped for persisting symptoms and onset of refractory severe hypokalaemia. Intravenous itraconazole was started at 400 mg daily on the first 2 days, followed by 200 mg daily for 12 days. The clinical course rapidly improved with symptom regression, although recovery from neutropenia occurred 2 weeks later. A second course of i.v. itraconazole (compassionate use) was given (same dosage and duration), for persisting pulmonary infiltrates (figure 1B). In September 2000, the patient was discharged in complete hematological and cytogenetic remission from ALL; itraconazole was continued orally (5 mg/Kg twice a day). A month later, she underwent surgical curettage of a peripheral residual nodule: pathologic examination and culture of the surgical section documented *Aspergillus fumigatus* infection. In January 2001 a course of consolidation chemotherapy was given, but the patient died of leukemia relapse resistant to salvage chemotherapy in June 2001; neither signs nor symptoms of pulmonary infection recurred after consolidation and salvage treatments.

Case report 2

In July 2000 a 43-year-old woman with ALL-L1 was admitted for induction chemotherapy including L-asparaginase, vincristine, daunorubicin and prednisone (GIMEMA 0496 Protocol). Standard antimicrobial prophylaxis was given. After 10 days of moderate neutropenia, she started having high fever followed by chest pain, cough, dyspnoea and moderate hypoxia, and received meropenem 3 gr daily, amikacin 1 gr daily and teicoplanin 400 mg daily. A week later, the persistence of symptoms and the appearance of pulmonary infiltrates (figure 1C) prompted antifungal treatment (i.v. itraconazole 400 mg daily for 2 days, followed by 200 mg daily for 12 days). After starting itraconazole the clinical course rapidly improved with symptom regression. No pathogens were found in the culture of bronchoalveolar lavage. Two weeks later recovery from neutropenia occurred and the patient was discharged. Itraconazole was continued orally (5 mg/Kg twice a day) for persisting pulmonary infiltrates, likely due to *Aspergillus* species (figure 1D). A few months later a CT-scan found no pulmonary infiltrates. She underwent consolidation and maintenance chemotherapy; neither signs nor symptoms of pulmonary infection recurred. The patient is in continuous complete hematological remission from ALL and off-therapy.

Only a few drugs are effective for treating invasive pulmonary aspergillosis, including conventional and lipid-based AMB, itraconazole, and, more recently, voriconazole and caspofungin (4,5). Few data are available on the use of i.v. itraconazole for invasive pulmonary aspergillosis in immunocompromised hosts (6). Our patients had a proven (case 1) or possible (case 2) invasive pulmonary aspergillosis (7,8); within the frame of a trial on the use of i.v. itraconazole in invasive fungal infections, this drug was introduced after informed consent as first-line treatment in case 2 and after failure of cAMB in case 1. During the following 2 weeks, even in the absence of neutrophil recovery, a complete disappearance of infectious symptoms occurred in both cases. Complete disappearance of the pulmonary infection was achieved after switching to high-dose oral itraconazole (Sporanox Oral Solution) (9), combined with surgical curettage in one case. At the dose used, i.v. itraconazole did not show any side-effect or hematological toxicity. In conclusion, this drug proved to be effective and well tolerated for the management of life-threatening invasive pulmonary aspergillosis. These observations warrant further investigations of i.v. itraconazole use in neutropenic patients with acute leukemia and *Aspergillus* infection.

Legend to figure:

Figure 1- Changing characteristics of invasive pulmonary aspergillosis on computed tomography scans. (A) Subpleural large parenchymal masses with a surrounding halo of ground-glass attenuation (halo sign), in the right lung; a small nodular lesion in the left lung. (B) Two weeks later, a fungus ball inside the right lesion. (C) Large triangular infiltrate in the left lung, and bilateral pleural effusion. (D) Two weeks later, cavitation described as air-crescent sign inside the parenchymal lesion.

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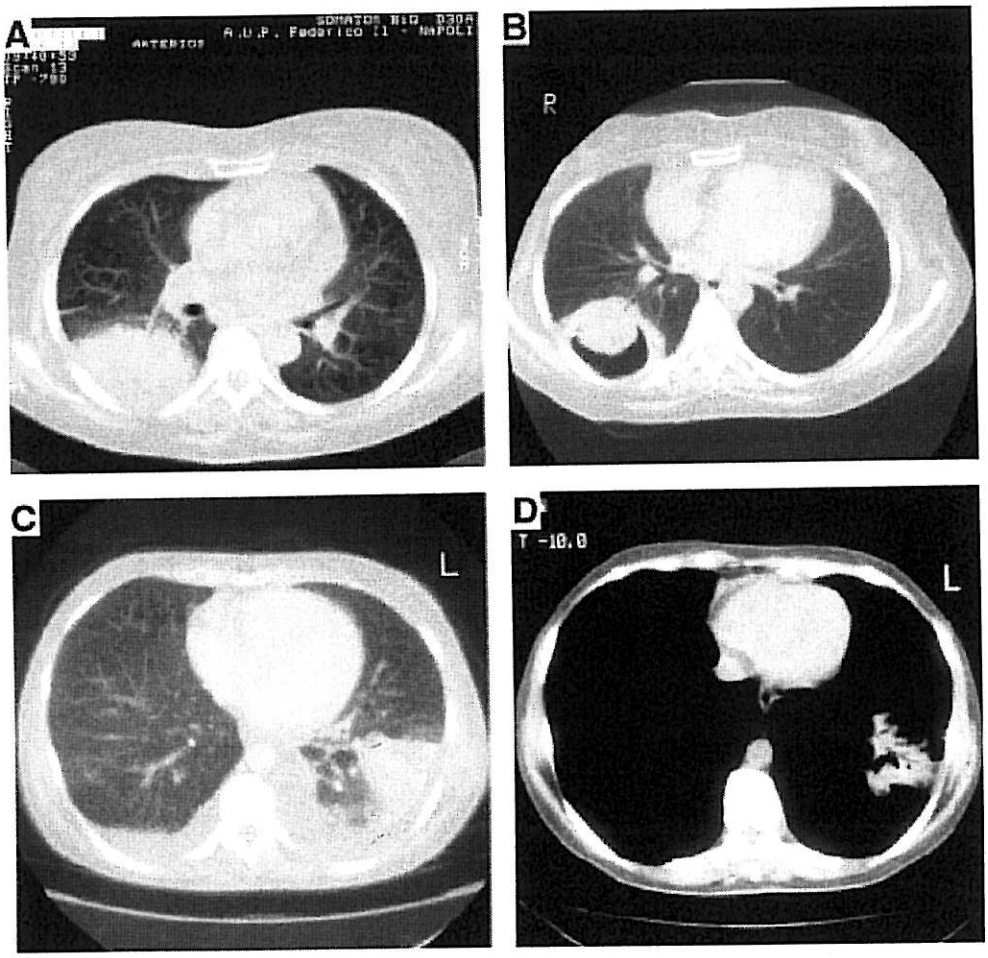
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Spleen enlargement following recombinant human granulocyte colony-stimulating factor administration for peripheral blood stem cell mobilization

Marco Picardi, Gennaro De Rosa, Carmine Selleri, Nicola Scarpato, Ernesto Soscia, Vincenzo Martinelli, Rosanna Ciancia, Bruno Rotoli

Background and Objectives. Recombinant human granulocyte colony-stimulating factor (rhG-CSF) is widely used to mobilize peripheral blood stem cells (PBSC) for autologous or allogeneic transplants. Such treatment may cause spleen enlargement; exceptionally, spontaneous spleen rupture has been reported. We investigated changes in spleen size during stem cell mobilization.

Design and Methods. We evaluated spleen size, comparing palpation with ultrasound (US)-evaluated longitudinal diameter and volume, in 13 healthy donors and 22 patients with a hematological malignancy who were undergoing PBSC mobilization with rhG-CSF-including regimens.

Results. Intraobserver and interobserver variability of US-calculated spleen volume was very low; the correlation between the volume calculated by US and that measured by 3-dimensional computed tomography was excellent. During mobilization, spleen enlargement was detected by palpation in 17% of subjects, by US-measured longitudinal diameter in 60%, and by US-calculated volume in 91%. The median increase in spleen volume was 300 mL (range, 54-820; $p<0.001$) in healthy donors and 135 mL (range, 0-413; $p=0.004$) in the group of patients; the enlargement correlated with white blood cell count elevation ($p=0.016$) but not with circulating CD34$^+$ cells. One month after the last administration of rhG-CSF, the median decrease was 160 mL (range, 35-800) in healthy donors and 58 mL (range, 0-310) in patients.

Interpretation and Conclusions. When evaluated by sensitive methods, rhG-CSF caused spleen enlargement in almost all individuals treated. US-calculated volume proved to be an excellent method, much better than longitudinal diameter, for detecting non-palpable splenomegaly induced by rhG-CSF.

Key words: spleen enlargement, rhG-CSF, peripheral blood stem cell collection, ultrasound scan.

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Peripheral blood stem cells (PBSC) are increasingly being used as an alternative to bone marrow for autologous or allogeneic transplants.[1] Recombinant human granulocyte colony-stimulating factor (rhG-CSF) alone or in combination with other drugs is highly effective in mobilizing stem cells, and available data regarding its short- and long-term toxicity have shown no serious adverse effects.[2,3] However, about one-third of neutropenic patients chronically treated with rhG-CSF develop palpable splenomegaly,[4] and there have been reports of spontaneous spleen rupture in rhG-CSF- or cyclophosphamide plus rhG-CSF-mobilized individuals,[5-7] or even in patients treated with rhG-CSF or rhGM-CSF after chemotherapy for acute leukemia or lymphoma.[8,9] Few data are available on changes in spleen size as a result of a brief course of rhG-CSF.[10,11] We tested the accuracy of ultrasound (US)-calculated spleen volume compared with palpation and US-measured longitudinal diameter in detecting changes in spleen size in two groups of subjects whose PBSC were mobilized by rhG-CSF-including regimens (healthy donors for allogeneic transplant, and patients scheduled for autologous transplant). We compared spleen volume changes in the two groups of subjects, and correlated the changes with the mobilizing regimen used and with circulating leukocyte and CD34$^+$ cell counts. In a subgroup of patients, we assessed interobserver variability of US measurements and examined the correlation between US-calculated and computed tomography (CT)-measured spleen volume. Finally, in 10 healthy volunteers we assessed reference ranges for US volume and intraobserver variability of US measurements.

Design and Methods

We prospectively studied 35 consecutive subjects (healthy donors or patients affected by a hematologic malignancy) who underwent mobilization to collect PBSC for allogeneic or autologous transplant. After written informed consent, the healthy donors received a mobilization regimen of s.c. rhG-CSF (Lenograstim, Italfarmaco, Rome, Italy) 263 μg twice a day, while patients received i.v. cyclophosphamide 7 g/m^2 plus s.c. rhG-CSF 263 μg once a day starting the day after the administration of cyclophosphamide (with the exception of patients with acute myeloid leukemia, who were mobilized with rhG-CSF only, 263 μg once a day, at recovery after consolidation chemotherapy). Flow cytometric counts of CD34$^+$ cells were monitored by a flow cytometer (FAC-

Scan, Becton Dickinson, San Jose, CA, USA) and expressed as cells/μL.[12] Circulating leukocytes, neutrophils and CD34+ cells were evaluated in all subjects on the day of the last rhG-CSF administration. PBSCs were collected by a double lumen venous catheter or venipuncture of both arms, performing large-volume apheresis with a continuous-flow cell separator (Spectra, COBE, Lakewood, CO, USA).

All spleen US scans were performed by the same operator, who used an EUB 525 Hitachi (Tokyo, Japan) instrument with a 2.5/3.5-MHz broadband curvilinear probe. Three scans were obtained for each subject: 1) the day before starting rhG-CSF, 2) the day of PBSC harvest, soon before collection, in mobilized donors (or the last day of rhG-CSF administration in the case of unsuccessful mobilization), 3) one month after rhG-CSF withdrawal. The spleen was scanned in the longitudinal and transverse planes by an intercostal and/or subcostal approach in subjects in the fasting state, in the supine or right-sided position, until complete organ visualization had been achieved. Longitudinal diameter, perimeter and area, defined as the maximum measurements with splenic borders and angles clearly defined, were measured, and the software of the US machine automatically calculated (area-length method: volume= $8 \times \text{area}^2/3 \times \pi \times$ longitudinal diameter) the volume (in milliliters), as already reported.[13] For each subject, the mean value of 3 measurements repeated on the same occasion was calculated and recorded for the final analysis.

In 10 healthy volunteers (matched for sex, age, and body-surface area with the cohort of subjects analyzed) we established reference values for US-calculated spleen volume and repeated the measurements 3 times at 1-week intervals to evaluate intraobserver reproducibility. In 3 unselected patients the US scan was repeated on 3 occasions (pre-, during-, and post-rhG-CSF course) by another operator unaware of the previous results and using the same US machine (interobserver reproducibility).[14] After additional informed consent, spleen CT scanning was performed in these 3 patients soon after the US examinations. Spleen axial images were obtained by a multirow helical instrument (Mx 8000; Marconi Medical Systems, Cleveland, OH, USA) to produce a 3-dimensional model (including length, width, thickness and cross-sectional area) used to calculate spleen volume automatically.[15] Technical parameters included a 6.5-mm slice width with identical reconstruction index, pitch 1, 200 mA, 120 kilovolt potential, and a rotation time of 0.75 seconds.

Statistical evaluations, including χ^2 testing, analysis of variance with Bonferroni's correction and Pearson's correlation, were performed with SPSS for Windows software (version 9.0, SPSS, Chicago, IL, USA).

Results

Characteristics of healthy donors and patients

As shown in Table 1, we analyzed 13 healthy donors and 22 patients affected by multiple myeloma (n=11), aggressive non Hodgkin's-lymphoma (n=4), acute myeloid leukemia (n=4) or Hodgkin's lymphoma (n=3) who had received chemotherapy courses 1 to 3 months before mobilization. The median age was 38 years (range, 28-55) and median body-surface area 1.8 m^2 (range, 1.6-2.1) for healthy donors, and 51.5 years (range, 18-63) and 1.8 m^2 (range, 1.5-1.98) for patients. No subject had palpable splenomegaly at entry to the study. During the study, screenings for infectious diseases possibly associated with splenomegaly (hepatitis A, B, and C viruses, human immunodeficiency virus 1/2, Epstein Barr virus, herpes simplex virus, varicella zoster virus, cytomegalovirus, toxoplasma sp.) and for the underlying hematologic malignancy were performed. No current viral or toxoplasma infection was detected, and in all patients the underlying hematologic disease was stable.

Spleen size assessment by different methods

In 10 healthy volunteers used for reference values, US-measured spleen longitudinal diameter ranged from 8 to 11.5 cm (median, 10) and US-calculated volume from 70 to 300 mL (median, 240). Intraobserver and interobserver reproducibility of spleen volume evaluation by US scan was excellent, with a Pearson value of 0.93 and 0.91, respectively. Spleen volume evaluation by US and CT scanning were well correlated, with a Pearson value of 0.94 ($p<0.001$) (Figure 1A).

Spleen size changes following rhG-CSF administration

In the 35 subjects analyzed during mobilization, splenomegaly was detected by palpation in 6, by US assessment of longitudinal diameter in 9, and by US assessment of volume in 27 (Figure 2). Compared to pre-rhG-CSF status, the spleen was found to be enlarged by palpation in 6, by US assessment of longitudinal diameter in 21 and by US assessment of volume in 32. Volume assessment had significantly higher sensitivity in detecting spleen enlargement than did the measurement of longitudinal diameter and palpation (91% of subjects versus 60% and 17%, respectively; $p=0.001$). Pre-rhG-CSF, spleen volume ranged from 81 to 380 mL (median, 254) in healthy donors and from 50 to 567 mL (median, 232) in patients. On the last day of rhG-CSF administration, spleen volume was enlarged in 13/13 healthy donors (median, 470 mL; range, 135-1200 mL) and in 19/22 patients (medi-

Table 1. Characteristics of patients and healthy donors.

Nr.	Sex	Age	Dx	Previous CHT	Mobilization CTX*	Mobilization G CSF°	WBC peak x10⁹/L	CD34+ cell peak/μL	Spleen volume (mL) before	Spleen volume (mL) during	Spleen volume (mL) after
1	F	33	Hdon		-	2×5	85.5	118	270	600	312
2	F	45	Hdon		-	2×6	56.5	70	287	370	300
3	M	38	Hdon		-	2×6	54.0	43	280	650	350
4	F	29	Hdon		-	2×5	53.4	66	254	406	246
5	F	28	Hdon		-	2×6	53.3	175	232	583	319
6	M	32	Hdon		-	2×7	51.6	66	380	1200	400
7	M	30	Hdon		-	2×8	50.0	10	170	470	390
8	F	35	Hdon		-	2×6	45.0	90	150	450	350
9	F	39	Hdon		-	2×6	44.8	90	250	350	280
10	F	41	Hdon		-	2×4	40.1	80	336^	450	400
11	F	55	Hdon		-	2×6	36.0	118	81	135	100
12	M	39	Hdon		-	2×6	30.6	98	200	660	400
13	M	38	Hdon		-	2×7	30.8	40	376	671	370
14	F	44	MM	Thal+D	+	1×12	30.0	10	240	520	280
15	F	63	MM	VAD	+	1×13	16.5	155	215	467	210
16	F	44	MM	Thal+D	+	1×10	14.3	52	220	360	310
17	F	54	MM	VAD	+	1×17	13.0	5	80	80	80
18	F	57	MM	VAD	+	1×13	10.0	211	50	130	100
19	F	53	MM	VAD	+	1×13	10.0	46	407	820	600
20	F	57	MM	VAD	+	1×13	9.8	78	180	560	250
21	F	46	MM	Thal+D	+	1×7	5.9	80	210	270	250
22	F	32	MM	VAD	+	1×12	2.4	153	350	350	350
23	F	53	MM	VAD	+	1×12	2.1	101	73	170	160
24	M	59	MM	Thal+D	+	1×12	1.8	30	225	458	300
25	M	47	NHL	CHOP	+	1×11	10.8	148	320	360	310
26	F	52	NHL	CHOP	+	1×13	9.0	10	418#	680	510
27	M	49	NHL	CHOP	+	1×13	7.5	37	66	114	70
28	M	55	NHL	CHOP	+	1×12	5.0	34	567#	770	600
29	F	35	AML	AML-12	-	1×6	16.8	35	370	510	360
30	M	51	AML	AML-12	-	1×6	16.4	51	104	176	110
31	F	55	AML	AML-12	-	1×5	16.0	97	240	370	360
32	M	40	AML	AML-12	-	1×13	3.0	4	260	260	260
33	F	24	HL	VEBEP	+	1×12	24.0	70	200	328	300
34	F	32	HL	VEBEP	+	1×8	20.0	30	280	490	320
35	M	18	HL	VEBEP	+	1×12	16.1	29	330	600	400

Hdon: healthy donor; MM: multiple myeloma; NHL: non-Hodgkin's lymphoma; AML: acute myeloid leukemia; HL: Hodgkin's lymphoma; Thal+D: thalidomide and dexamethasone; VEBEP: an ABVD-like regimen including vinblastine, etoposide, bleomycin, epirubicin and prednisone; AML-12: induction and consolidation according to the EORTC-GIMEM Protocol.
**Cyclophosphamide 7 g/m²; °Vials per day x number of days; ^a donor with β-thalassemia trait; #patients with spleen involved by lymphoma.*

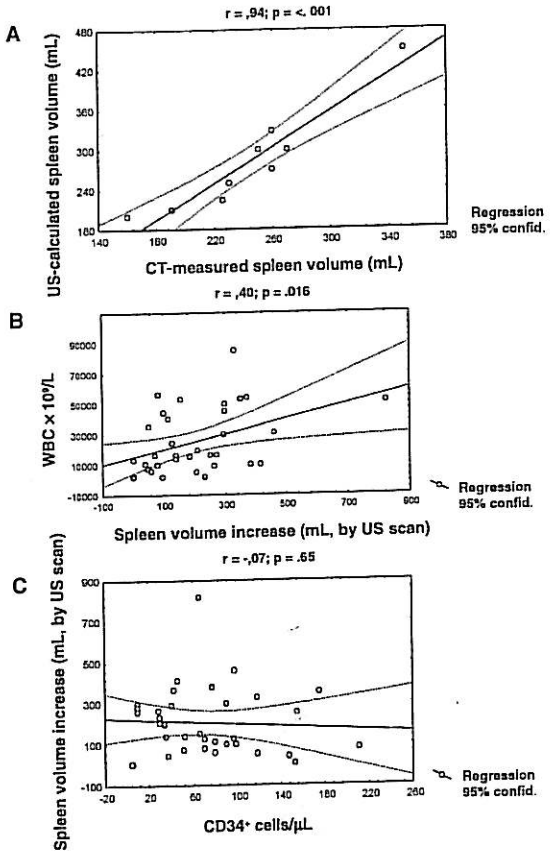

Figure 1. Statistical correlations. (A) Correlation between US-calculated volume and 3-dimensional CT-measured volume in 3 patients before, during, and after rhG-CSF administration. (B) Correlation between white blood cell count elevation and spleen volume increase in the whole cohort of subjects analyzed (n=35) on the last day of rhG-CSF administration. (C) Absence of correlation between spleen volume increase and circulating CD34⁺ cells in the whole cohort of subjects analyzed (n=35) on the last day of rhG-CSF administration.

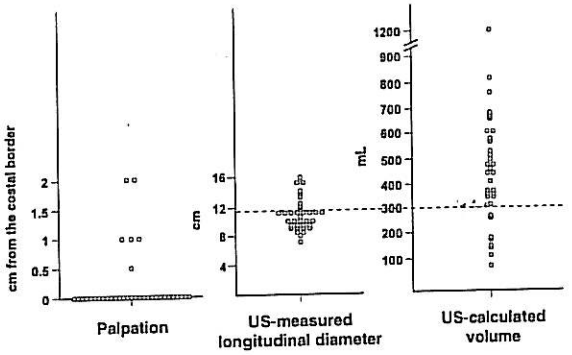

Figure 2. Spleen size soon after the last dose of rhG-CSF administration as detected by different methods. The dotted line is the upper limit of normal values.

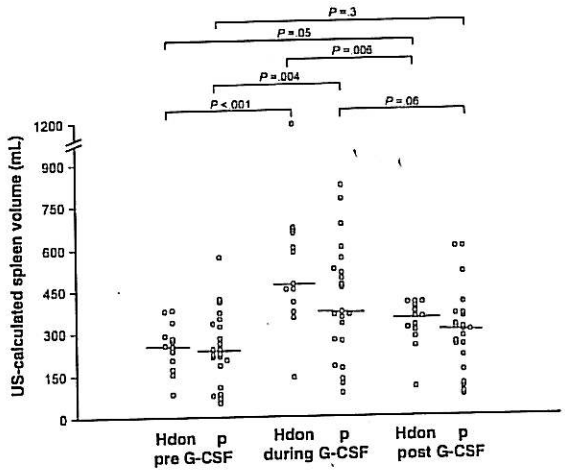

Figure 3. Spleen volume modifications following rhG-CSF administration. Spleen volume was evaluated by US before, during, and after rhG-CSF administration in 13 healthy donors (Hdon) and 22 patients (*p*) undergoing PBSC mobilization.

an, 365 mL; range, 80-820 mL); values were significantly higher than before rhG-CSF in both groups (healthy donors, $p<0.001$; patients, $p=0.004$). The difference in percent increase in spleen volume between healthy donors (median 122%; range, 29-230) and patients (median 66.5%; range, 0-211) was statistically significant ($p=0.02$) (Figure 3).

PBSC mobilization and collection

Overall, 30 subjects mobilized and underwent a single PBSC apheresis after a median rhG-CSF treatment of 6 consecutive days in healthy donors and of 12 days in patients. One healthy donor and 4 patients were poor mobilizers. The healthy donor (#7 in Table 1) had a circulating leukocyte peak of 50×10^9/L and spleen enlargement from 170 to 470 mL; patient #14 had multiple myeloma with leukocyte peak of 30×10^9/L and spleen enlargement from 240 to 520 mL; patient #17 had multiple myeloma with a leukocyte peak of 13×10^9/L without spleen enlargement; patient #26 had non-Hodgkin's lymphoma, with a leukocyte peak of 9.0×10^9/L and spleen volume increased from 418 to 680 mL; and patient #32 had acute myeloid leukemia, with neither circulating leukocyte elevation nor spleen enlargement. A single patient (#22) with multiple myeloma was a good mobilizer, showing neither leukocyte elevation nor spleen enlargement. Overall, spleen volume enlargement

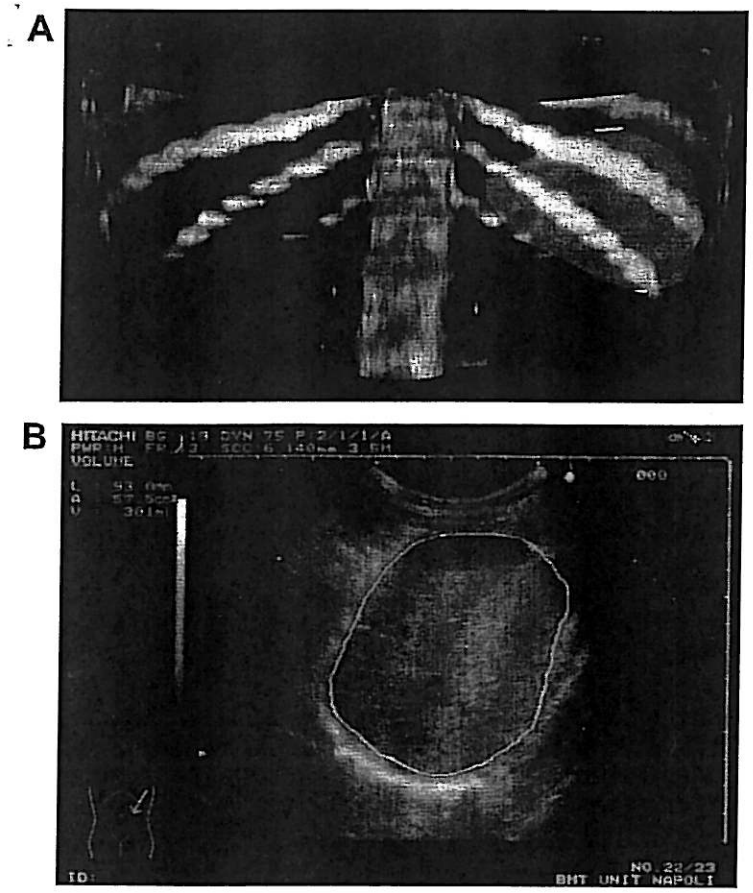

Figure 4. Spleen images obtained by three-dimensional CT scanning (A) and by US scanning (B). In this representative patient (#33) spleen volume was 300 mL by CT scan and 300 mL by US scan.

was detected in 32/35 rhG-CSF-treated subjects (91%), and in 29/30 mobilizers (97%).

Circulating cells and spleen size changes

On the day of the last rhG-CSF dose, leukocyte and neutrophil counts were significantly higher in healthy donors than in patients, although the number of circulating CD34+ cells was similar (Table 2). Spleen volume increase correlated with the increase in white blood cell count (p= 0.016; r=.4) (Figure 1B); by contrast, no correlation existed between spleen volume increase and the rise in circulating CD34+ cell count (Figure 1C). Indeed, white blood cell and CD34+ cell increases were not correlated (p=0.48, r=.12).

Spleen enlargement reversal

One month after the last dose of rhG-CSF, spleen volume had regressed to between 100 and 400 mL (median, 350) in healthy donors and to 70 and 600 mL (median, 300) in patients; there was a borderline statistical difference (p=0.05) between the first and the third US examination in the group of healthy donors.

Spleen size change-related symptoms or complications

Even upon specific questioning, no subject reported any discomfort or pain in the splenic area during or after mobilization; US images always showed splenic parenchyma to be homogeneous, with no nodules or hematoma.

Discussion

There are anecdotal reports of spleen enlargement after rhG-CSF administration for PBSC collection. This was systematically investigated in a

Table 2. Peripheral blood values in the two groups of subjects soon after the last dose of rhG-CSF.

	Healthy donors median (range)	*Patients median (range)*	*P*
Leukocytes × 10^9/L	50.0 (30.6-85.5)	10.4 (1.8-30.0)	<.001
Neutrophils × 10^9/L	40.2 (24.0-68.0)	8 (1.4-19.2)	<.001
CD34+ cells /μL	80 (10-175)	48.5 (4-211)	NS

series of 91 healthy donors by Platzbecker *et al.*, using one-dimensional measurements:[10] after s.c. rhG-CSF 7.5 μg/kg/day for 5 days, US measurement of longitudinal and diagonal diameters showed that spleen size increased by a factor of 1.1, with no correlation with white blood cell elevation. An attempt to calculate spleen volume changes indirectly was also made, resulting in a supposed median volume increase of about 30%. In our series, the number of individuals with spleen enlargement after rhG-CSF administration rose from 17% and 60% as detected by palpation and US-measured longitudinal diameter, respectively, to >90% when the volume was taken into account. Multidimensional US spleen volume estimation showed a median size increase of 122% in the group of healthy donors. Low intraobserver and interobserver variability of measurements, and the excellent correlation with 3-dimensional CT-measured volume proved the high reliability of US-calculated volume for sizing the spleen (Figure 4).

The mechanisms by which splenic tissue enlarges during rhG-CSF administration are still unclear. They may include: i) intrasplenic accumulation of circulating granulocytes and myeloid precursors; ii) extramedullary myelopoiesis; and iii) intrasplenic trapping and/or proliferation of stem cells. In a few instances of splenectomy during mobilization with rhG-CSF, histological analyses documented intrasplenic infiltration by mature and immature myeloid cells;[5-7] animal studies suggested a massive migration of myeloid precursors from the marrow to the spleen via the blood, which was reversed one month after the end of rhG-CSF administration.[16-19] Myeloid accumulation could be due to modification of the adhesion molecule pattern induced by rhG-CSF on the cell surface of myeloid cells as well as of their receptors on splenic stromal cells.[17,20] In our study, the extent of spleen enlargement during rhG-CSF correlated with the increase in white blood cell count but not with that of circulating $CD34^+$ cells, thus fitting with the hypothesis of myeloid cell accumulation and arguing against stem cell homing and proliferation. These findings are consistent with those reported by Stroncek *et al.*[11]

Spleen enlargement was significantly greater in healthy donors than in patients. The difference observed between the two groups can be attributed essentially to the different schedule of rhG-CSF administration (double daily dose in healthy donors, although the cumulative dose was about the same in the two groups) and to residual myeloid suppression in the patients, who received rhG-CSF soon after high doses of cytotoxic drugs. Indeed, even peak white blood cell counts were significantly different in the two groups of individuals studied. Since the daily dose of rhG-CSF seems to be the major determinant for both white blood cell elevation and spleen enlargement, caution should be taken in scheduling high-dose rhG-CSF, especially in healthy donors.[21-23]

By one month after the end of rhG-CSF administration, spleen volume had decreased in both groups of subjects, suggesting that the enlargement is a temporary phenomenon.

It is noteworthy that no individual had any subjective symptoms of rapid spleen enlargement, not even the normal donor whose spleen size increased in a few days from 400 to 1200 mL; the absence of pain may be detrimental, since spontaneous splenic rupture could occasionally occur without any premonitory symptoms. US-calculated volume may help to identify donors with greater spleen enlargement, thus needing close monitoring.

In conclusion, in virtually all individuals submitted to stem cell mobilization a brief course of rhG-CSF induced significant spleen volume enlargement, which was directly correlated with an increase in circulating leukocyte count. There is a need to investigate whether different mobilizing regimens, including rhG-CSF in different pharmaceutical forms (e.g., pegfilgastrim) or other cytokines, have the same effect on spleen size.

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Pre-publication Report & Outcomes of Peer Review

Contributions

MP, GDR and BR were the main investigators who designed the study. MP performed the ultrasound examinations and wrote the paper. NS performed the collections of PBSC. ES performed the computed tomography examinations. CS, VM, and RC were responsible for the clinical care of analyzed subjects. All the authors gave their critical contribution to the manuscript. BR revised the paper and gave final approval for its submission. Primary responsibility for the paper: MP; primary responsibility for Tables 1, 2 and Figures 1-3; MP, BR; primary responsibility for Table 4: ES, MP.

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Manuscript processing

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In the following paragraphs, Professor Anderlini summarizes the peer-review process and its outcomes.

What is already known on this topic

Recombinant granulocyte colony-stimulating factor (rhG-CSF) is now frequently administered to normal stem cell donors to mobilize and collect peripheral blood stem cells for allogeneic transplantation.

Splenic enlargement and, rarely, non-traumatic rupture have emerged as adverse events related to rhG-CSF administration to healthy donors, although data on this complication remain sketchy.

What this study adds

The study by Picardi *et al.* expands on what is presently known, providing valuable information for physicians caring for these donors..

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Intestinal toxicity during induction chemotherapy with cytarabine-based regimens in adult acute myeloid leukemia

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Background: Cytotoxic regimens used in induction treatments for acute myeloid leukemia (AML) almost always include standard or high-dose cytarabine (Ara-C). During or soon after induction therapy, leukemic patients frequently develop gastroenteric complications, characterized by abdominal pain and diarrhea. The association of these symptoms with fever and melena is typical of necrotizing enterocolitis (NE), a life-threathening condition that can be documented by ultrasound abdominal scan.

Patients and methods: We analyzed retrospectively the clinical course of 169 adult patients with AML treated by standard dose Ara-C-containing induction regimens, either by continuous venous infusion (group 1) or subcutaneous injection (group 2). Ultrasonography was employed as early diagnostic tool in a majority of patients with gastroenteric complications. Bowel wall thickening was accurately measured and used to confirm the diagnosis of necrotizing enterocolitis.

Results: In the first group of 115 patients (median age, 51 years), gastroenteric complications were observed in 55 patients (48%), and 10 patients (9%) received diagnosis of NE, which was fatal in four. Patients with NE had a median age older than that of patients without gastroenteric symptoms, and a more prolonged neutropenia. In the second group of 54 patients (median age, 60 years), gastroenteric events were observed in 14 patients (26%), and no case of NE was recorded.

Conclusions: This retrospective analysis shows that NE is a serious complication occurring mainly in patients treated by Ara-C administered as continuous i.v. infusion.
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Keywords: acute myeloid leukemia; cytarabine; intestinal toxicity; necrotizing enterocolitis

Introduction

Cytarabine (Ara-C) is one of the most powerful cytotoxic drugs available for the treatment of acute leukemias. Ara-C-based regimens are the gold standard for induction therapy of acute myeloid leukemias (AML); in combination with anthracyclines and etoposide, it is able to induce 60–80% complete hematological responses.[1–3] The pharmacokinetic and pharmacodynamic properties of Ara-C led to its use with different dosages and schedules: low, standard or high dose, by subcutaneous or i.v. administration, or even by continuous intravenous infusion. Thus, Ara-C may be considered a flexible drug. A prolonged exposure to Ara-C, which can be attained *in vivo* by 24 h continuous infusion, is likely to increase its cytotoxic effect, both on leukemic cells and normal tissues.[4]

Several extrahematologic complications may be observed during and after Ara-C administration: drug-related fever, chemical conjunctivitis (more frequent with the highest doses), skin rash, palmar erythema, oral mucositis, neurotoxicity and gastrointestinal complications such as nausea, vomiting and diarrhea. Some of these complications, such as gastrointestinal (GI) damage, may be very severe and life-threatening. In this setting, neutropenic necrotizing enterocolitis (NE) is a rare but well-recognized intestinal complication occurring after cytotoxic treatment in patients with hematological malignancies. It is characterized by bowel inflammation with wall thickening, edema and necrosis, usually observed in the terminal ileum (ileotyphlitis), cecum or right colon.[5,6] Generally, NE is characterized by abdominal signs of severe gut injury (pain, diarrhea, melena) associated with fever.[7] Although severe neutropenia caused by intensive chemotherapy is retained as the main etiologic factor for NE development, even direct toxic effects on mucosal cells is to be considered.[4]

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Antimicrobial prophylaxis and therapy

Microbiological specimens from pharynx, nose and stool were obtained before starting treatment and then weekly until hematological recovery. Cotrimoxazole (160/800 mg b.i.d., orally) or ciprofloxacin (500 mg b.i.d., orally) as antibacterial, and fluconazole (150 mg/day, orally) or itraconazole (200–400 mg/day, orally) as antimycotic prophylaxis were given to all patients. At the appearance of fever, culture from blood (central line and peripheral vein) and swabs from pharynx, nose and stool were performed, and empiric broad-spectrum antibiotic treatment (cephalosporin plus aminoglycoside) was started. A glycopeptide (vancomycin or teicoplanin) against Gram-positive bacteria was added if appropriate. In case of abdominal complications, meropenem (1 g every 8 h, i.v.) instead of cephalosporin, and metronidazole (500 mg every 12 h, i.v.) as anti-anaerobic were employed. In patients nonresponding to this treatment (ie, fever >38°C persisting for more than 96 h) amphothericin deoxycholate (1–1.5 mg/kg/day, i.v.) was started.

Ultrasonographic (US) examination

Bedside US of the four abdominal quadrants was performed by a hematologist trained in diagnostic ultrasonography in 58 patients who showed abdominal complications during or soon after the induction cytotoxic treatment. An EVB 525, Hitachi (Tokyo, Japan) instrument, with 2.5/3.5 MHz curvilinear probe was used. Liver, gallbladder, spleen and bowel were investigated. Bowel wall thickness was evaluated carefully, and a thickness ⩾4 mm was considered a pathologic finding.

Necrotizing neutropenic enterocolitis (NE)

Neutropenic patients showing fever, abdominal pain and diarrhea with or without intestinal bleeding, in whom sonographic findings showed bowel wall thickening ⩾4 mm, were diagnosed as having NE. These patients were treated with intensive supportive management including fasting, total parenteral nutrition, meropenem and metronidazole as stated above, and nasogastric suction if required.

Table 3 Clinical features of patients with NE compared with that of patients who showed other gastroenteric (GI) events and of patients without GI complications

	Patients with NE (n=10)	*Patients with GI complications (n=45)*	*Patients without GI complications (n=60)*
Gender: M/F	2/8	23/22	30/30
Age, median (range)	52 (30–59)	44 (16–72)	49 (15–77)
Other drugs			
DNR/Ida/Mito	3/4/3	22/14/8	38/7/10
Daunoxome	—	1	5
Etoposide	9	32	23
ANC<500 days	18	14	14
Plt<20 000 days	22	14	12

All patients received Ara-C by c.i., associated with daunorubicin (DNR) or idarubicin (Ida) or mitoxantrone (Mito), ±etoposide.

Statistical methods

For statistical analysis, we used the SPSS software. Possible differences between the two groups were evaluated using the χ^2 test. Any $P<0.05$ was considered significant.

Results

Patients treated by Ara-C continuous infusion (Group 1)

In this group of 115 patients, the duration of severe neutropenia (ANC <500/μl) and thrombocytopenia (platelets <20 000/μl) was 15 and 12 days, respectively. Fever ⩾38°C, mainly of unknown origin (FUO), was observed in 81 patients, for a median of 5 days (range 3–10 days). Moderate to severe abdominal symptoms occurred in 55 patients (48%); abdominal pain was associated with diarrhea (grade 2–3 WHO) in 48 cases (42%) and with melena (grade 4 WHO) in 15 patients (13%). US scan, performed in 46 patients, showed typical signs of NE in 10 cases, often localized at the caecum wall (ileothyphlitis). In these patients, bowel wall thickening ranged from 4 to 6 mm; other relevant US findings were ileum and proximal colon overdistension and moderate peritoneal effusion. NE occurred between day +15 and day +20 from the start of chemotherapy.

When we compared these 10 patients with the group of patients ($n=45$) who had GI complications without NE and with the other 60 patients who did not show GI complications, there was a prevalence of female patients, a slight difference in median age, and a more prolonged neutropenia and thrombocytopenia (Table 3).

In patients with abdominal symptoms *Escherichia coli*, *E. faecalis* and *Aspergillus flavus* were obtained from superficial swabs and/or blood cultures, while serum toxin assay for *Clostridium difficile* was always negative.

The outcome of patients with abdominal symptoms differed according to the presence or absence of diarrhea and melena and to the severity of the US findings. Overall, grade 3–4 intestinal injury was resolved by early adequate medical management in 16 patients, and was fatal in four. Death occurred because of severe intestinal bleeding (one case), mycotic invasion followed by blood dissemination due to Aspergillus (two cases), septic shock due to *E. coli* (one case).

Patients treated by subcutaneous Ara-C (Group 2)

Even in this group of 54 patients, neutropenia (ANC <500/μl) and thrombocytopenia (platelets <20 000/μl)

Table 4 GI complications in AML patients treated by regimens including Ara-C administered by continuous infusion (group 1) or s.c. (group 2)

	Group 1 (n=115)	*Group 2 (n=54)*	*χ^2 test (P)*
Abdominal symptoms (all WHO grades)	55 (48%)	14 (26%)	0.0069
Diarrhea (grade 2–3)	48 (42%)	9 (16%)	0.0013
Melena (grade 4)	5 (4%)	6 (11%)	0.75
Necrotizing enterocolitis	10 (9%)	0	0.0255
Induction death			
Total	7	6	0.253
Due to gastroenteric bleeding	0	3	0.013
Due to fatal NE	4	0	0.173
Time to ANC $>500/\mu l$ (days, median)	15	15	ns
Time to Plt $>20\times10^3/\mu l$ (days, median)	12	12	ns
Days with fever $\geqslant38°C$, median	5	4	ns

lasted a median of 15 and 12 days, respectively. Fever >38°C lasting a median of 4 days (range 2–20 days) occurred in 32 patients. Gastroenteric complications (all WHO grades) were observed in 14 patients (26%), with diarrhea in nine cases (17%) associated with melena in six (11%). Only three patients required intensive medical management with TPN, nasogastric suction and somatostatin. GI bleeding was fatal in three out of six patients who had this abdominal event. In this group, US scan was performed in 12 patients and no patient received diagnosis of NE. Microbiological stool cultures showed the presence of Gram− (*E. coli*) and Gram+ (*E. faecalis*, *Staphylococcus epidermidis*) bacteria; *C. difficile* was not detected by toxin assay.

When we compared the two groups of patients retrospectively studied by the χ^2 test, a significantly higher incidence of abdominal complications, diarrhea and NE emerged for the group of patients who had received Ara-C by c.i., although the group receiving Ara-C by s.c. injection included more patients with poor prognosis factors (older, ineligible for trials) (Table 4).

Discussion

In the last 10 years, cytotoxic regimens for AML have included Ara-C with variable doses and schedule of administration.[1,9–11] We have compared gastrointestinal complications in AML patients who had received standard dose Ara-C by continuous infusion with those who had received a similar drug dosage by s.c. injection.

In the group of patients treated by continuous infusion, a more prolonged toxic exposition both for hematopoietic cells and nonhematological tissues occurred, and this may have caused more frequent complications in the GI tract. Among GI complications, necrotizing enterocolitis is a rare life-threatening event reported after chemotherapy in patients with acute leukemias.[5,6,12,13] Even though abdominal pain, diarrhea and fever are frequent symptoms in patients treated by intensive chemotherapy, only a minority of them fulfill the diagnosis of NE according to US bowel examination. The reported incidence of NE during induction treatment for acute leukemias in adults ranges from 2 to 16%.[5,6,13] A recent prospective study by Gorschluter *et al.*[7] on 36 patients with leukemia who underwent 62 episodes of severe chemotherapy-induced neutropenia, confirmed a low incidence of NE, but a high mortality rate in patients in which it did occur.

In our entire series, GI complications occurred in 69/169 patients (41%), and NE was diagnosed in 10 cases (5.9%, 15% of those with GI symptoms); however, when the patients were stratified according to type of Ara-C administration (group 1: c.i.; group 2: s.c.) GI complications occurred in 48% in group 1 and in 26% in group 2, and NE was observed only in group 1 patients, with an incidence of 8.7%. Intensity and duration of neutropenia were the same in the two group of patients; this suggests that GI complications and NE occurrence were related to the degree of toxic mucosal damage rather than to infectious complications.

No statistical difference was found in relation to the different anthracyclines used; however, it is to be noticed that no patient out of six who received liposomal daunorubicin had NE.

Within the group of patients treated with Ara-C by c.i., when we compared the patients who developed NE with the patients who did not there was a small difference in median age and a more prolonged neutropenia and thrombocytopenia (Table 3). In addition, the combination of Ara-C c.i. with etoposide was used more frequently in patients developing NE.

In patients with GI complications, early detection of specific sonographic abdominal images can address the correct diagnosis and the proper treatment.[14] Medical management of NE includes total parenteral nutrition, nasogastric suction, antibiotics and antimycotics, somatostatin or octreotide, while no beneficial effects have been reported with glutamine oral supplementation.[15,16] In our series, NE was resolved by early diagnosis and early medical treatment in six cases, while four patients died because of severe infection or bleeding. This retrospective analysis points out to more frequent and severe abdominal complications in patients treated by Ara-C as continuous infusion.

A word of caution is needed in interpreting these results, because the group of patients treated by Ara-C c.i. received the drug for more days compared to the group with Ara-C s.c. However, it is unlikely that modest differences in Ara-C total dose may be responsible for increased toxicity, in a setting of relatively low doses; indeed, the hematological toxicity, measured as number of days of cytopenia, was exactly the same.

In conclusion, it is still unclear whether dose and schedule of Ara-C administration have a relevant impact on AML outcome; possible benefits in terms of antileukemic activity need to be balanced towards increased toxicity.

In neutropenic patients, drug-induced alterations of the bowel wall may also be responsible for hematogenous bacterial dissemination, leading to a septic status.

We analyzed retrospectively the incidence and type of gastroenteric complications in 169 adult AML patients submitted to induction treatment in our Hematology Unit, according to the schedule of Ara-C administration (continuous venous infusion versus subcutaneous treatment).

Patients and methods

We reviewed the clinical records of 169 adult patients who had received *de novo* diagnosis of AML between January 1985 and December 2000. The diagnosis of AML was made according to standard morphologic and cytofluorimetric findings; subtype classification was performed according to the FAB revised criteria.[8] Patients with acute promyelocytic leukemia were excluded from this study. All patients had been treated with an Ara-C containing induction regimen, and were divided into two groups according to the schedule of drug administration: continuous venous infusion (c.i.) (Group 1) or subcutaneous injection (s.c.) (Group 2). Group allocation was not random: a majority of patients were enrolled in international or national trials (EORTC, GIMEMA), the designs of which always included Ara-C by continuous infusion; the remaining patients were treated according to standard protocol including Ara-C by subcutaneous injections, because not eligible for the current trial, or because no trial was active in that period.

Continuous infusion (Group 1)

As first-line induction therapy, 115 patients had been treated by a polychemotherapy regimen in which Ara-C was administered by continuous infusion (ie, 24 h/day), according to various trials: EORTC 8A/8B ($n=45$); EORTC-GIMEMA AML 10 ($n=58$) and AML 13 ($n=6$); GIMEMA-elderly AML ($n=6$). There were 55 males and 60 females, with a median age of 51 years (range 15–77). Median Ara-C daily dose was 100 mg/m^2, for a median of 7 days. In association with Ara-C, 58 patients received daunorubicin, 25 patients received idarubicin and 26 patients received mitoxantrone; six patients were treated with liposomal daunorubicin (Daunoxome®). Etoposide was used for patients treated according to AML10 and AML 13 trials. Patient characteristics are summarized in Table 1, and protocol regimens are shown in Table 2.

Subcutaneous injection (Group 2)

In all, 54 patients, 24 males and 30 females, had been treated by an induction regimen containing Ara-C by subcutaneous injection every 12 h for 5 days (DATE, ICE). Several patients in this group had been considered ineligible for a multicentric study because of age, previous myelodysplasia or comorbidities. The median age was 60 years (range 15–78). Median Ara-C daily dose was 100 mg/m^2 for 5 days. In combination with Ara-C, 20 patients received daunorubicin (45 mg/m^2 for 3 days) and 6-thioguanine (100 mg/m^2 for 5 days), whereas 34 patients received idarubicin (8 mg/m^2 for 3 days). Etoposide (100 mg/m^2 for 3 days) was added in the induction treatment for all patients. The characteristics of this group of patients are also listed in Table 1.

Table 1 Characteristics of the patients whose data were retrospectively collected

	Group 1 (Ara-C c.i.)	*Group 2 (Ara-C s.c.)*
n	115	54
Age, median (range)	51 (15–77)	60 (15–78)
Gender (M/F)	55/60	24/30
FAB subtype		
M0–M1	28	11
M2	36	19
M4	31	11
M5(a/b)	17	7
M6	2	6
M7	1	—
Ara-C		
Median daily dose, mg/m^2	100	100
Median of days	7	5
Treatment protocol		
EORTC 8A/8B	45	—
EORTC-GIMEMA AML10	58	—
EORTC-GIMEMA AML13	6	—
GIMEMA, elderly AML	6	—
DATE	—	20
ICE	—	34

Table 2 Induction regimens applied

EORTC 8A/8B	Daunorubicin, 45 mg/m^2 d 1→3 ARA-C, 200 mg/m^2 c.i. d 1→7
AML10	Daunorubicin, 50 mg/m^2 or idarubicin, 10 mg/m^2 or mitoxantrone, 12 mg/m^2 d 1, 3, 5 VP16, 100 mg/m^2 d 1→5 ARA-C, 100 mg/m^2 c.i. d 1→10
AML13	Mitoxantrone, 7 mg/m^2 d 1, 3, 5 VP16, 100 mg/m^2 d 1→3 ARA-C, 100 mg/m^2 c.i. d 1→7
GIMEMA elderly	Daunoxome, 80 mg/m^2 d 1→3 ARA-C, 100 mg/m^2 c.i. d 1→7
DATE	Daunorubicin, 45 mg/m^2 d 1→3 6TG, 100 mg/m^2 d 1→5 ARA-C 100 mg/m^2 s.c. d 1→5 VP-16 100 mg/m^2 d 1→3
ICE	Idarubicin, 8 mg/m^2 d 1,3,5 ARA-C 100 mg/m^2 s.c. d 1→5 VP-16 100 mg/m^2 d 1→3

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Hepatitis B virus reactivation after fludarabine-based regimens for indolent non-Hodgkin's lymphomas: high prevalence of acquired viral genomic mutations

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Background and Objectives. Chemotherapy can cause hepatitis flare-up through viral reactivation in patients who have had contact with hepatitis viruses. Few data are available on the genotype of the reactivated viruses.

Design and Methods. In 40 consecutive adult patients with indolent non-Hodgkin's lymphoma (NHL) receiving fludarabine-based front-line chemotherapy, we performed a prospective study on viral hepatitis reactivation and analyzed the genotype of the reactivated viruses. Before chemotherapy, 4 patients were healthy carriers of hepatitis B surface antigen (HBsAg), 2 had HB core antigen antibodies (anti-HBc), 6 anti-HBs and 6 anti-HCV; 22 were seronegative.

Results. Hepatitis flare-up occurred in the 4 HBsAg-positive patients and in 1 anti-HBc-positive patient at a median of 1 month (range 1-4) after chemotherapy, when the CD4/CD8 ratio was still inverted. HBV reactivation was documented in all 5 instances (HBV-DNA 2-8×10^6 copies/mL). Two of the 5 patients responded to lamivudine, whereas 1 died of acute liver failure and 2 had persistent severe hepatitis. HBV genome sequencing at hepatitis flare-up showed that deviation from the closest related published sequences was 1.0% and 1.1% in the 2 lamivudine-responsive patients, and 1.5%, 1.8% and 1.7% in the 3 lamivudine-resistant patients. The polymerase open reading frame (ORF) and the HBs ORF of lamivudine-resistant strains contained several novel amino acid substitutions.

Interpretation and Conclusions. These results suggest that fludarabine treatment of HBV-infected patients is frequently associated with acute hepatitis due to viral reactivation, and that lamivudine may be less effective in this situation than in other settings of immunocompromised hosts because of the emergence of resistant mutant strains.

Key words: HBV, fludarabine, NHL, viral genomic mutations.

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Roughly a third of the world's population will contract hepatitis B virus (HBV) infection during their life.[1] The outcome of HBV infection is the result of a complex, as yet not fully understood, viral-host interaction, which may give rise to a wide spectrum of clinical conditions going from healthy carrier status to acute and/or chronic liver disease.[2,3]

HBV infection is prevalent in Asia, Africa, Latin America and Southern Europe, where the percentage of persistent HB surface antigen (sAg) carriers in the general population ranges from 2% to 20%.[4,5] Thus, in these countries it is not infrequent that a HBV carrier is a candidate for antineoplastic chemotherapy. Acute hepatitis due to HBV reactivation is a complication in this setting: 14 to 55% of HBsAg-positive patients undergoing chemotherapy may experience acute liver disease related to enhanced HBV replication.[6,7] A survey of Chinese patients undergoing chemotherapy showed that the frequency of HBV reactivation was highest in patients with non-Hodgkin's lymphoma (NHL), and this was attributed to the highly immunosuppressive steroid-based regimens used.[8] The clinical consequences of HBV reactivation range from mild liver dysfunction to massive necrosis and liver failure with a 5%-12% mortality rate.[9]

Fludarabine, a nucleoside analog used as a single agent or combined with other drugs for a variety of hematologic malignancies, is very effective in the treatment of indolent NHL.[10] It exerts its cytotoxic effect by penetrating the DNA of dividing cells where it induces a wide range of helix distorting lesions that are not repaired by the nucleotide excision repair complexes.[11] However, the efficacy of fludarabine against neoplasias with a low growth fraction , such as chronic lymphocytic leukemia and indolent NHLs, indicates that other mechanisms are operating, likely determining proneness to apoptosis.[12] A well known effect of fludarabine treatment is profound and prolonged immunosuppression with a relevant decrease of $CD4^+$ and $CD8^+$ lymphocytes, which predisposes to opportunistic infections[13,14]

To our knowledge, there are no data about acute hepatitis due to viral reactivation in NHL patients treated with fludarabine-containing regimens. We evaluated the incidence, risk factors, etiology, morbidity and mortality of acute liver damage in a series of patients undergoing fludarabine-based front-line chemotherapy for indolent NHL. Additional data on viral mutation rate were obtained from the study of the genome sequence of the virus strains responsible for hepatitis flare-ups.

Table 1. Clinical features and chemotherapy protocols of the 40 patients studied.

NHL type	*n*	*Hepatitis virus serological status at NHL diagnosis*	*NHL treatment*
Follicle center	26	HBsAg = 1, anti-HBc = 1 anti-HBs, anti-HBc, anti-HBe = 5 anti-HCV = 4 Seronegative = 15	Flu + Mito = 18 Flu = 8
Nodal small lymphocytic	10	HBsAg = 2, anti-HBc = 1 anti-HBs, anti-HBc, anti-HBe = 1 anti-HCV = 2 Seronegative = 4	Flu = 5 Flu + Mito = 3 Flu + Ida + Cy = 2
Marginal zone	4	HBsAg = 1 Seronegative = 3	Flu + Ida + Cy = 3 Flu = 1

Flu indicates intravenous fludarabine (25 mg/m²/daily for 5 days) for 6 courses; Flu + Mito, intravenous fludarabine (25 mg/m²/daily for 3 days) and mitoxantrone (10 mg/m² for 1 day) for 6 courses; and Flu + Ida + Cy, intravenous fludarabine (25 mg/m²/daily for 3 days), idarubicin (14 mg/m² for 1 day) and cyclophosphamide (200 mg/m²/daily for 3 days) for 6 courses.

Design and Methods

Patients and study design

Over the past 4 years, we prospectively studied 40 consecutive previously untreated patients (22 women and 18 men; median age 50 years, range 40-82) affected by grade I follicular, nodal small lymphocytic or marginal zone B-cell NHL, according to the REAL/WHO classification[15] (Table 1). All patients were scheduled to receive 6 monthly courses of fludarabine-based chemotherapy regimens that did not contain steroids. Diagnostic tests were performed before the start of each course of treatment, and monthly after chemotherapy completion. Before starting chemotherapy, all patients were assessed for serum HBsAg and HBeAg, and for antibodies against HBs, HBe, HBc, Hδ, HCV, HAV, cytomegalovirus, Epstein Barr virus and herpes simplex virus using commercially available kits; HBV-DNA (sensitivity of the assay: >10^2 copies/mL) and HCV-RNA were measured by a quantitative polymerase chain reaction (PCR) procedure (Cobas Amplicor Roche Diagnostic Systems, Basel, Switzerland). The same tests were performed again in patients who developed a hepatitis flare-up (defined as a greater than 3-fold elevation of serum ALT and/or AST above the upper normal limit) together with flow cytometric assessment of CD4, CD8 and CD56 in circulating lymphocytes. Lamivudine (100-200 mg daily) alone or in combination with interferon-α (10 MU, subcutaneously three times a week) was administered to patients with hepatitis flare-up due to HBV; liver ultrasound and biopsy were performed in patients who did not show serological improvement, despite the antiviral therapy.[16]

Sequence analysis of HBV-DNA

Viral DNA was purified from 200 μL of the patient's serum, withdrawn at the time of HBV reactivation, using a QIAamp Blood Kit (Qiagen, Chatsworth,

Table 2. Primers used to amplify overlapping sequences of HBV genomes isolated from the patients' sera.

	Strand	*Sequence*	*Annealing temperature*	*Alignment to HBV reference sequence (no. X65257)*
1F	+	5'GGGTCACCATATTCTTGGG3'	50°C	2816-2834
1R	–	5'CA(A/G)AGACAAAAGAAAATTGG3'	50°C	821-805
2F	+	5'GTCTGCGGCGTTTTATCA3'	50°C	383-400
2R	–	5'GGAGTTCCGCAGTATGGATCGG3'	50°C	1284-1263
3F	+	5'CAACTCCTAGCAGCTTGTTTTGC3'	65°C	1278-1300
3R	–	5'GTGCCCCCCGTGGT3'	65°C	1531-1518
4F	+	5'TCTTTTGGAGTGTGGATTCGC3'	65°C	1263-1283
4R	–	5'CAGGTACAGTAGAAGAATAAAGCCCA3'	65°C	2513-2489
5F	+	5'TCAATCGCCCGCTCG3'	65°C	2409-2423
5R	–	5'GGATAGAACCTAGCAGGCATAATTAATT3'	65°C	2658-2631

Table 3. Clinical features and outcome of the five NHL patients who had HBV reactivation.

Pt.Sex/Age (years)	NHL DX	NHL therapy*	Serological status at NHL diagnosis	Acute hepatitis flare-up		HBV status at reactivation		Hepatitis treatment	Hepatitis outcome
				AST/ALT peak (IU/L)	weeks†	DNA copies (*10^6/mL)	sAg/eAg		
1 M/52	NSL	Flu + Ida + Cy	HBsAg+	1000/1200	9	5	+/-	Lamivudine	No improvement
2 F/60	NSL	Flu	HBsAg+	400/800	4	8	+/-	Lamivudine + α-interferon	No improvement
3 F/57	FC	Flu	Anti-HBc+	400/900	16	2	+/+	Lamivudine + α-interferon	Normalization in 48 weeks
4 F/40	MZ	Flu + Ida + Cy	HBsAg+	2000/2400	4	8	+/-	Lamivudine	Normalization in 5 weeks
5 F/52	FC	Flu + Mito	HBsAg+	800/900	4	5	+/-	Lamivudine	Normalization in 4 weeks

*NSL indicates nodal small lymphocytic; FC, follicle center; and MZ, marginal zone. *See Table 1. †Time from NHL treatment completion.*

CA, USA). Most of the HBV genome including the complete polymerase and HBs open reading frame (ORF) was amplified by a PCR in five separate reactions using partially overlapping primer couples (Table 2). PCR was performed using 20 ng aliquots of DNA in a mixture containing 10 mM Tris HCl (pH 8.3), 2 mM $MgCl_2$, 50 mM KCl, 0.2 mM of each deoxyribonucleotide, 2.5 U of Taq polymerase and 0.5 mM of each specific primer in a final volume of 50 μL. The time/temperature profile of the amplification reactions was: denaturation at 94°C for 1 min, annealing at primer specific temperature (Table 2) for 1 min, and extension at 72°C for 1 min, for 35 cycles. PCR-amplified products were cloned in a plasmid vector (TOPOCloning, Invitrogen, Carlsbad, CA, USA) and both strands of at least five independent clones of each amplification product were sequenced using automated DNA sequencing (ABI-310, Applera, Forster City, CA, USA).

The ABI sequence editor software was used to reassemble the whole viral genome sequence of each patient. The genomic sequences were compared with the HBV sequences available in the GeneBank and EMBL databases. The predicted translations of the five viral reading frames corresponding to polymerase, surface (S, PreS1, and PreS2), core, precore and X proteins, were compared to the SWISS-PROT resources. All genomic and protein sequence analyses were performed with the Blast and the Clustalw software available at the URL *http://bioinfo.biogem.it*. We followed the HEP DART International Committee recommendations for genotype comparisons and for the nomenclature of the mutations found in the genomic sequences of viral isolates.[17]

Results

Acute hepatitis development

Of the 40 patients analyzed, pre-treatment assessment of viral serological status showed that 12 were HBV-positive, 6 were HCV-positive, and the others were seronegative for both viruses. Of the 12 HBV-positive patients, four were healthy carriers of HBsAg (normal liver function tests and serum HBV-DNA negative by PCR), two were anti-HBc-positive without anti-HBs, and the remaining 6 were anti-HBs-positive (together with anti-HBe and/or anti-HBc) (Table 1). All the anti-HCV-positive patients had histologically-documented active chronic hepatitis, and HCV-RNA sequences were detected in their serum by PCR. In addition, 25 patients were positive for anti-HAV, 20 for anti-cytomegalovirus, 18 for anti-Epstein Barr virus and 15 for anti-herpes simplex virus-1.

All patients received the scheduled 6 courses of chemotherapy; none received hemoderivatives, or antiviral prophylaxis before, during or after chemotherapy.

Acute hepatitis was diagnosed in five patients after a median of 7 months from the start of chemotherapy (1 month from the completion of chemotherapy). All episodes of acute hepatitis were due to HBV reactivation (positive conversion of serum HBV-DNA assay) and were correlated with the pre-treatment serological status (Table 3). Indeed, all the four HBsAg-positive patients and one of the two anti-HBc-positive patients developed signs of acute liver damage. There were no signs of hepatitis in the 6 anti-HBs-positive patients, in the 6 anti-HCV-positive patients or in the remaining 22

Table 4. Peripheral blood lymphocytes in the 40 NHL patients at diagnosis and during chemotherapy, and lymphocyte subpopulations of the five patients at HBV reactivation.

	N. of patients		*Peripheral blood lymphocytes* *Total*	*CD4*	*CD8*	*CD56*
Pre-treatment	40	median range	1600 (893-5628)	na	na	na
After 3 courses of treatment	40	median range	770 (336-2150)	na	na	na
At treatment completion	40	median range	572 (296-1500)	na	na	na
At onset of acute hepatitis	5	median range	1200 (600-1600)	240 (132-353)	593 (354-810)	216 (108-592)

na indicates data not available.

patients (Figure 1). In particular, no hepatitis flare-ups were observed in patients who had had contact with HAV and with minor hepatotropic viruses (cytomegalovirus, Epstein Barr virus, herpes simplex virus-1). The drugs used in combination with fludarabine did not seem to influence the viral flare-up. In fact, of the five patients with HBV reactivation, two had received fludarabine as a single agent, one fludarabine plus mitoxantrone, and the remaining two fludarabine plus idarubicin and cyclophosphamide (Table 3). Interestingly, in all patients viral reactivation was observed at the time of immunological reconstitution; however, in all cases the CD4/CD8 ratio was still inverted (Table 4).

Clinical features and outcome of acute hepatitis

All five patients had a severe form of acute hepatitis. The median serum peaks were: AST 800 IU/L (range, 400-2000), ALT 900 IU/L (range, 800-2400), total bilirubin 12 mg/dL (range, 6-22) and HBV-DNA 5×10^6 copies/mL. Consequently, lamivudine was used as first-line treatment in all five. In two patients (#4 and #5 in Table 3) lamivudine rapidly induced normalization of liver function tests and disappearance of HBV-DNA from serum. HBV replication persisted in the remaining three patients. Ultrasound scan ruled out focal liver lesions in all three. Biopsies from patients #1 and #2, 32 and 40 days after acute hepatitis onset, respectively, showed marked hepatocyte lysis, moderate portal space fibrosis and inflammation, a number of portal-portal bridges, and intracytoplasmic HBsAg/HBcAg/HBV-DNA in the majority of hepatocytes, thereby confirming the viral etiology of the liver disease. Two of these three patients died. Patient #1 died of acute liver failure 7 weeks after starting lamivudine. Patient #2, who also received interferon-α, died of a second malignancy (intestinal perforation due to colon adenocarcinoma) two months after the onset of hepatitis, without any sign of liver function improvement. Interferon-α was added to lamivudine in patient #3; liver function tests normalized and serum HBV-DNA disappeared 12

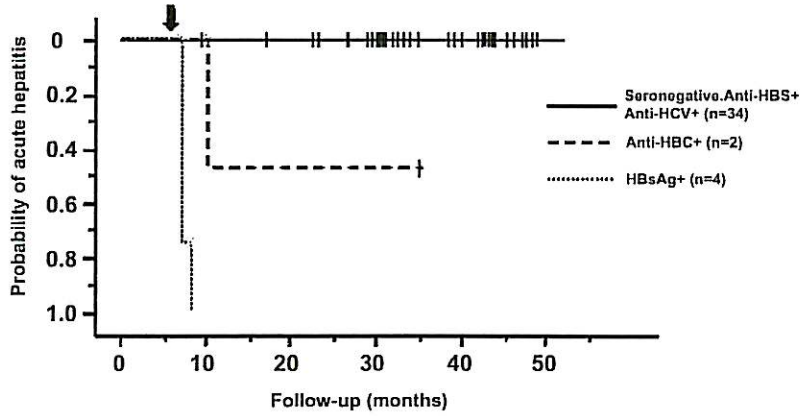

Figure 1. Actuarial risk of hepatitis flare-up in fludarabine-treated NHL patients. Patients are stratified according to the pre-treatment serological status (see text for details). The vertical arrow indicates the time of fludarabine treatment completion.

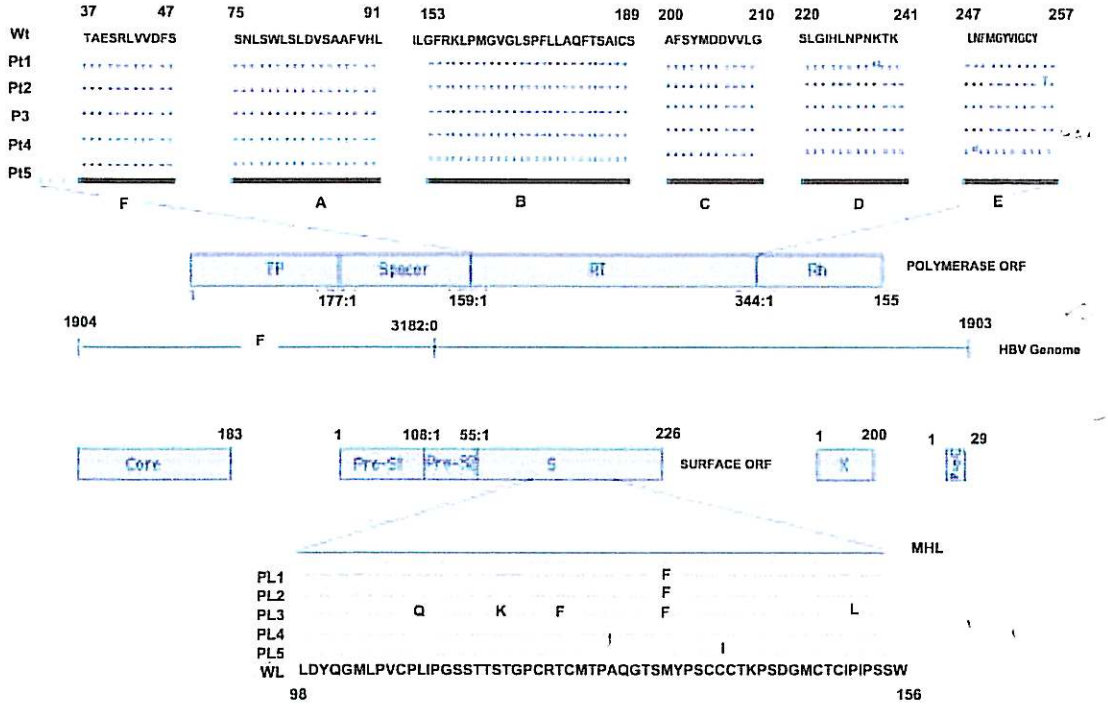

Figure 2. Amino acid mutations at the HBV conserved regions in the five NHL patients. Schematic representation of the HBV genome with its five open reading frames. The amino acid sequences of conserved regions of the reverse transcriptase and the major hydrophilic loop of the surface antigen are shown together with the amino acid substitution found in the patients. Amino acid numbering in the case of reverse transcriptase follows recommendations of the consensus proposals for HBV polymerase nomenclature.[17]

may lead to massive hepatocyte destruction.[6]

In the present study, fludarabine-based cytotoxic regimens were administered without steroids as front-line treatment in 40 consecutive patients affected by indolent NHL. Consistent with the high prevalence of hepatitis virus infections in Southern Europe, 30% of patients were HBV-positive and 15% had HCV active replication with chronic hepatitis. No hepatitis flare-up occurred after fludarabine treatment in the anti-HBs-positive patients, in the anti-HCV-positive patients or in patients seronegative for both viruses, whereas life-threatening acute hepatitis occurred in all four HBsAg-positive patients and in one of the two anti-HBc-positive patients. All five patients had normal liver function tests and no detectable levels of serum HBV-DNA before starting chemotherapy –conditions that can be defined as *healthy carrier* and *occult infection*, respectively.[20,21] This means that before chemotherapy, our patients had a very low level of circulating HBV-DNA ($\leq 10^2$ copies/mL), which was not detectable by the standard diagnostic tests. Alternatively, they could have harbored variable amounts of HBV-DNA exclusively in the liver, and this began to replicate upon the breakdown of immunosurveillance.[22,23] The clinical signs of hepatitis were manifested at reconstitution of circulating lymphocytes, but at a time when the CD4/CD8 ratio was still inverted (Table 4). Fludarabine treatment in NHL patients has been reported to cause a profound and prolonged CD4 depletion, lasting months after the termination of therapy.[24,25] Incomplete recovery of CD4 cells might explain the severity of the hepatitis observed in our patients. Although the hepatocellular injuries caused by HBV are mainly immune-mediated (CD8 cytotoxic lymphocytes directly recognize and lyse liver cells that have HBV-derived peptides on their surface), non-cytotoxic (CD4 lymphocyte-dependent) mechanisms are required for viral clearance.[5,26] The rate of spontaneous genomic mutations reported in HBV carriers is $\leq 0.7\%$.[27] Because HBV reverse transcriptase lacks proof-reading activity it has a high mutation rate, i.e., between 10^{-5} to 10^{-6} per site per year.[28] The consequent accumulation of sequence variants is called *sequence or strain evolution*. Because the virus mutation rate was higher in our patients than expected from spontaneous sequence evolution, one may suspect that fludarabine affects this mutation rate. The-

Table 5. Mutations found in the prevalent HBV strains of NHL patients at hepatitis flare-up.

Patient	*#Closest matched HBV sequences (Gene Bank no.)*	*Geno/ serotype*	*Total nucleotide mutations (%)*	*Total amino acid mutations (%)*	*Mutations at the MHL domain of HBs antigen*	*Mutations at conserved RT domains (A-F)*
1	X65257	D/ayw	1.5	2.3	Y134F	N238H
2	X65257	D/ayw	1.8	3.9	Y134F	C256T
3	V01460	D/ayw	1.7	3.7	L109Q T118K C124F Y134F S154L	
4	V01460	D/ayw	1.0	1.6	–	–
5	V01460	D/ayw	1.1	1.8	T140I	N246H

months after the onset of hepatitis.

Patients #3, #4 and #5 are currently thriving and off-therapy; they had seroconversion from HBsAg to anti-HBs and normal liver function tests at a median of 3.4 years from the onset of acute hepatitis.

HBV genomic mutations

None of the five patients with post-chemotherapy hepatitis flare-up had detectable circulating HBV genomic molecules at the time of NHL diagnosis. We were, therefore, able to analyze the genomic sequences only at the time of reactivation. Because of genetic diversity among the HBV genotypes A to F, there is no consensus sequence that could serve as standard for all comparisons. To measure the degree of variability in these sequences, we compared them to the closest related published sequence, which was then considered the reference sequence, and deviations from the reference sequence were considered to be mutations. In all five cases, the genomic sequences had the highest degree of homology with genotype D sequence, corresponding to the ayw serotype (Table 5). We found two types of HBV clones in each patient: a prevalent clone and a minor clone. The prevalent clone was considered the etiological agent of the hepatitis flare-up. The genomic sequences of the etiological clones deviated from the reference sequence by a mean of 1.42%, with a high degree of variability among the isolates. In each patient, the minor clone sequence matched the reference sequence much closer than the etiological strain. Interestingly, we found a rough correlation between the clinical response to lamivudine and the degree of genomic mutations of the corresponding etiological viral isolate. The deviation from the closest sequences was 1.0% and 1.1% for the isolates from, respectively, patients #4 and #5, who rapidly responded to lamivudine, whereas it was 1.5%, 1.8%, and 1.7% in, respectively, patients #1, #2 and #3, who did not respond to lamivudine (Table 5). When we translated the observed mutations into amino acid substitutions, we found that the difference between responders and non-responders was evident also at the polypeptide level. Indeed, the mean rate of amino acid substitutions was lower in patients #4 and #5 than in patients #1, #2, #3, both at the polymerase ORF and at the HBs ORF (2.7% vs 4.0%, and 1.25% vs 3.2%, respectively; Figure 2 and Table 5). It is noteworthy that all non-responders had the tyrosine→phenylalanine substitution at position 134 of the MHL domain of the surface ORF, and all the amino acid substitutions of the highly conserved regions were found only in the prevalent etiological clones and never in the minor clones (*data not shown*). As expected in our geographical area, we found stop codon mutations at the pre-core ORF in the isolates of four patients with HBV reactivation, which is consistent with the absence of HBeAg (Table 3).

Discussion

In four large series of HBsAg-positive patients treated with cytotoxic agents for hematologic malignancies, the rate of HBV reactivation ranged from 14% to 55%.[7-9,18] Inclusion of steroids in the chemotherapy regimens led to the highest rate of hepatitis flare-up and of fatal liver failure[3,7] probably because the HBV genome contains a glucocorticoid-responsive element that may act as an enhancer of viral replication.[19] More than 50% of hepatitis flare-ups due to HBV reactivation occur in the window between the end of chemotherapy and the complete recovery of immunocompetence.[7] After widespread viral infection of hepatocytes during immunosuppression, the rebound in T-cell function and number

oretically, fludarabine might enter hepatic cells and, given its similarity to purine nucleotides, it could be incorporated into HBV-DNA during viral replication thereby leading to proof-reading errors and, consequently, to the accumulation of mutations. Another, not necessarily alternative, mechanism is that the profound immunosuppression induced by fludarabine might favor a high rate of HBV replication, thereby increasing the probability of spontaneous genomic mutations. Upon restoration of immunocompetence, immune system effectors might select the predominant strain because of its replication capacity; in addition, lamivudine may favor the expansion of drug-resistant mutants. In our patients, the finding of a minor clone with only a few mutations seems to support the latter model.

There was a high incidence of non-responders to lamivudine in our small series of patients. Only two patients showed prompt normalization of liver tests and HBV-DNA disappearance, whereas patient #1 died of liver failure, patient #2 died of unrelated causes without any evidence of clinical response, and patient #3 recovered from the liver disease after 10 months of combined antiviral treatment. In the three lamivudine-resistant patients, the correlation between rate of genomic sequence mutations in the prevalent strain and response to the drug was confirmed by the finding of a high number of amino acid substitutions at two highly conserved regions, namely the MHL region of the surface antigen and the conserved A to F region of polymerase.[17] In particular, two patients had an amino acid substitution at the catalytic domains of reverse transcriptase, which are highly conserved among various viruses including HIV.[29,30] Lamivudine binding to a specific target on the polymerase (a pocket within the reverse transcriptase/polymerase domain) is crucial for the drug's powerful antireplicative activity. Most mutations associated with lamivudine resistance were found to occur at the 6 highly conserved domains of reverse transcriptase.[17,31] The mutations are usually selected for during treatment, increasing progressively with the duration of treatment, ranging from 15% to 30% after 12 months of treatment and exceeding 50% after 3 years.[32,33] Patients #1 and #2 had punctiform mutations of the polymerase gene, suggesting that nucleotide replacements may lead to conformational changes of the binding site, thus preventing access to lamivudine.[34] Patient #3 had 6 amino acid substitutions at the MHL domain, which is the target of the neutralizing antibodies.[35]

In conclusion, our study indicates that among patients who harbored HBV, HBsAg-positive and anti-HBc-positive patients constitute a subset at risk of severe hepatitis due to viral reactivation after fludarabine treatment. The incidence of hepatitis exacerbations in these patients is higher than that reported for patients treated with other types of chemotherapy regimens, including those containing steroids.[7-9,18] The absence of viral reactivation in our HCV-infected patients and the high reactivation rate in HBV-infected patients indicate that the mechanisms damaging the liver are substantially different in the two diseases. Specific prophylaxis does not seem necessary to prevent HCV reactivation during and after fludarabine therapy. By contrast, lamivudine administration may be needed to prevent or to treat HBV reactivation in immunocompromised patients.[36,37] However, the high rate of genome mutations and amino acid substitutions at critical conserved domains and the prolonged CD4 depletion observed in our series could be important factors for the viral reactivation, for the severity of the hepatitis and for the lack of response to lamivudine. Clinical trials are needed to determine the efficacy of other potent antiviral drugs and of combination treatments in the management of fludarabine-related HBV reactivation.[38,39]

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Pre-publication Report & Outcomes of Peer Review

Contributions

MPi, FP, FS and BR were the main investigators who designed the study and wrote the paper. FS, FP, CQ, ADG and BdD performed the molecular analyses. MPi, ADR, MPe and RC were responsible for the clinical care of analyzed patients. All the authors gave their critical contributiuon to the manuscript. BR and FS revised the paper and gave final approval for its submission. We thank Jean Ann Gilder for editing the manuscript. Primary responsibility for the paper: FP; primary responsibility for all Tables and Figures: CQ.

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In the following paragraphs, Professor Cazzola summarizes the peer-review process and its outcomes.

What is already known on this topic

Chemotherapy can cause hepatitis flare-up through viral reactivation in patients who have had contact with hepatitis viruses. Few data are available on the genotype of the reactivated viruses.

What this study adds

Fludarabine alone or in combination with other (non-steroid) antineoplastic drug(s) was a powerful trigger of severe hepatitis due to HBV reactivation. A high incidence of genomic mutations was found in the strains of HBV responsible for the hepatitis flare-ups, and there was a high incidence of non responders to lamivudine in this small series of patients with HBV reactivation.

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Randomized Comparison of Power Doppler Ultrasound–Directed Excisional Biopsy With Standard Excisional Biopsy for the Characterization of Lymphadenopathies in Patients With Suspected Lymphoma

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Authors' disclosures of potential conflicts of interest are found at the end of this article.

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ABSTRACT

Purpose
The sensitivity of lymph node excisional biopsy requires validation. Power Doppler ultrasound (US) helps predict the malignant status of lymphadenopathies. We used power Doppler US to select for biopsy the lymph node most suspected of malignancy.

Patients and Methods
One hundred fifty-two patients having lymphadenopathies with clinical suspicion of lymphoma were divided into two well-matched groups and randomly assigned to undergo either standard or power Doppler US–directed lymph node excisional biopsy.

Results
Histology showed a malignancy in 64% of patients in the standard group (lymphoma, 49 patients; carcinoma, two patients) and in 87% of patients in the US-assisted group (lymphoma, 62 patients; carcinoma, one patient). There were significantly fewer biopsy-related complications in the assisted group than in the standard group. During the follow-up of the patients with lymph nodes reported as being reactive, 14 of 29 patients in the standard group were rebiopsied and were found to have lymphoma (13 patients) or carcinoma at the subsequent lymph node histology, whereas none of the patients in the assisted group (nine patients) required a second biopsy. Thus, biopsy provided false-negative results for malignancy in 21% of patients affected by lymphoma in the standard group and never in the assisted group ($P < .01$).

Conclusion
Power Doppler US is an accurate tool for screening lymphadenopathies to be removed by excisional biopsy in patients with suspected lymphoma.

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INTRODUCTION

In case of clinical suspicion of lymphoma, a lymph node enlargement requires histologic assessment to define a correct diagnosis and to develop a proper treatment plan. Prebiopsy evaluation of enlarged cervical, supraclavicular, axillary, or inguinal lymph nodes is usually left to physical examination alone; a careful and thorough palpation of superficial lymph node regions, performed by a physician experienced in the management of patients with lymphoma, is considered to provide sufficient information to schedule an excisional biopsy.[1] However, the possible presence of enlarged reactive or necrotic

lymph nodes and of nonpalpable but histologically significant malignant lymph nodes may impair the success of an excisional biopsy.[2,3] New approaches to this procedure, based on imaging-assisted methods are now available.

Power Doppler ultrasound (US) is a recent imaging technique that is able to accurately define the anatomic site of a lymph node, with its morphologic (including size, shape, and hilar and cortical deformation) and vascular characteristics.[4,5] Compared with the standard color Doppler, the properties of power Doppler US are homogeneous noise appearance, less direction and velocity dependence, less temporal variance, improved vessel contrast, and higher sensitivity. Power Doppler US clearly assesses in vivo intranodal angioarchitecture, mimicking angiography, distinguishing arterial and venous vessels, and calculating velocimetric parameters of the vessel flow.[4-6]

Angiogenesis is recognized as being critical for solid tumor growth, invasion, and metastasis. The various steps of neoplastic angiogenesis, such as basement membrane disruption, endothelial cell migration and proliferation, and tube formation, lead to the development of abnormal vascularization, with stenoses, occlusion, and/or dilation and/or arterovenous shunts.[7] The findings of increased endothelial cell and vessel proliferation in bone marrow or enlarged lymph nodes pointed to a possible role of neoangiogenesis in the pathogenesis of multiple myeloma or B-cell lymphomas.[8,9] With appropriate and standardized methodology,[10] power Doppler US has proven useful to identify malignant lesions because it detects more flow signals than gray-scale and color Doppler US, thus better differentiating between benign and malignant superficial lymphadenopathies.[4-6,11,12] We performed a randomized comparison of power Doppler US–directed excisional biopsy with standard excisional biopsy to characterize superficial lymphadenopathies in patients with clinical suspicion of lymphoma.

PATIENTS AND METHODS

Study Design and Patient Characteristics

During the past 4 years, 152 consecutive subjects (82 men and 70 women; median age, 44 years; range, 15 to 82 years) who were referred for superficial lymph node enlargement of unknown origin entered onto the study. Of these patients, 40, 60, and 52 had palpable lymph nodes in a one, two, or three anatomic regions, respectively. Clinical indication to perform an excisional biopsy was the only inclusion criterion. Patients affected by Epstein-Barr virus, cytomegalovirus, herpes simplex virus, rubella, toxoplasma, or tuberculosis infection were excluded. A minority of patients had already had computed tomography scans, and the findings of deep-seated lymph nodes had strengthened the suspicion of a malignant systemic disease. The study was a single-center trial involving two study groups and was approved by the local ethics committee. Patients were randomly assigned to receive lymph node biopsy using one of two methods, standard excisional biopsy (nonassisted group) or excisional biopsy under power Doppler US direction (assisted group). The primary aim of the study was to evaluate the capacity to predict the lymph node status, which was measured in terms of the percentage of cases of malignant involvement detected by power Doppler US–directed excisional biopsy versus standard excisional biopsy. Additional aims were the evaluation of biopsy-related complications and the discovery of a malignant disease during the follow-up of patients who had had the first biopsy negative for malignancy. The overall diagnostic accuracy was defined as the rate of correct patient classification, on the basis of having or not having lymph nodes positive for malignancy during the follow-up. Patients were informed of the aims of the study, the potential results of the procedures, and the meaning of the randomization, and signed a consent form before the operation. All biopsy procedures were performed by one of three surgeons experienced in lymph node resection, according to standard methods.[13] To avoid imbalance in infectious risk, patients in both groups received a short course of antibiotic prophylaxis (amoxicillin plus clavulanic acid, 2 g/d orally for 4 days) starting the day of biopsy.

Biopsy Procedure in the Nonassisted Group

In a day-hospital regimen or as in-patients and under local or general anesthesia (at surgeon's discretion), biopsy was directed to the region containing the most superficial and/or largest lymph node, as suggested by the physical examination. The lymph nodes were harvested through skin-crease incision obtained by free-hand methods.

Biopsy Procedure in the Assisted Group

Patients underwent US exploration of all superficial lymph node areas, including those apparently not involved in the disease, 24 hours before biopsy, and any abnormal (for size, shape, or hilus conformation) lymph node underwent power Doppler US. The information yielded was used to select the site of biopsy. Examinations were carried out by the same operator (M.P., a hematologist trained in diagnostic US),[14,15] using a high-resolution US Hitachi instrument equipped with power Doppler (EUB 6500; Hitachi, Tokyo, Japan) and a 13-6 MHz broad-band linear array transducer (EUB 54 M probe; Hitachi). Lymph nodes were assessed by gray scale to define their anatomic site, depth, size, shape, and hilus and by power Doppler to investigate the intranodal vascular pattern. Shape was studied with the long-to-short axis ratio (L/S) and defined as round for L/S values between 1 and 1.5 and oval for L/S values between 1.5 and 2, as described by other authors.[5] Settings for power Doppler were standardized for the highest sensitivity in the absence of apparent noise, using high-pass filter at 50 Hz, pulsed repetition frequency at 650 to 800 Hz, moderate-to-long persistence, and a slow-sweep technique. Under these conditions, the lowest possible measurable blood velocity was defined below 5 cm/sec. The method for appropriate gain optimization was in accordance with the criteria described by Bude and Rubin.[12] Intranodal vascular mapping was categorized as central/hilar type, peripheral type, mixed type (central/hilar and peripheral vessel signal), and chaotic type (vessel signal chaotically distributed within the node), in accordance with other authors.[5,11] As for Doppler spectral analysis, the resistive index (RI) value of arterial vessels (peak systolic velocity-end diastolic velocity/peak systolic velocity, as defined by Pourcelot)[16] was calculated by sampling at least three different intranodal sites (periphery, interior, and center/hilus); each RI measurement was determined after at least three stable consecutive cycles of waveform.

For each enlarged lymph node, the mean value of three measurements was calculated.

The main criterion used for selecting the node to be biopsied was the RI value; for each patient, the lymph node with the highest RI mean value was labeled and selected as target for biopsy. When more nodes had similar RI values, additional selection criteria were round shape, hilus absent, and intranodal hypervascularization.

In 12 patients, the selected lymph node was studied by repeated power Doppler US assessments on two occasions at a 1-hour interval by the same operator (intraobserver reproducibility) and by another operator unaware of the previous result, always using the same US machine (interobserver reproducibility).[17,18] The target area for biopsy was marked on the skin with indelible ink surrounding the probe contour, and the size and deepness of the lymph node were recorded. In the day-hospital regimen or as in-patients and under local or general anesthesia (at surgeon's discretion), the lymph nodes were harvested through skin-crease incision guided by the skin markings indicating the power Doppler US–selected lymph node.

Histopathologic Evaluation

Histopathologic examination was performed in a single pathology unit by three expert hematopathologists who were blinded to the patient's clinical condition, to the excision method, and to the histologic results of the other operators. Lymph node samples were routinely fixed in formalin and embedded in paraffin. The histologic sections were stained according to standard methods (hematoxylin and eosin and Giemsa). All cases of lymphoma were diagnosed by a combination of morphologic and immunohistochemical (using a large panel of monoclonal antibodies) assessment and were classified according to the current Revised European-American Lymphoma and WHO criteria.[19,20] Distinction between lymphomas with indolent or aggressive clinical behavior was made as reported by other authors.[21,22] Epithelial metastatic tumors were identified by monoclonal antibodies to cytokeratin. Overall, biopsies were categorized as either positive for malignancy (samples containing adequate number of cells with morphologic atypia and immunohistochemical evidence of monoclonality) or negative for malignancy (samples containing adequate number of cells with no evidence of malignancy). Patients classified as having a histologic result negative for malignancy underwent strict follow-up by clinicians blinded to the excision method used for biopsy.

Statistical Analysis

Statistical evaluations, including χ^2 (P was expressed as Yates corrected) and Student's t test, analysis of variance with Bonferroni correction, Pearson correlation, and log-rank test (to compare curves representing event-free survival), were performed with SPSS for Windows software (version 9.0; SPSS, Chicago, IL).

RESULTS

Of the 152 patients randomly assigned to a study group, 80 (53%) received standard excisional biopsy, and 72 (47%) received power Doppler US–directed excisional biopsy (a few patients were lost to follow-up after randomization and before biopsy, and this occurred by chance more frequently in the assisted group). Both groups were well matched at entry with respect to age and sex (Table 1). A total of 116 lymph nodes were removed and examined from the 80 patients in the nonassisted group, whereas only one lymph node was removed from each of the 72 patients in the assisted group. There was no significant difference between the two groups regarding the size of the lymph nodes removed. Patients in the nonassisted group had slightly more cervical and inguinal biopsies, whereas patients in the assisted group had slightly more supraclavicular and axillary biopsies.

Table 1. Patient Characteristics in the Two Study Groups

Characteristic	Nonassisted Group (No.)	US-Assisted Group (No.)	*P*
Total patients	80	72	
Sex			
Male	42	40	NS
Female	38	32	
Age, years			
Median	45	43	NS
Range	17-82	15-78	
Biopsy site			
Cervical	48	34	.01
Supraclavicular	6	13	.05
Axillary	6	16	.02
Inguinal	20	9	.01
No. of lymph nodes removed	116	72	< .001
Diameter of examined lymph nodes,* cm			
Median	1.8	2.0	NS
Range	0.4-6.0	0.4-7.0	

Abbreviation: NS, not significant.
*Long axis.

Histology

Of the 80 patients in the nonassisted group, 51 (64%) had lymph nodes positive for malignancy (B-cell non-Hodgkin's lymphoma [NHL], 26 patients; Hodgkin's disease [HD], 23 patients; and metastatic carcinoma, two patients), and 29 (36%) had lymph nodes negative for malignancy (described as benign lymphoid hyperplasia in all patients, with steato-fibrotic and/or necrotic changes in 18 of the patients). Of the 72 patients in the assisted group, 63 (87.5%) had lymph nodes positive for malignancy (B-cell NHL, 29 patients; T-cell NHL, four patients; HD, 29 patients, and metastatic carcinoma, one patient), and nine (12.5%) had lymph nodes negative for malignancy (benign lymphoid hyperplasia). There was complete agreement among the three pathologists on the histologic diagnosis (Table 2).

Overall, the 38 patients with lymph nodes negative for malignancy (defined as reactive or inflammatory) were observed for a median of 11 months (range, 1 to 40 months). During the follow-up, for 14 of 29 patients in the nonassisted group, the clinicians required a second lymph node biopsy, and a malignancy was finally detected. The second

Table 2. Histologic Characterization of Removed Lymph Nodes in the Two Study Groups

Histology	No. of Patients	
	Nonassisted Group	US-Assisted Group
Aggressive non-Hodgkin's lymphoma		
Diffuse large B-cell lymphoma	12	9
Follicular (grade 3) lymphoma	0	3
Mantle-cell lymphoma	4	7
Other B-cell lymphomas	1	4
T-cell lymphoma	0	4
Indolent non-Hodgkin's lymphoma		
Follicular (grade 1 to 2) lymphoma	6	5
Small lymphocytic lymphoma	3	1
Hodgkin's disease	23	29
Metastatic carcinoma	2	1
Nonmalignant findings		
Benign lymphoid hyperplasia	29	9

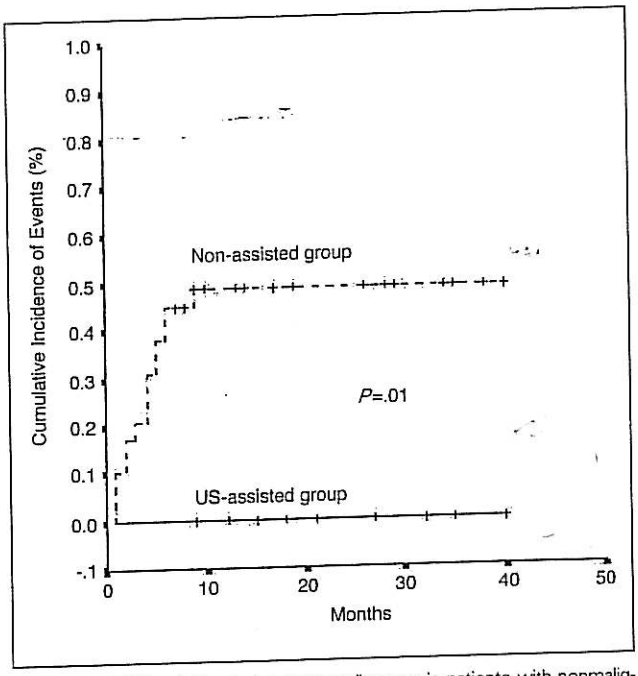

Fig 1. Probability of developing overt malignancy in patients with nonmalignant findings at the first lymph node biopsy in the nonassisted group (n = 29) and in the ultrasound (US)-assisted group (n = 9). Months = months from the first biopsy.

biopsy, which was performed after a median of 4 months (range, 1 to 9 months) from the first biopsy, demonstrated HD in seven patients, NHL in five patients, melanoma in one patient, and Rosai-Dorfman disease in one patient (with a severe clinical course requiring cytotoxic treatment as a lymphoma; Table 3). In contrast, none of the nine patients who had had diagnosis of a benign lesion at the first biopsy in the assisted group required a second biopsy or developed a malignancy, with a median follow-up of 21 months (range, 9 to 40 months; $P = .01$; Fig 1).

Therefore, the overall diagnostic accuracy of lymph node status in the nonassisted group was 82% (ie, results accurate in 66 of 80 patients), with a sensitivity of 78% (51 of 65 patients with lymph nodes positive for malignancy were identified) and a false-negative rate of 22% (14 of 65 patients with lymph nodes positive for malignancy were not identified). By contrast, the overall diagnostic accuracy and the sensitivity of the lymph node status in the assisted group

Table 3. Findings in 14 Patients in the Nonassisted Group Who Underwent a Second Biopsy

Patient No.	No. of Months Between the Two Biopsies	Biopsy Site		Size of the Removed Nodes (cm)*		Histologic Diagnosis	
		First	Second	First	Second	First	Second
1	1	Cervical	Cervical	1.0	2.0	Reactive†	Grade 1 follicular NHL
2	1	Inguinal	Supraclavicular	2.0	2.5	Reactive	Nodal small lymphocytic NHL
3	1	Axillary	Cervical	1.5	3.0	Reactive†	Anaplastic large cell NHL
4	2	Inguinal	Axillary	2.0	4.0	Reactive	Grade 1 follicular NHL
5	2	Cervical	Supraclavicular	1.5	2.8	Reactive†	Nodular sclerosis HD
6	3	Cervical	Supraclavicular	2.0	4.0	Reactive†	Nodular sclerosis HD
7	4	Inguinal	Axillary	1.8	3.8	Reactive	Grade 1 follicular NHL
8	4	Cervical	Supraclavicular	1.5	4.0	Reactive†	Melanoma
9	4	Inguinal	Cervical	1.0	4.2	Reactive†	Mixed cellularity HD
10	5	Cervical	Cervical	2.0	2.0	Reactive	Mixed cellularity HD
11	5	Cervical	Cervical	2.5	5.0	Reactive†	Mixed cellularity HD
12	6	Cervical	Supraclavicular	2.0	4.7	Reactive†	Nodular sclerosis HD
13	6	Inguinal	Axillary	3.0	4.8	Reactive	Nodular sclerosis HD
14	9	Inguinal	Axillary	3.3	4.5	Reactive†	Rosai-Dorfman disease

Abbreviations: NHL, non-Hodgkin's lymphoma; HD, Hodgkin's disease.
*Long axis.
†With intranodal steato-fibrotic and necrotic changes.

were 100% (ie, no false-negative cases; Fig 2). There was a statistically significant difference between the two groups regarding diagnostic accuracy and sensitivity ($P < .001$).

Power Doppler US Results

The average time required for power Doppler US examination was 30 minutes (range, 20 to 50 minutes). Intraobserver and interobserver reproducibility of intranodal vascular mapping and RI measurements were excellent. Of the 12 lymph nodes tested for reproducibility, 11 (91%) were classified identically by the same observer at two power Doppler US examinations 1 hour apart ($r = 0.9$), and 10 (83%) were classified identically by observers A and B ($r = 0.88$).

For each lymph node removed, shape and size were classified identically by the US operator and the pathologist. This finding consistently demonstrated that the surgeon had removed the indicated lymph node. Depth of the selected lymph nodes was between 1 and 4 cm. As for morphologic characteristics, malignant lymph nodes had a median of the long axis of 2 cm (range, 0.4 to 7.0 cm), were round in 50 cases and oval in 13 cases, and had hilus absent in 42 cases and present in 21 cases. Vascular mapping of malignant lymph nodes was mixed in 30 cases, chaotic in 24 cases, peripheral in six cases, and central/hilar in three cases (Fig 3). There were nine lymph nodes (12.5%) classified as suspected of malignancy by power Doppler US, which were shown to be reactive at histology. Of these nodes, the median of the long axis was 1.9 cm (range, 0.8 to 3.0 cm), six were oval, three were round, five were hilus present, and four were hilus absent; vascular mapping was of the hilar type in four, mixed in three, and peripheral in two. The median RI value of aggressive NHL (0.85; range, 0.7 to 0.98) was significantly ($P < .01$) higher than the median values of HD (0.74; range, 0.6 to 0.96), indolent NHL (0.71; range, 0.68 to 0.87), and benign lymphoid hyperplasia (0.68; range, 0.6 to 0.77; Fig 4). The metastatic carcinoma RI value was 0.8. Overall, the positive predictive value for malignancy of the parameters studied was as follows: for gray-scale US: round shape, 79%; hilus absent, 67%; and size $\geq$ 2 cm, 48%; and for vascular mapping by power Doppler US: mixed type, 48%; chaotic type, 38%; peripheral type, 10%; and central/hilar type, 5%. As for RI, considering a cutoff value $\geq$ 0.8, the predictive value for malignancy was 48%.

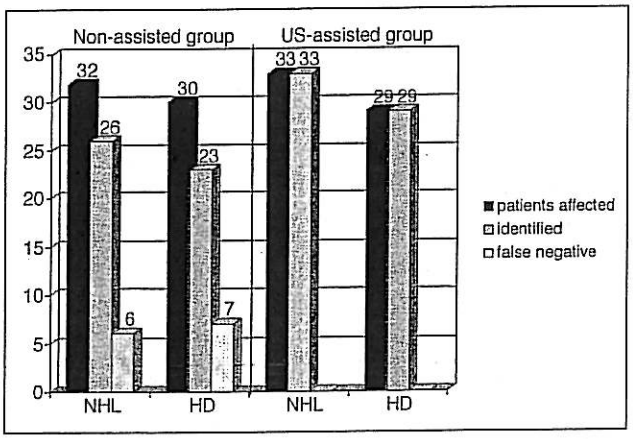

Fig 2. Diagnostic accuracy in detecting lymph nodes involved by lymphoma in the two study groups. US, ultrasound; NHL, non-Hodgkin's lymphoma; HD, Hodgkin's disease.

Biopsy Procedures and Complications

Sixteen patients underwent biopsy (axillary, n = 6; supraclavicular, n = 6; and cervical, n = 4) under general anesthesia, with an average hospitalization of 2.5 days (all in the nonassisted group). All other patients underwent biopsy in a day-hospital regimen under local anesthesia. The procedures were equally distributed among the three surgeons; no surgeon had more complications compared with the others. Patients who received non-US–directed biopsy had significantly more pain, numbness, or paresthesia and larger scars than patients who underwent US-directed biopsy. Moreover, 11 patients in the nonassisted group and no patient in the assisted group developed lymphorrhea; all patients recovered from the complication after one or more liquid aspirations (between 20 and 50 mL for each patient; Table 4).

DISCUSSION

The aim of this study was to determine the ability of power Doppler US to predict the presence of intranodal malignancy, thus improving the diagnostic accuracy of excisional biopsy in patients who have enlarged lymph nodes with clinical suspicion of lymphoma. The enlargement may often involve more than one lymph node; because the biopsy procedure has only a diagnostic purpose, the surgeon will select the easiest to reach lymph nodes (usually those seated superficially in a cervical or inguinal region). However, not all lymph nodes may be involved by the main disease entity; there is a risk of removing satellite reactive lymph nodes, thus missing the primary diagnosis of a malignant disease present in another node, which is sometimes deeper seated or even seated in a different anatomic area.[23] An affected lymph node may also undergo necrosis and/or steatofibrotic changes, which could avert the pathologist from the correct diagnosis.[3] These are all potential sources of inaccuracy in standard excisional biopsy. Preliminary reports indicating that power Doppler US might predict the presence of malignancy in superficial or deep-seated lesions have recently appeared.[4-6,11,24] In the present randomized study, we used power Doppler US to identify as biopsy target the most suspected area of malignancy. The selected lymph node was fully characterized as far as anatomic location, depth, size, shape, hilus alterations, and intranodal angioarchitecture were concerned. Intranodal hypervascularization and arterial vessels with relatively high RI value fulfilled

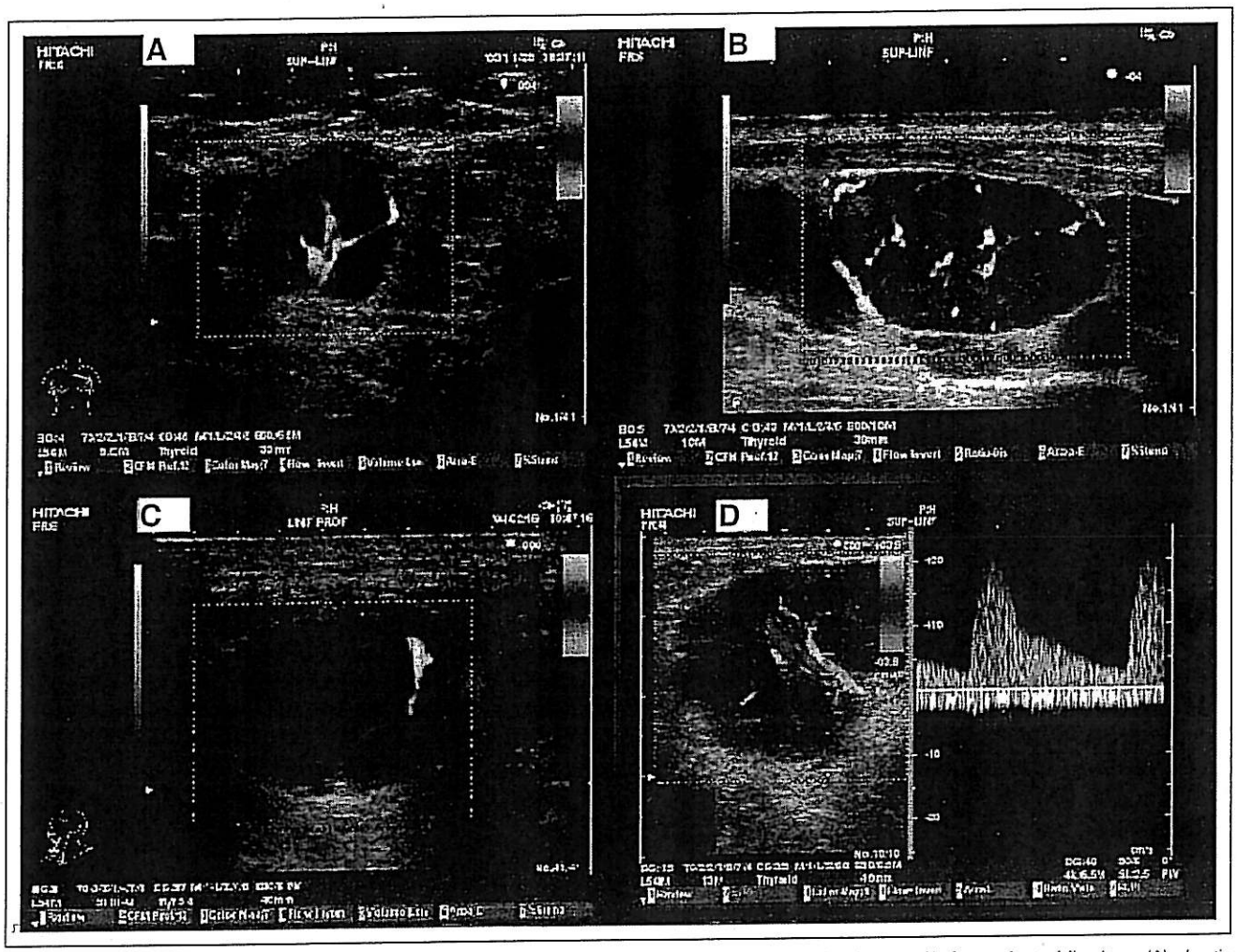

Fig 3. Angioarchitecture of lymph nodes affected by an aggressive lymphoma as revealed by power Doppler ultrasound in four patients. Mixed type (A), chaotic type (B), peripheral type (C), and hilar type with aberrant resistive index value (D).

the selection requirements. Lymph nodes showing no perfusion or low vascularization (tiny flow signals), likely caused by steato-fibrotic and/or necrotic changes of intranodal tissue, were avoided. The US examination was carried out by members of the hematology staff trained in diagnostic ultrasonography; the low intraobserver and interobserver variability of power Doppler assessments proved the high reliability of the examination. The result of this study shows that power Doppler US–directed excisional biopsy is significantly more effective in identifying lymph nodes positive for malignancy than standard excisional biopsy. We would like to underline the excellent tissue samples obtained in the US-directed group, which were informative and successful (tissue adequate for a correct histologic diagnosis), establishing the specific etiology of the enlargement, in all instances. Event-free survival of patients with negative biopsy for malignancy was significantly better in the assisted group than in the nonassisted group. Fourteen patients, in whom a few months earlier a non-US–directed biopsy had provided results of a benign adenopathy, were found to have a malignant disease involving other lymph nodes. Of them, 11 patients underwent rebiopsy in an anatomic area different from that biopsied the first time. It is reasonable to assert that the malignancy was already present at the time of the first biopsy and could have been detected by selecting a more significant lymph node had a power Doppler US study been performed. The higher prevalence of biopsy in inguinal areas in the nonassisted group may not be incidental. Supraclavicular and axillary nodes are often deep seated and less detectable by physical examination and, thus, are less frequently selected by the clinician or the surgeon as target for the biopsy; in contrast, these nodes can be more specific for the basic malignant disease and, thus, more frequently selected for the biopsy by the US operator.

By summing the number of patients who received diagnosis of malignancy at the first or second biopsy in the nonassisted group (51 + 14 = 65 of 80 patients), we found a percentage of malignancy similar to that observed in the

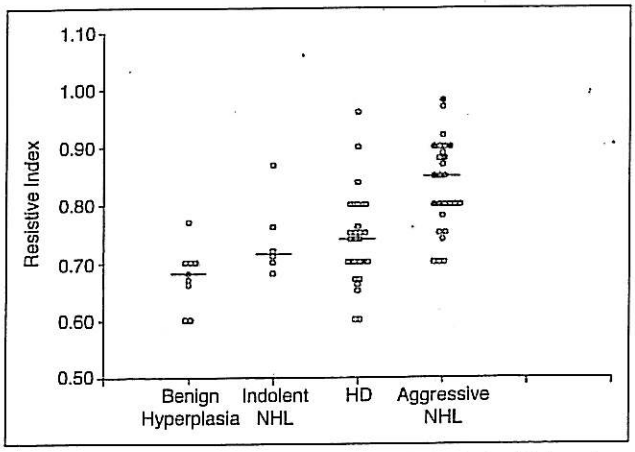

Fig 4. Power Doppler ultrasound measured resistive index (RI) in various patient categories. A correlation between RI and clinical aggressiveness emerges. Mantle-cell lymphoma (shaded circles) behaves as an aggressive lymphoma. NHL, non-Hodgkin's lymphoma; HD, Hodgkin's disease.

assisted group (63 of 72 patients), in whom all diagnoses were made by a single biopsy. These data confirm that standard excisional biopsy may carry a significant number of false-negative results. The false-negative rate was slightly higher in HD than in NHL patients; it was also higher for inguinal nodes (30%) compared with other sites (axillary, 17%; cervical, 8%; and supraclavicular, 0%).

Quantitative assessment of intranodal vascularization provided relevant information. High RI values (rapid systolic flow and poor telediastolic component) were predictive of an aggressive malignant disease (aggressive NHL or metastatic carcinoma), whereas lower RI values were found in HD, indolent NHL, and benign lymphoid hyperplasia. Interestingly, mantle-cell lymphoma, a small-cleaved lymphocytic lesion with a severe clinical course, which was considered as a low-grade lymphoma until a few years ago,[22] showed RI values in the range of aggressive diseases (median RI, 0.88) that were even higher than the RI values of diffuse large B-cell lymphoma (median RI, 0.8), grade 3 follicular lymphoma (median RI, 0.8), and other B- or T-cell aggressive lymphomas (median RI, 0.85; Fig 4). The mechanism by which lymphoma lesions have such diversified angiopatterns is still unclear. In aggressive NHLs, the magnitude of neoangiogenesis is probably the most relevant factor. In situ data obtained by transmission electron microscopy in B-cell NHL showed that angiogenesis, defined as formation of new vessels and remodeling of existing vessels, increases with tumor progression (in terms of increasing malignancy grading); the network of new vessels with irregular diameter and defective wall structure leads to abnormal flow and, hence, to the aberrant Doppler spectral patterns.[9] Similar findings were reported in bone marrow during progression from monoclonal gammopathy of undetermined significance to multiple myeloma.[8] In indolent NHL and in HD, this mechanism could be less operative, making the differentiation from reactive or inflammatory lesions less clear cut. In such instances, power Doppler US findings of round shape, hilus absent, and intranodal hypervascularization with chaotic feature may be additional selection criteria, as described by other authors.[5,11] In a small fraction of patients in the assisted group (12.5%), power Doppler US examination suggested malignancy that was neither confirmed subsequently at histology nor occurred during the follow-up. This means that power Doppler US study is highly sensitive but not absolutely specific.

Table 4. Compliance to Biopsy in the Two Study Groups

Complication	Non-Assisted Group (%)	US-Assisted Group (%)	*P*
Pain on operated site*			
No	30	78	< .001
Yes, mild and transient	33	11	
Yes, continuous	37	11	
Numbness on operated site			
No	20	88	< .001
Yes	80	12	
Swelling on operated site			
No	86	100	.003
Yes	14	0	
Aesthetic appearance of biopsy scar†			
Acceptable	30	83	< .001
Unpleasant	70	17	

*Postoperative pain was evaluated as absent, mild (not requiring analgesia), or continuous (requiring analgesia).
†As judged by the patients themselves 1 month after biopsy.

Power Doppler US–directed excisional biopsies were carried out always in a day-hospital regimen and under local anesthesia, whereas some patients in the nonassisted group needed general anesthesia and ward admission. The better tolerance of US-directed biopsies (less pain, less swelling, and more acceptable aesthetic scars) can be attributed to the perfect knowledge by the surgeon of the site and depth of the node to be removed, which lead to a precise incision and to the removal of a single node, thus avoiding larger cuts and intraoperative maneuvers.

In conclusion, our study provides evidence justifying the use of power Doppler US assistance as part of the work-up before performing a biopsy of superficial lesions suspected of lymphoma. This method improves the diagnostic accuracy and safety of excisional biopsy for the characterization of lymphadenopathies. It reliably provides adequate tissue for a correct histologic diagnosis, obviating the risk of underdiagnosis, which may cause a harmful diagnostic delay, and reducing postbiopsy morbidity and hospitalization costs.

Authors' Disclosures of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

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Interferon alfa treatment for pregnant women affected by essential thrombocythemia: Case reports and a review

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KEY WORDS
Essential thrombocythemia
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Objectives: In the past essential thrombocythemia was considered a disease of the elderly. At present, the number of young people suffering from this disease is growing, with a slightly higher frequency in females. We investigated the effects of interferon alfa therapy in these patients.
Study design: We describe 9 pregnancies in 4 women affected by essential thrombocythemia.
Results: Four pregnancies were carried out without interferon alfa therapy, and resulted in 2 intrauterine deaths, 1 spontaneous abortion, and 1 neonatal death. Interferon alfa was given during another 5 pregnancies; among them, 2 ended in preterm deliveries with normal infants, and 3 in full-term deliveries. The literature is reviewed.
Conclusion: Our cases and published series suggest that fetal outcome is improved by therapy, and that interferon alfa may be the best therapeutic option.

Essential thrombocythemia (ET) is a clonal hematopoietic disorder[1] that can be diagnosed in adherence to the Polycythemia Vera Study Group (PVSG) criteria.[2] Pregnancies in women affected by ET can be complicated by recurrent abortion, fetal growth restriction, stillbirth, and placental abruption.[2] The increasing number of young people affected, and the slight female preponderance suggest the elaboration of appropriate guidelines for pregnant women. Present therapeutic approaches in pregnant patients vary from no treatment to treatment with platelet reductive agents as monotherapy or in combination with antithrombotic medications (acetylsalicylic acid [ASA]).[2]

We describe 4 women affected by ET. From 1991 to 2001, they had 9 pregnancies; during 4 of them, they were left untreated or under ASA treatment as a result of patient decision or resistance to therapy, while in the course of the subsequent 5 pregnancies, they received interferon alfa (α-IFN) treatment, with or without ASA.

Case report

The clinical characteristics of our patients at diagnosis are described in Table I. Information about platelet count, type, dose, and length of therapy for all of our cases are summarized in Table II.

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Table I Patient characteristics at diagnosis of ET

Patient	Age	Plt × $10^3/\mu L$	Spleen volume by US scan*	Cytogenetics	bcr/abl	BM fibrosis
1	25	812	NA	46,XX	Absent	Absent
2	22	732	115 mL	46,XX	Absent	Absent
3	28	720	150 mL	46,XX	Absent	Absent
4	25	1200	1270 mL	NA	Absent	NA

Plt, Platelet; *US*, ultrasound; *BM*, bone marrow; *NA*, not available.
* Normal spleen volume is considered 60 to 200 mL.[32]

Table II Patient obstetric history

			Platelet count*								
Patient	Pregnancy	Age at pregnancy	Start of pregnancy	Peak	Nadir	At delivery	α-IFN therapy schedule	Length of therapy	ASA	GA at delivery	Pregnancy outcome
1	I	24	814	NA	NA	NA	None		Yes	36 wks	IUD
	II	25	467†	560	362	280	3 MU, 3/W	From preconception to delivery	Yes	34 wks	A&H
	III	30	825	NA	NA	875	None		Yes		SA
	IV	31	650	NA	NA	635	None		Yes	30 wks	NND
2	I	23	440†	399	328	315	3 MU, 1/W	From preconception to delivery	No	FT	A&H
	II	27	458†	399	263	334	3 MU, 1/W	From preconception to delivery	Yes	FT	A&H
3	II‡	28	310†	317	265	380	3 MU, 1/W	From 14wks to delivery	Yes	36 wks	A&H
4	I	30	800	NA	NA	NA	None		Yes	27 wks	IUD
	II	35	1742	1503	641	400	3 MU, 3/W	From 8wks to delivery	Yes	FT	A&H

GA, Gestational age; *NA*, not available; *IUD*, intrauterine death; *A&H*, alive and healthy; *SA*, spontaneous abortion; *NND*, neonatal death; *FT*, term.
* $\times 10^3/\mu L$.
† The first pregnancy occurred before ET diagnosis.
‡ Pregnancy started during α-IFN treatment.

Patient 1

This patient had thrombocytosis (platelet count 814 × $10^3/\mu L$) since 1992. At her first pregnancy (October 1992 to May 1993), intrauterine death occurred while she was receiving only ASA. In August 1993, a diagnosis of ET was made at our institution, and α-IFN therapy at the dose of 3 international megaunits (MU), 5 days per week, was started. In November 1993, still under α-IFN therapy, she became pregnant again. After counselling, α-IFN was continued, and ASA (100 mg daily) was added, starting from 12 weeks of gestation. At 34 weeks of gestation we opted for cesarean section because of mild asymmetric intrauterine growth restriction (IUGR) with reduced amniotic fluid, and pathologic umbilical artery Doppler with absence of diastolic flow. A healthy female newborn of 2150 g weight (below the 25th percentile) with Apgar score 8 and 9 at 1 and 5 minutes, respectively, and normal number of platelets was delivered. Post partum, a modest rebound from 440 × $10^3/\mu L$ to 651 × $10^3/\mu L$ after operation was recorded. α-IFN therapy was discontinued to allow the patient to breast-feed. After 6 months, α-IFN therapy was resumed because of increasing platelet count. It was then definitively stopped because of insufficient response (the platelet count was constantly over 800 × $10^3/\mu L$). The patient had 2 more pregnancies under treatment with only ASA (100 mg daily). The first pregnancy, in October 1998, resulted in a spontaneous abortion after a few weeks of gestation; the other pregnancy, in July 2000, was complicated by symmetric IUGR, severe oligohydramnios, and absence of diastolic flow in the umbilical artery Doppler. A cesarean section was performed at 30 weeks of gestation. The Apgar score was 3 and 6 at 1 and 5 minutes, respectively, and the infant died after a few days from prematurity.

Patient 2

This woman was diagnosed with ET in March 1997, and soon started α-IFN therapy (3 MU 5 days per week) because of her history of bleeding episodes. In June 1997, she became pregnant. After counselling, she agreed to continue α-IFN therapy, and the dose was

Table III Summary of pregnancy outcome according to treatment (our series included)

Treatment	Reference	No. of pregnancies	SA (%)	IUD (%)	NND (%)	Ectopic pregnancy (%)	Elective abortion (%)	Pregnancy outcome FD (%)	Pregnancy outcome A&H (%)
None	1,3–5,7,10–13, 16–20,22,25	88	40	4	1	1	4	50 (57)	38 (43)
ASA	1,5,10–13,15,19–20, 22,24–26	86	25	7	1			33 (38)	53* (62)
ASA + IFN	6,7,14	8			1			1 (13)	7 (88)
IFN	2,5,21,23,27–28	11	2					2 (18)	9† (82)
ASA + dipyridamole	12,26	1		1				1	
ASA + heparin	2,5,11,20,26,29	9	1					1 (11)	8 (89)
ASA + heparin + AT-III antagonist	20	2							2
Hydroxyurea	10,12	2					1	1	1
ASA + hydroxyurea	1	1							1
Platelet pheresis	12,16,18	3				1		1	2
ASA + platelet pheresis + IFN	4	1							1
ASA + platelet pheresis	11,12	4							4
Others	10	2							2
Total		218	68 (31)	12 (6)	3 (1)	2 (1)	5 (2)	90 (41)	128 (59)

SA, Spontaneous abortion; *IUD*, intrauterine death; *NND*, neonatal death; *FD*, fetal death; *A&H*, alive and healthy.

* Down syndrome (1 case).

† Bone and genital malformation (1 case).

reduced to 3 MU 3 days per week starting from 20 weeks of gestation. ASA was not prescribed because of patient's history of bleeding. She had an uneventful pregnancy and was delivered of a normal male newborn weighing 3070 g at 41 weeks of gestation, with Apgar scores 8 and 9 at 1 and 5 minutes, respectively. The baby's platelet count was normal. The patient did not breast-feed in order to continue the therapy with α-IFN (3 MU once per week). In July 2002, she became pregnant again and continued the maintenance treatment. After an uneventful pregnancy, she was delivered of a healthy male baby weighing 3000 g at 40 weeks of gestation, with Apgar scores 8 and 9 at 1 and 5 minutes, respectively, and normal platelet count. She interrupted the therapy to breast-feed; the follow-up of both children reports good health.

Patient 3

This patient was 28 years old when ET was diagnosed; she had had her first pregnancy before ET appearance in 1996, at the age of 25 years. Being symptomatic with headache and erythromelalgia, she was treated with α-IFN (3 MU 3 times per week). As soon as the second pregnancy was documented (at 14 weeks of gestation), the dose was reduced and ASA (100 mg daily) was added. After counselling, this treatment was continued throughout the pregnancy. The pregnancy was uneventful, with normal growth of the fetus. At 36 weeks of gestation she was delivered of a healthy female newborn, weighing 3050 g, with Apgar scores 8 and 9 at 1 and 5 minutes, respectively, and normal platelet count. Lactation was pharmacologically suppressed to continue the treatment with α-IFN. No rebound thrombocytosis was observed after delivery. After a few months, the therapy was stopped for patient's decision (platelets count 372 $\times$ $10^3/\mu L$). At present, the follow-up shows normal baby development without clinical complications.

Patient 4

This woman was diagnosed with ET in 1991 when she was 25, and was given α-IFN (3 MU 3 times per week) until 1995, when she decided to stop the therapy. In July 1996, intrauterine fetal death occurred at 27 weeks' of gestation. In August 2000, she became pregnant again, and after counselling, at 8 weeks of gestation, therapy with α-IFN was started again, combined with ASA, 100 mg daily. This treatment was continued throughout the pregnancy. The pregnancy was uneventful with normal fetal development. At 40 weeks of gestation the patient was delivered of a female newborn, weighing 3210 g, with Apgar scores 7 and 9 at 1 and 5 minutes, respectively. The baby's platelet count was normal. α-IFN was interrupted to allow nursing, but it needed to be resumed after 15 days because the platelet count was increasing ($896 \times 10^3/\mu L$). The patient is still under α-IFN therapy, and the baby is growing healthy.

Comment

The issue of when and how to treat patients with ET is of particular concern in young patients because they will

be receiving therapy for many years, with increasing risk for treatment-related side effects. ET in pregnancy has been reported to be complicated by recurrent abortion, intrauterine death, stillbirth, premature delivery, preeclampsia, and fetal growth restriction caused by placental infarction resulting from thrombosis. Maternal complications, such as bleeding or thrombotic events,[2] may also occur during pregnancy or post partum. To improve pregnancy outcome and reduce maternal complications, the use of antiplatelet drugs and/or cytoreductive agents is often considered, but the optimal treatment is still controversial. Some believe that pregnancy outcome is not therapy dependent, and thus no treatment is needed during pregnancy.[3] On the other hand, antiproliferative drugs such as hydroxyurea could have adverse effects on the fetus (abortion, congenital malformations, or intrauterine growth restriction).[2-4] Following early reports on safety and efficacy during pregnancy in women with ET,[5] α-IFN is increasingly being used during pregnancy, although pregnancy is still listed as a contraindication to α-IFN treatment. α-IFN is not mutagenic in vitro or teratogenic in animal studies; it does not reduce fertility.[4-7] The abortifacient effects observed in rhesus monkey occur at doses markedly higher than those used in therapy.[5,8] Some reports have shown that IFN and IFN-like molecules are produced by murine and human placenta (trophoblast interferons). They are expressed for a short period in high concentrations, and have antiluteolytic, antiviral, antiproliferative, and immunomodulatory effects through receptors on the endometrial epithelium.[9]

The treatment by ASA has a logical basis in order to reduce ischemic placental damage. It is reported to be effective in many vaso-occlusive manifestations, but its efficacy in reducing pregnancy complications has not been proven.[3] It may increase the risk of hemorrhage, thus being indicated in symptomatic ET pregnant women without history of bleeding. According to some authors, management of the ET patient should be based on risk stratification[3,10] because patients over age 60 years or who have a previous history of thrombosis are at high risk. We think that even pregnancy should be considered a risk factor, especially if the patient has experienced complications during a previous pregnancy. All 4 of our cases needed to be treated both because of their being symptomatic since the onset of disease, and because of their obstetric history, as summarized in Table II.

From a review of the literature and including our series, 218 pregnancies in 114 women affected by ET have been analyzed in 27 reports.[1-7,10-29] Fetal outcome was the following: 128 live births (59%) (including 16 preterm deliveries and 1 postterm delivery), 67 miscarriages, 12 stillbirths, 5 elective abortions, 2 ectopic pregnancies, 3 neonatal deaths, and 1 incompetent cervix. Seven babies suffered from IUGR, 1 from Down syndrome, and 1 from IUGR with bone and genital malformation. It is difficult to draw general rules from these data because of treatment heterogeneity and absence of risk stratification. Of the 114 women, 23 (19%) were symptomatic before pregnancy. α-IFN treatment was scheduled in 20 of the 218 pregnancies but was continued until delivery in only 17. As summarized in Table III, the percentage of live babies was higher (69%) in treated (any treatment) than in untreated pregnancies (43%). In all but 3 patients treated with α-IFN (as single agent or combined with ASA), pregnancy resulted in live births without complications for either fetus or mother, thus resulting in effective and safe deliveries in 85% of the pregnancies in which it was administered. In our own series, α-IFN treatment led to term delivery in the same women who experienced poor outcome when α-IFN was not or could not be given (Table II), a finding that can be considered as an internal control. Even though normal pregnancies are described in absence of treatment, we think that all pregnant women with ET should be treated because the real number of patients suffering from complications of ET may be underestimated.[2] As for metabolism and possible side effects of α-IFN in pregnancy, no conclusive data are available. Pons et al[30] studied the pharmacokinetics of α-IFN in pregnant women in 1995 and demonstrated that α-IFN was undetectable both in the amniotic fluid and in fetal blood, and that the pharmacokinetic parameters did not differ from that in nonpregnant women.[30] Thus, at the doses used, α-IFN does not seem to cross the placenta and cannot cause any damage to embriogenesis or to the developement of the immunologic system in the fetus.[2] As for safety of breast-feeding during IFN therapy, in our patients, we either stopped IFN treatment or suppressed lactation after delivery, although there is no evidence of breast-feeding danger during by IFN. A slight elevation of IFN concentration was reported by Kumar[31] in the milk of lactating women treated by high-dose (30 MU iv) IFN. In a patient of our series (#1) who continued breast-feeding during IFN treatment, a formal quantitative assay of IFN concentration in the milk was prevented by intrinsic milk toxicity on the cells used for the assay; however, the milk from the patient was more toxic to the target cells than the milk from a healthy control subject.

In conclusion, optimal management of ET patients is still poorly defined, and there are no established protocols. Normally, only patients belonging to the high-risk group need to be treated. However, we feel that during pregnancy all women are at risk of complication and should be treated. We suggest α-IFN to reduce platelet count, combined with ASA if no hemorrhagic complication is present.

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Hematopoietic Stem Cells

High number of circulating CD34⁺ cells in patients with myelophthisis

Six patients with bone marrow micrometastases from solid cancers presented with increased numbers of circulating CD34⁺ cells; the CD34⁺ cell counts were very high in some cases. By contrast, no patient with metastatic cancer without bone marrow involvement showed raised numbers of circulating hemopoietic progenitors.

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The circulating CD34 (cCD34⁺) cell count is assessed daily to monitor stem cell mobilization in patients undergoing stem cell harvest from peripheral blood. Another condition with raised numbers of cCD34⁺ cells is idiopathic myelofibrosis,[1] a finding suggesting that a normal bone marrow microenvironment is essential for the binding of CD34⁺ cells to stroma. Myelophthisis is a peculiar bone marrow microenvironment alteration caused by the presence of a high number of non-hematopoietic neoplastic cells, impairing hematopoiesis by largely unknown mechanisms. Space occupation is not the main mechanism, since micrometastases do not colonize the whole marrow; rather, marrow is usually hypocellular, with some degree of fibrosis revealed at bone biopsy.[2,3] We investigated the number of cCD34⁺ cells in patients with myelophthisis.

We studied eleven patients with diffuse metastatic cancer (Table 1). Myelophthisis was suspected in patients presenting uni-, bi-, or trilineage cytopenia not due to chemotherapy, and was documented by the finding of non-hematopoietic neoplastic cells in a bone marrow aspirate and/or biopsy. Peripheral blood, collected in K_3EDTA, was processed within 3 hours of venipuncture. CD34⁺ cells were detected by a combination of two monoclonal antibodies, fluorescein isothiocyanate-conjugated anti-CD45 and phycoerythrin-conjugated anti-CD34 (Becton Dickinson, Boston, MA-USA); c-kit expression was also measured. Fifty microliters of blood were incubated with the antibodies for 20 min in tubes containing a known number of microbeads. Cells were identified by FACSCalibur (Becton Dickinson, Boston, MA, USA) using scattering and fluorescence methods, and analyzed following the sequential gating strategy recommended by the International Society for Hematotherapy and Graft Engineering (ISHAGE). To exclude any influence of drugs, patients were studied before or at least three months after chemotherapy; none of the patients had received growth factors.

Of the eleven patients with metastatic cancer of various origin, six showed bone marrow micrometastases, suggested by cytopenia and documented by bone aspirate and/or biopsy, while five patients had no sign of bone marrow involvement. Marrow fibrosis (surrounding the metastatic lesions) was detected in the three patients with myelophthisis who underwent bone biopsy. cCD34⁺ (c-kit⁺) cell counts were elevated in all 6 patients with myelophthisis, and were particularly high in four. In contrast, CD34⁺ cells were almost undetectable in the patients with metastatic cancer without bone marrow involvement (Table 1). The mean number of cCD34⁺ cells was 49.2 and 1.2 cells/μL in the two groups, respectively. Among patients suffering from various types of disorders and tested for cCD34⁺ cells in our institution, the number of cells detected was second highest in myelophthisis; the highest number was found in idiopathic myelofibrosis (Figure 1).

Stem cell mobilization by growth factors and/or chemotherapy is caused by modifications of membrane-bound molecules that enable detachment of cells from the stroma. The only disease in which a high number of cCD34⁺ cells has been described in the absence of any treatment is idiopathic myelofibrosis;[1,4,5] both an altered bone marrow microenvironment and stem cell surface

Table 1. Circulating CD34⁺cells in metastatic cancer patients with or without bone marrow involvement.

	Case				Blood count					
	Age	Gender	Type of cancer	Hb g/dL	WBC $\times10^3$/L	N $\times10^3$/L	Plt $\times10^3$/L	Cancer cells in bone marrow	Previous therapy	CD34+ /μL
1	46	F	Breast	8.2	4.400	2.200	63	yes	None	60.6
2	34	M	Lung	7.9	5.310	3.240	163	yes	Cis-platinum	19.4
3	53	F	Stomach	8.4	17.000	14.620	49	yes	None	48.2
4	66	M	Lung	7.7	14.600	10.366	24	yes	None	149.4
5	53	M	Stomach	8.2	3.040	1.824	70	yes	None	8.1
6	50	F	Breast	9.4	15.500	7.500	51	yes	None	9.7
7	71	M	Stomach	11.1	6.410	4.295	265		5-FU	0
8	64	M	Lung	14.7	7.790	6.465	199		Taxotere	4.0
9	59	M	Lung	13.4	9.920	6.448	191		Cis-platinum	2.0
10	77	M	Colon	13.8	3.930	2.974	95*		5-FU	0
11	64	M	Stomach	11.9	8.600	6.450	237		5-FU	0

*: *low count due to hypersplenism secondary to hepatitis C virus-related chronic hepatitis.*

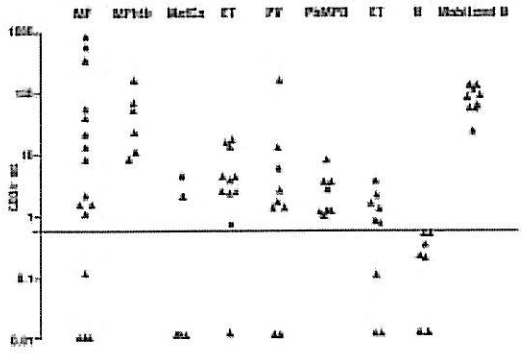

Figure 1. Circulating CD34⁺ cells in patients with metastatic cancer (with or without bone marrow involvement) or with various types of myeloproliferative disorders. MF: idiopathic myelofibrosis; MPhth: myelophthisis; MetCa: patients with diffuse metastatic cancer without bone marrow involvement; ET: essential thrombocythemia; PV: polycythemia vera; Ph- MPD: Philadelphia negative myeloproliferative disorders; ST: secondary thrombocytosis; N: 10 healthy subjects; Mobilized N: normal subject mobilized by granulocyte colony-stimulating factor for allogeneic donation; Solid line: upper limit in normal individuals.

abnormalities may underlie this phenomenon. A modest increase of cCD34⁺ cells, attributed to granulocyte-monocyte colony-stimulating factor (GM-CSF) production by the neoplastic tissue, has been described in patients with head or neck cancer.[6] We found that patients harboring cancer cells in the bone marrow had a mean of 49.2 cCD34⁺ cells/μL, 50 times more than in patients with metastatic cancer without bone marrow involvement. This may be the consequence of various not mutually exclusive mechanisms, including: (i) a stromal alteration, evidenced in these patients by a frequent dry tap bone marrow aspiration and some degree of fibrosis;[7-3] (ii) growth factors with mobilizing properties produced locally by the neoplastic cells; (iii) local production of molecules other than growth factors involved in stem cell mobilization. As far as concerns this last possibility, it could be relevant to note that a cleaved molecule of soluble urokinase-type plasminogen activator (uPA) receptor seems to be involved in both the metastasizing capacity of cancer cells[7] and in stem cell mobilization.[8]

Our series is too small to draw any conclusion on a possible relation between degree of cytopenia and number of cCD34⁺ cells; the type of primary tumor does not seem to be relevant. It is intriguing that two patients with raised cCD34⁺ cell counts had low hemoglobin and platelet levels but elevated neutrophil counts, suggesting a common mechanisms for high cCD34⁺ and white cell counts, as seen during mobilization. Several issues in this setting need to be elucidated (e.g., cytokine levels in blood and bone marrow, pattern of adhesion molecules and *in vitro* growth of cCD34⁺cells). From a clinical point of view, our observations suggest that high cCD34⁺ cell count may be indicative of bone marrow involvement in patients with metastatic cancer.

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Key words: circulating CD34 positive cells, myelophthisis, metastatic cancer.

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Red Cell Disorders

Association of the G-463A myeloperoxidase polymorphism with infection in sickle cell anemia

Infections constitute a principal cause of morbidity and mortality in sickle cell anemia (SCA). Here we present evidence to suggest that a polymorphism (G-463A MPO) in the gene encoding the myeloperoxidase (MPO) enzyme, important for the host defense system, may significantly increase susceptibility to infection in SCA.

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Sickle cell anemia is characterized by morphologically abnormal red cells, vaso-occlusion with ischemic tissue injury and susceptibility to infection. Infections, such as pneumonia, osteomyelitis, meningitis, urinary infections and septicemia, constitute a common cause of hospitalization in patients. While many patients have reduced splenic function, the mechanisms that render SCA patients more susceptible to infection are unclear. The severity of SCA varies greatly between individuals and this phenotypic variability is generally attributed to so-called genetic modulators.[1] Myeloperoxidase (MPO) is a lysosomal enzyme found in neutrophils and monocytes

Estimation of bulky lymph nodes by power Doppler ultrasound scanning in patients with Hodgkin's lymphoma: a prospective study

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The accuracy of standard methods in estimating bulky lesions requires validation. We used clinical/computed tomography (CT) evaluation and power Doppler ultrasound (US) to detect bulky disease in 137 consecutive Hodgkin's lymphoma patients, and analyzed the prognostic relevance of each method. Bulky disease was detected by clinical/CT evaluation in 47% of the patients and by power Doppler US in 20%. After treatment, at multivariate analysis power Doppler US-selected bulky disease was the parameter that best correlated with freedom from treatment failure ($p<0.001$). Power Doppler US, a readily available imaging technique, provides a better prognostic classification by detecting true bulky disease more accurately.

Key words: Hodgkin's lymphoma, power Doppler ultrasound scan, bulky disease.

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Size and extension of lymphadenopathies are important factors in defining the prognosis and designing the most appropriate treatment in Hodgkin's lymphoma, bulky disease and advanced stage being recognized adverse prognostic factors.[1] In many institutions, clinical and computed tomography (CT) examination are considered to provide sufficient information to map disease sites and estimate tumor burden.[1] However, non-palpable histologically significant malignant lymph nodes or enlarged reactive or necrotic lymph nodes may bias such a staging procedure.[2] High-resolution ultrasound (US) with power Doppler is a recent imaging technique that accurately defines the morphologic and vascular characteristics of a lymph node. Power Doppler US has proven useful to identify malignant lesions, detecting more flow signals than gray-scale and color Doppler US.[3,4] We tested the accuracy of power Doppler US in detecting bulky lymph nodes in patients with Hodgkin's lymphoma at initial presentation, verifying its prognostic relevance.

Design and Methods

Study design

In four years, 137 consecutive newly diagnosed Hodgkin's lymphoma patients were submitted to staging procedures. The lymph nodes most suspected of having bulky characteristics were detected using clinical/CT scan examination and power Doppler US in all patients. The study aim was to compare the value of power Doppler US-defined bulky disease with that of clinical/CT-defined bulky disease in predicting freedom from treatment failure (FFTF). In addition, other clinical variables routinely used as prognostic factors were evaluated in the statistical analysis.[5] Patients were informed of the study aim, and signed a consent form according to the Helsinki declaration.

Clinical/CT scan evaluation

Lymph node evaluation was performed by physical examination and CT using a multirow helical instrument and i.v. contrast medium (Mx 8000; Marconi Medical Systems, Cleveland, OH, USA). For each patient, the region containing a bulky disease, defined as any lymph node mass with a long axis ≥5 cm, was looked for and recorded.

Power Doppler US procedure

Patients underwent US exploration of all superficial lymph node areas and any abnormal lymph node was examined by power Doppler, using a high-resolution US instrument equipped with power Doppler (EUB 6500; Hitachi, Tokjo, Japan) and a 13-6 MHz broad-band linear probe. Lymph nodes were assessed by gray-scale to define their anatomic site, depth, size, shape and hilus and by power Doppler to investigate their intranodal vascularization.[6] Size was studied by measuring the perimeter, cross-sectional diameter and area (defined as the maximum measurements with nodal borders and angles clearly defined); thereafter, the volume in mL was automatically calculated by the US machine software. As for Doppler spectral analysis, the resistive index (RI) of

arterial vessels was calculated as defined by Pourcelot.[7] The presence of an abnormal vascular pattern combined with a RI value ≥0.65 fulfilled the requirement for intranodal hypervascularization. The combination of volume ≥30 mL and intranodal hypervascularization was the main criterion used to define a lymph node as bulky. In 13 patients the bulky lymph node was studied by repeated US volume measurements on two occasions at 1-hour intervals by the same operator (to test intraobserver reproducibility) and by another operator unaware of the previous result, always using the same US machine (to test interobserver reproducibility). The same 13 patients underwent whole-body fluorine-18-fluoro deoxyglucose positron emission tomography (FDG-PET)/CT scans, to examine the correlation between bulky lymph nodes selected by power Doppler US and those selected by PET/CT [the superficial mass with the highest standardized uptake value (SUV) and with 3-dimensional volume ≥30 mL].[8]

Therapeutic plan and response evaluation

All patients underwent ABVD-like chemotherapy courses repeated every 4 weeks. The response to chemotherapy was defined according to standardized criteria.[9,10] All patients considered responders received involved field radiation with a linear accelerator (planned dose 32Gy, without boost on initial bulky sites).

Statistical analysis

Univariate and multivariate analyses based on the Cox proportional hazards regression model were carried out to assess the prognostic factors significantly contributing to FFTF. Other statistical evaluations included χ^2, unpaired Student's t test, and analysis of variance with Bonferroni's correction. SPSS for Windows (version 12.0; SPSS, Chicago, IL, USA) was the software used.

Results

The patients' characteristics are summarized in Table 1. All patients received six courses of chemotherapy, in a median time of 6 months. A total of 125 patients (91%) achieved complete responses and underwent radiotherapy as planned. Patients were then observed for a median of 20 months (range, 7-43); 117 of them remained in sustained complete remission. Overall, a total of 20 patients suffered from events: five patients did not respond to chemotherapy, seven had a partial response, and eight relapsed.

Clinical/CT scan results

Sixty-five patients (47%) were assigned to the bulky group, having superficial (n=33) and/or mediastinal (n=44) lymph node masses. Twelve simultaneously had superficial and mediastinal masses. The median of long axis measurements of the masses was 7 cm (range, 5-12). No patient had abdominal bulky lymph nodes. The remaining 72 patients had lymph nodes with a diameter < 5 cm.

Table 1. Characteristics of the entire study population.

Characteristics	*No.*	*(%)*
Total patients	137	
Sex		
Male	79	(57)
Female	58	(43)
Age, years		
Median	30	
Range	15-74	
Histology		
Nodular sclerosis	96	(70)
Mixed cellularity	34	(25)
Lymphocyte Predominance	7	(5)
No. of nodal sites involved		
2	47	(34)
3	50	(36)
> 3	40	(30)
Contiguous extranodal involvement*	22	(16)
Ann Arbor Stage		
I	14	(10)
II	90	(66)
III	28	(20)
IV	5	(4)
Splenic involvement°	20	(14)
B symptoms	75	(55)
Erythrocyte sedimentation rate ≥ 50 mm	80	58)
Mediastinal bulky	44	(32)
Clinical/CT-selected superficial bulky disease	33	(24)
Power Doppler US-selected bulky disease	26	(20)

**an extranodal extension of the disease confined to a single lung lobe, or to sites such as pericardium/pleura/chest wall/pharynx, contiguous to the lymph nodes involved; °focal lesion(s) visible on CT, FDG-PET and/or US scanning.*

Power Doppler US results

The average time required for the power Doppler US examination was 30 minutes (range, 20-50). Intraobserver and interobserver measurement reproducibility was excellent, with a Pearson's value of 0.9 and 0.88, respectively. Twenty-six patients (20%) were assigned to the power Doppler US-defined bulky group. The median of the selected lymph node volume measurements was 37.5 mL (range, 30-180); depth was between 1 and 4 cm, and the site was cervical in four, supraclavicular in five, axillary in 13, pectoral in two, and inguinal in two. Lymph nodes were round in 18 cases and oval in eight; the hilus was absent in 16 cases and truncate in ten. Intranodal vascular mapping was mixed in 13 cases, chaotic in ten, peripheral in two, and central/hilar in one (Figure 1). At Doppler spectral analysis, the median RI value was 0.74 (range 0.65-0.95). There was complete agreement on bulky disease identification between power Doppler-US and PET/CT scans in 13 patients studied by both methods; the volume of the PET/CT-selected bulky lymph nodes ranged from 30 to 60 mL and the SUV from 7 to 13. The remaining 111 patients had superficial lymph nodes with a volume < 30 mL or ≥30 mL without hypervascularization.

Overall, the designation of superficial sites containing bulky disease was concordant between clinical/CT scans and power Doppler US in 18 patients and discordant in 23 patients.

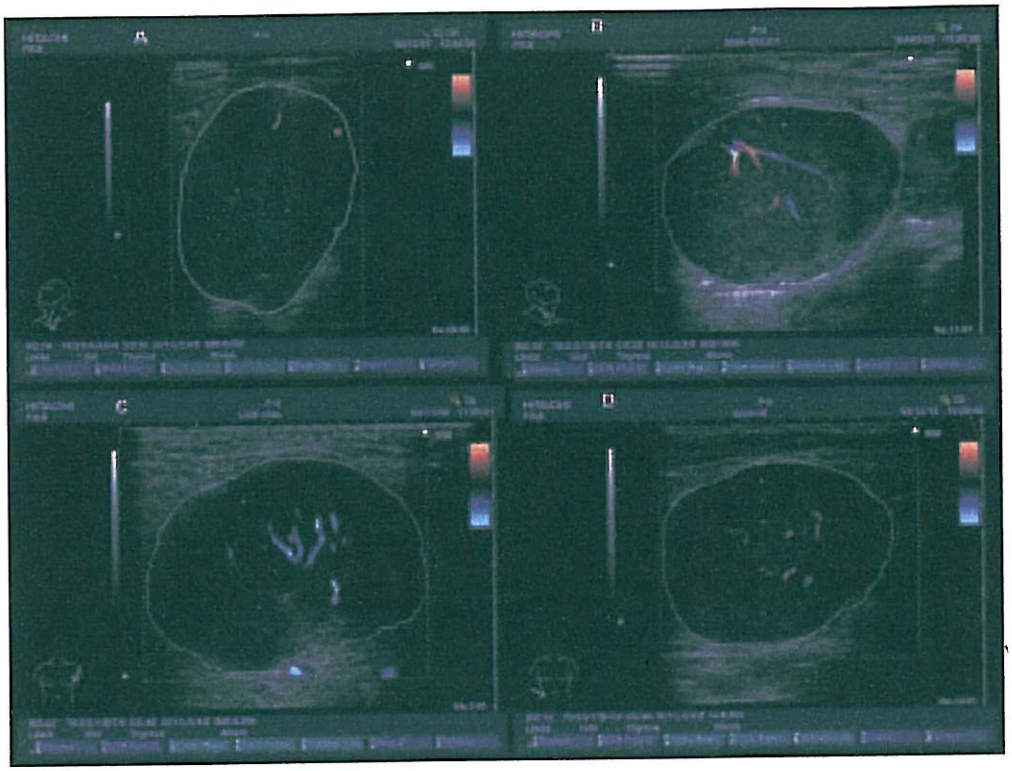

Figure 1. Power Doppler ultrasound (US) features. Peripheral type (A), mixed type (B), chaotic type (C), and central type (D) vascularization in bulky lymph nodes (volume ≥30 mL) as revealed by power Doppler US scanning in patients with Hodgkin's lymphoma.

Risk factors for FFTF

In a univariate analysis for prognostic factors, power Doppler US-selected bulky disease surpassed the mediastinal and superficial bulky disease selected by clinical/CT scan evaluation and any other analyzed factor as a significant predictor of FFTF. When a multivariate analysis was performed, only power Doppler US-selected bulky disease and advanced stage retained statistically significant prognostic value, and again power Doppler US was the best indicator of prognosis. Mediastinal bulky disease retained borderline statistical significance (Table 2).

Discussion

Stringent criteria for defining bulky disease are still controversial. According to various authors, bulky disease is considered any node mass whose largest diameter is at least 5 cm, 7 cm, or 10 cm.[1,11-13] However, one-dimensional measurement is often inaccurate in reliably determining lymph node size. On the other hand, not all enlarged lymph nodes are involved by the main disease entity; there is a risk of considering as malignant satellite lymph nodes that are reactive, necrotic or steato-fibrotic. We designed the present prospective study to test the hypothesis that a combined study of lymph node volume and angioarchitecture by power Doppler US may provide a more accurate estimation of bulky disease in patients with Hodgkin's lymphoma. The low intraobserver and interobserver variability of US assessment of volume, and the strong correlation between bulky lymph node selected by power Doppler US and PET/CT scanning (that measure both mass volume and activity) confirmed the high reliability of the method. The results of this study show that power

Table 2. Search for factors predicting freedom from treatment failure: univariate and multivariate analyses.

Factor	FFTF (%)	p univariate	HR	95% CI	p multivariate	HR	95% CI
Female/Male	82 (78)	0.73	1.1	0.46-3.01	0.2	0.51	0.18-1.44
Age, years: <45/≥45	78 (69)	0.097	0.48	0.2-1.14	0.92	0.94	0.27-3.27
Stage: I/II vs III/IV	86 (68)	**0.002**	3.88	1.63-9.26	**0.02**	3.61	1.25-10.41
B symptoms: No/Yes	95 (67)	**0.003**	6.02	1.78-20.3	0.26	2.2	0.56-8.6
Nodal sites involved: <3/≥3	87 (70)	0.25	1.56	0.735-3.29	0.41	0.58	0.16-2.11
Contiguous extranodal involvement*: No/Yes	82 (67)	**0.02**	2.79	1.17-6.64	0.61	1.31	0.46-3.77
Erythrocyte sedimentation rate: <50/≥50	86 (70)	0.17	1.8	0.771-4.22	0.95	1.04	0.35-3.07
Mediastinal bulky: No/Yes	88 (62)	**0.03**	2.56	1.11-5.93	0.06	3.07	0.93-10.16
Clinical/CT-selected superficial bulky: No/Yes	82 (78)	0.64	1.25	0.49-3.2	0.11	0.34	0.09-1.3
Power Doppler US-selected bulky: No/Yes	88 (47)	**<0.001**	7.9	3.37-18.5	**<0.001**	9.86	3.1-31.39

**an extranodal extension of the disease confined to sites contiguous to the lymph nodes involved.*

Doppler US leads to a better prognostic classification than does clinical/CT scan evaluation and surpasses several other commonly evaluated risk factors, predicting FFTF more properly.

The reason why lymph nodes estimated by different methods may have variable prognostic significance has anatomical bases. Clinical/CT scan evaluation using one-dimensional measurement, without informations on the vascular characteristics of the lymph node, is generally unable to differentiate between viable tumor and necrosis, inflammation or fibrosis in the mass. In power Doppler US-selected lymph nodes, the magnitude of neoangiogenesis is probably the most relevant finding. The various steps of neoplastic angiogenesis lead to the development of abnormal vascularization, with defective wall structure, stenoses, occlusion, vessel dilation or arterovenous shunts.[14] The neoangiogenesis network is recognized as being critical for tumor growth, invasion and metastasis. *In situ* data in lymphoma tissue showed that angiogenesis increases with tumor progression (in terms of increasing grade of malignancy).[15,16]

In conclusion, power Doppler US may provide more standardized and uniform criteria for detecting true bulky disease. These results need to be confirmed in large prospective studies, which may include the use of contrast agent enhanced US.[17] Our data support the concept of clinical heterogeneity in patients with Hodgkin's lymphoma. Neoangiogenesis-induced hypervascularization of bulky lymph nodes may be implicated in more aggressive behavior of the disease; thus, patients with true bulky disease may benefit from more intensive treatment.[11-13]

MP performed the ultrasound examinations, and prepared the manuscript; BR supervised the analysis and the interpretation of data, and prepared the manuscript; RC, ADR, BM, FP performed the physical examinations; GC and PZ performed the histological examinations; DD conducted all the statistical analyses; RL performed the biopsies of lymph nodes; EN, CS, and MS performed CT and PET examinations. All authors contributed to the interpretation of the data, revised the manuscript, and approved its final version. The authors declare that they have no potential conflicts of interest. Work supported by grants from Associazione Italiana contro le Leucemie (Salerno), INTAS, AIRC (Milano), CNR PF Biotecnologie (Roma), MURST-COFIN (Roma), and Regione Campania, Italy.

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Severe Reactivation of Hepatitis B Virus Infection in a Patient with Hairy Cell Leukemia: Should Lamivudine Prophylaxis be Recommended to HBsAg-Negative, Anti-HBc-Positive Patients?

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Abstract

The reverse seroconversion to hepatitis B virus infection has been sporadically described in onco-haematological patients receiving cytotoxic therapy or allogeneic bone marrow transplantation and can be associated with the development of acute icteric hepatitis. We present a male HBsAg-negative, anti-HBc-positive patient with Hairy Cell Leukemia who developed acute B hepatitis more than 1 year after the last course of 2-CdA and 6 months after splenectomy, while the patient was receiving therapy with αIFNr. The acute B hepatitis promptly responded to lamivudine therapy followed by viral clearance.

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Introduction

Reactivation of hepatitis B virus (HBV) infection has been described in patients with antibodies against HBV (anti-HBs and/or anti-HBc) undergoing chemotherapy or bone marrow transplantation for lymphoprolipherative disorders [1, 2]. In these patients antibodies against HBV may be lost, and reappearance of markers of active viral replication (HBsAg, anti-HBcIgM, HBeAg and HBV-DNA) may occur. We report a case of HBV reactivation occurred in a patient who had undergone chemotherapy and splenectomy for hairy cell leukemia, although he had been treated with alpha interferon just before the onset of viral reactivation.

Case Report

A 71-year-old Italian male received diagnosis of Hairy Cell Leukemia (HCL) in July 2001; at that time he tested negative for HBsAg and anti-HBs, but positive for anti-HBc IgG at the concentration of 40 Paul Ehrlich Unit/ml (Anti-HBc Elecsys- Roche Diagnostics), hallmark of a resolved past HBV infection. He was treated with 2-chlorodeoxyadenosine (2-CdA) until April 2002 (overall nine administrations). On February 2002, while still receiving 2-CdA, he was re-tested for HBV markers, and the former serological pattern was confirmed; serum aminotransferase levels were in the normal range (Table 1).

In January 2003, the patient underwent splenectomy because persistence of a marked splenomegaly (the spleen occupying more than the left abdomen overtaking the xiphoumbilical line up to the right). Four months later, in May 2003, the patient started recombinant alpha interferon (αIFNr) at the dose of 3 Million Units (MU) daily 6 days/week as maintenance treatment for HCL; this treatment was untoward by side effects (muscular pain). In July 2003, while still receiving αIFNr therapy, the patient developed fever (peak up to 38 °C) and skin icterus; he stopped αIFNr therapy and was admitted to our hospital. Biochemical tests showed a marked flare up of aminotransferases (AST 1075 U/L, ALT 808 UI/L, normal value up to 40 UI/L) and of bilirubin (total bilirubin 23.55 mg/dl, conjugated bilirubin 17.89 mg/dl). The patient was re-tested for serum HBV markers: he was found positive for HBsAg, anti-HBc IgM, anti-HBc IgG, HBeAg; HBV-DNA detected by polymerase chain reaction (PCR) was 145,000 copies/ml; serum markers for Hepatitis D, Hepatitis A and Hepatitis C Virus (HCV-RNA by PCR) infection were negative. The patient started therapy with lamivudine at the dose of 100 mg daily p.o. Both aminotransferase and bilirubin levels markedly decreased in a few days; 7 days later, HBV-DNA levels decreased (from 145,000 to 58,000 copies/ml), whereas HBsAg, anti-HBc IgM and HBeAg persisted positive; 15 days later, HBV-DNA fell to 1,900 copies/ml, anti-HBc IgM and HBeAg disappeared (without seroconversion to anti-HBe). One month later, aminotransferase levels returned in the normal range and HBsAg was cleared, although the patient was anti-HBs-negative; HBV-DNA was negative (< 200 copies/ml). Two months later, anti-HBs appeared. Lamivudine therapy was stopped 1 month after HBsAg to anti-HBs

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Table 1
Liver function tests and HBV markers in a patient with Hairy Cell Leukemia who experienced reactivation of HBV infection.

	Jul 2001	Feb 2002	Jan 2003	May 2003	Jul 22 2003	Jul 28 2003	Aug 4 2003	Aug 12 2003	Aug 18 2003	Aug 25 2003	Sep 17 2003	Apr 2004	Oct 2004
Therapy		2CdA	Surg	αIFNr		LAM	LAM	LAM	LAM	LAM	LAM	αIFNn	αIFNn
AST UI/L	24	24	18	NT	1075	538	138	121	48	30	31	18	19
ALT UI/L	14	14	10	NT	808	524	197	160	64	30	18	11	10
T Bil mg/dl	0.70	0.77	0.55	NT	23.55	13.1	4.23	3.23	2.09	1.63	0.59	0.69	1.12
D Bil mg/dl	0.13	0.11	0.10	NT	17.98	8.85	2.70	1.99	1.25	0.9	0.23	0.15	0.75
GGT UI/L	42	71	63	NT	151	196	203	175	136	112	92	39	38
HBsAg	Neg	Neg	NT	NT	Pos	NT	Pos	Pos	NT	Neg	Neg	Neg	Neg
Anti-HBs	Neg	Neg	NT	NT	Neg	NT	Neg	Neg	NT	Neg	Pos	Pos	Pos
Anti-HBc IgG	Pos	Pos	NT	NT	Pos	NT	Pos	Pos	NT	NT	NT	Pos	Pos
Anti-HBc IgM	Neg	Neg	NT	NT	Pos	NT	Pos	Neg	NT	Neg	Neg	NT	Neg
HbeAg	Neg	Neg	NT	NT	Pos	NT	Pos	Neg	NT	Neg	Neg	NT	NT
Anti-HBe	Neg	Neg	NT	NT	Neg	NT	Neg	Neg	NT	Pos	Pos	NT	NT
HBV-DNA (copies/ml)	NT	NT	NT	NT	145×10^3	NT	58×10^3	1.9×10^3	NT	< 200	< 200	NT	< 200

2-CdA: 2-chlorodeoxyadenosine; Surg: surgery (splenectomy); αIFNr: recombinant alpha interferon; LAM: Lamivudine; αIFNn: natural alpha interferon; AST n.v. 0-40; ALT n.v. 0-40; T Bil: total bilirubin n.v. up to 1.1 mg/dl; D Bil: direct (conjugated) bilirubin n.v. up to 0.25 mg/dl; GGT n.v. 0–50; NT: not tested

seroconversion. In January 2004 treatment by natural alpha IFN (αIFNn) was started, at the dose of 3 MU daily 5 days/week. The treatment was well tolerated; in April and October 2004 blood count, serum aminotransferase and bilirubin levels were within normal limits; the patient tested negative to HBsAg, and positive to anti-HBs and anti-HBc Ig; HBV-DNA was negative (October 2004). At physical examination, the patient showed mild latero-cervical lymph node enlargement, and hepatomegaly.

Discussion

The reverse seroconversion to HBV infection, i.e., HBV reactivation in patients with previously detected antibodies against HBV (anti-HBs and/or anti-HBc), has been already described in patients receiving cytotoxic therapy [3] or allogeneic bone marrow transplantation [1, 4–7]; it has to be distinguished from acute exacerbation of chronic HBV infection, i.e. enhanced viral replication and liver damage in an HBsAg-positive-patient. Although often asymptomatic, the reverse seroconversion from anti-HBs to HBsAg can be associated with the development of acute icteric hepatitis [6].

The case herein reported is intriguing because HBV infection reactivation occurred more than 1 year after the last course of 2-CdA (and 6 months after splenectomy), while the patient was receiving therapy with αIFNr. The development of acute B hepatitis in a patient under treatment with IFN confirms the poor efficacy of αIFN in modifying the natural course of acute B hepatitis. To our knowledge, there are no data suggesting a role of splenectomy for the onset of HBV reactivation.

The clinical features of icteric B hepatitis promptly resolved with lamivudine therapy; bilirubin and aminotransferase levels dramatically decreased, and the serum markers of HBV replication (anti-HBc IgM, HBeAg and HBV-DNA) were cleared in 1-month period; lastly, the patient seroconverted from HBsAg to anti-HBs in less than 2 months. This data confirm the efficacy of lamivudine as well as its safety in patients with onco-haematological disorders because the drug has no immuno-modulating effects and does not interfere with mithocondrial DNA or marrow cell progenitor [3, 6].

This case raises two important questions. First, the risk of reactivation is a relevant issue in countries such as Italy, where there is a large "historic" cohort of adults who, before the mandatory vaccination against HBV of all new born babies and adolescents was implemented, had been exposed to HBV and are anti-HBs- and/or anti-HBc-positive [8]. Thus, we think that HBsAg-negative patients with lymphoproliferative disorders have to be accurately tested for all serum markers of HBV, and those who have markers of a previous exposure to HBV (anti-HBs and/or anti-HBc IgG) should be accurately monitored (like that HBsAg-positive patients) if treated with chemotherapy. The second question regards the prophylaxis of HBV reactivation in subjects with markers of previous exposure

to HBV infection at the time of receiving immunosoppressive or cytotoxic drugs; while HBsAg-positive patients are recommended to start lamivudine before immunosuppression or soon after signs of acute exacerbation [9], no guidelines are available for patients who have serological antibodies against HBV [10]. We wonder if also in these patients the use of lamivudine could represent the best way to prevent, and not only to treat, HBV reactivation. Alternatively, patients should be closely monitored for HBV replication markers so that lamivudine treatment can be started as soon as signs of HBV reactivation appear.

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